

The anti-inflammatory effects of Morin hydrate in atherosclerosis is associated with autophagy induction through cAMP signalling

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Abstract

Although the previous trials of inflammation have indicated that morin hydrate (MO) hold considerable promise, understanding the distinct mechanism of MO against inflammation remains a challenge. **Methods and results:** This study investigated the effect of MO in atherosclerosis in ApoE^{-/-} mice and underlying cell signaling of MO effect in inflammation in human umbilical vein endothelial cells (HUVECs). Administration of MO significantly reduced serum lipid level, inflammatory cytokines (TNF- α and ICAM-1), and atherosclerotic plaque formation in vivo. MO presence attenuated the expression of TNF- α -induced inflammatory cytokines (ICAM-1, COX-2, and MMP-9), and remarkably enhanced microtubule associated protein 1 light chain 3 beta 2 (MAP1LC3B2) expression and sequestosome 1 (SQSTM1/p62) degradation in HU-VECs. These MO effects were significantly prevented by the presence of autophagic inhibitors, 3-methyladenine (3-MA), or chloroquine (CQ), as well as siRNA suppression of ATG5 and BECN1. MO increased intracellular cAMP levels and activated cAMP-PKA-AMPK-SIRT1 signaling in vivo and in vitro. These changes resulted in increased expression of autophagy-related protein MAP1LC3B2 and decreased secretion of inflammatory cytokines (ICAM-1, COX-2, and MMP-9). **Conclusion:** Our results suggest that anti-AS and anti-inflammatory effects of MO are largely associated with its induction of autophagy through stimulation of cAMP-PKA-AMPK-SIRT1 signaling pathway.

Keywords:

Atherosclerosis / Autophagy / Inflammation / cAMP-PKA-AMPK-SIRT1 signaling pathway / Morin hydrate

1. Introduction

Atherosclerosis is the most common pathological process associated with serious cardiovascular diseases [1], such as stroke, limb ischemia, and myocardial infarction [2]. Accumulating evidence reveal that an inflammation process, in which lipid particles and immune cells are gradually accumulated in subendothelial regions resulting in narrowing of the arterial lumen and thrombosis, is associated with development of atherosclerosis [1]. This inflammation process leads to endothelial secretion of inflammation cytokines and other acute-phase reactants such as IL-1, IL-2, IL-6, IL-8, IL-12, TNF- α , interferon- γ , and

platelet-derived growth factor [3, 4]. And these inflammation cytokines drive endothelial dysfunction, subclinical lesions, the late plaque formation, and disruption of atherosclerotic plaque. Currently, agents directly acting on the inflammatory cascade in atherosclerosis (AS) are now investigated widely, raising hope for advances in the treatment of atherosclerosis.

Various studies have shown that flavonoids such as morin hydrate (2', 3, 4', 5, 7-pentahydroxyflavone) have a good anti-inflammatory effect in atherosclerosis [5–7]. Morin hydrate (MO) is a bioflavonoid isolated as a yellowish pigment from a number of fruits, vegetables, and herbs of the Moraceae family [8]. MO has been reported to exert a variety of beneficial pharmacological effects including protection of cardiovascular cells and hepatocytes against oxidative injury [5,6], inhibition of xanthine oxidase activity [9], and apoptosis induction in human colon cancer cells (HCT-116) [10]. MO has also shown to improve inflammation in human epithelial cells by down-regulating nuclear factor- κ B, mitogen-activated protein kinase signaling pathways, sphingosine kinase 1/sphingosine 1-phosphate signaling pathway, etc. [11,12]. Recently, several studies have shown that autophagy acted as a negative regulator of inflammation in macrophages [13, 14]. Autophagy is a degradation process that delivers cytoplasmic material to lysosomes via double-membraned organelles termed autophagosomes (an enclosed portion of the cytoplasm) [15]. Autophagy, occurring in all cells to maintain mammalian homeostasis [16], has been shown to protect against neurodegeneration, inflammation in cardiovascular disease and cancer [17]. Moreover, autophagy has been reported to contribute to the removal of misfolded proteins in endothelial cells and prevent the risk of atherosclerosis. The presence of MO has recently been shown to increase the level of intracellular cAMP [18], one of the most important secondary messengers and activates PKA in mammalian cells [19, 20]. Activation of cAMP/PKA signaling pathway in endothelial cell is involved in induction of autophagy [21]. AMP-activated protein kinase (AMPK), a downstream kinase of PKA signaling pathway and an intracellular energy sensor kinase [22], has also been reported to regulate autophagy under normal and stress conditions [23]. AMPK can initiate autophagy to exert neuroprotection against focal cerebral ischemia and hence diminish ischemia related cell death [24]. AMPK was reported to be required for activation of silent information regulator of transcription 1 (SIRT1) [25] that could attenuate endothelial inflammatory reactions by inducing autophagy through deacetylation of several autophagy-related genes [26]. The aim of this study was to evaluate the anti-inflammatory effects and underlying mechanism of MO *in vitro* in human umbilical vein endothelial cells and *in vivo* in mice.

2. Materials and methods

2.1 Animals

All animal care and experimental procedures complied with Institutional Guidelines of Animal Care and Use Committee at Shandong University (Shandong, China). This project was conducted in the School of Pharmacy, Shandong University as per the approval granted by the Animal Ethics Committee of Shandong University (NO. ECAESDUSM 201) A total of 18 male 6-week old apolipoprotein E (ApoE)^{-/-} mice and 18 male 6-week old C57 mice were purchased from Peking University Resources Centre (Permission number: SCXK 2011-0012).

The animals were maintained in constant temperature-controlled rooms ($25 \pm 2^{\circ}\text{C}$) with controlled lighting (12 h light-dark cycle). C57 mice were received normal diet, and ApoE^{-/-} mice were received a high fat diet (0.25% cholesterol and 15% cocoa butter) for 16 weeks.

2.2 General procedures

Both the ApoE^{-/-} mice and C57 mice were randomly divided into three groups (n = 6/group): vehicle (2% tween80) group, 30 mg/kg/d and 100 mg/kg/d MO groups. Male mice were treated with MO every day via gavage at the dose of 30 mg/kg/d or 100 mg/kg/d for ten consecutive weeks. At the end of the experiment, body weight was obtained, and then all mice were starved for 12 h and anesthetized by Urethane. Blood samples were taken from the retro-orbital sinus of each mouse into a heparin-containing tube. Serum lipid levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) were measured by biochemistry automatic analyzer (Roche Cobas Integra 800, Basel, Switzerland). The concentrations of serum inflammatory cytokines (TNF- α and ICAM-1) were measured by ELISA kits according to the manufacturer's instructions. Then, all mice were rapidly perfused through the left ventricle with PBS, followed by 4% paraformaldehyde. For en face analysis, the aortas from the ascending arch to the iliac bifurcation were prepared and cleaned of peripheral tissue, opened longitudinally, pinned flat, stained with Oil-Red O and washed by 75% alcohol for examination of pathological changes and lipid deposition. Then the areas of the aorta and lesions were quantized by the software Image pro plus. The ratio of lesion area (R) was calculated as $R = \frac{\text{the areas of lesion aorta}}{\text{the areas of all aorta}}$. In addition, aortas tissues were lysed in lysis buffer (100 mM Tris-HCl, pH 6.8, 2% m/v SDS, 20% v/v glycerol, 200 mM β -mercaptoethanol, 1 mM PMSF, and 1 g/mL aprotinin) and the expression of proteins (PKA, AMPK, p-AMPK, and SIRT1) were determined by Western blotting method.

2.3 Experimental procedures

2.3.1 Cell culture

The human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord veins (Qilu Hospital, Shan-dong University). HUVECs were maintained in endothelial cell medium (ECM, ScienCell) supplemented with 5% heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin (P/S), and endothelial cell growth supplement (ECGS) at 37°C in a humid atmosphere (5% CO₂-95% air) and were harvested by brief incubation in 0.25% w/v EDTA-PBS. All experiments were conducted with cells within 3–6 passages.

2.3.2 Cytotoxic assay

HUVECs (1×10^4 per well) were seeded in 96-well plates and cultured till 80–90% confluence before introduction of various concentrations of TNF- α (Sigma-Aldrich) (5, 10, 20, 50, 100, 200, and 400 ng/mL) for 4 h. Next, the cells were treated with various MO (Sigma-Aldrich) concentrations (1, 5, 10, 20, 50, 100, 200, and 400 μM) for 24 h. The control group was treated with 0.1% DMSO. After removal of the culture medium, the cells were washed with PBS. Ten percent of CCK-8 solution (Dojindo, Japan) was then added to the

plates for 24 h at 37°C. Light absorbance was measured at 450 nm on Thermo Multiskan GO microplate reader (Thermo, USA). Triplicate experiments with quintuplicate samples were performed.

2.3.3 ELISA assay HUVECs (1×10^4 per well) were seeded in 96-well plates and cultured till 80–90% confluence before exposure to TNF- α (5, 10, 20, and 50 ng/mL) for 4 h. Some groups were pretreated with different concentrations of MO (1, 10, and 20 μ M) for 4 h before exposure to 10 ng/mL TNF- α for an additional 4 h. The level of soluble intercellular adhesion molecule 1 (sICAM-1) in the culture supernatant was measured using an ELISA kit (eBioscience) according to the manufacturer's instructions. In some experiments, the cells were stimulated with or without 10 μ M MO for 4 h, followed by additional 4 h incubation with or without 10 ng/mL TNF- α . Cells were then treated with 10 μ M KH7 (Cayman) (an inhibitor of ADCY) for 1 h or 10 μ M Rolipram (Selleckchem) (an inhibitor of PDE4) for 1 h. Intracellular levels of cAMP in HUVECs and atherosclerotic plaques were measured also by an ELISA kit (R&D systems) according to the manufacturer's instructions.

2.3.4 Western blot

HUVECs (5×10^5 per well) were seeded in 6-well plates and exposed to increasing concentrations of TNF- α (5, 10, 20, and 50 ng/mL) or MO (1, 10, and 20 μ M) for 4 h and then treated with 10 ng/mL TNF- α for 4 h. The control group was treated with 0.1% DMSO. The cells were then treated with 3 μ M CQ (Sigma-Aldrich), 5 mM 3-MA (Sigma-Aldrich), 5 mM Nicotinamide (Beyotime Biotechnology, China), 2 μ M EX527 (Sigma-Aldrich), 10 μ M KH7, or 10 μ M Rolipram for 2 h. The cells were then treated with or without TNF- α (10 ng/mL) for another 4 h. Target proteins were detected using 1:1000-diluted primary antibodies against ICAM-1 (Zhongshan Jinqiao Biotechnology, China), COX-2 (Cell Signaling Technology), MMP-9 (Cell Signaling Technology), MAP1LC3B (microtubule associated protein 1 light chain 3 beta 2) (Sigma-Aldrich), SQSTM1/p62 (sequestosome 1) (Cell Signaling Technology), BECN1 (Cell Signaling Technology), ATG5 (Epitomics, USA), PKA (Wuhan Huamei biotech, China), p-AMPK (Wuhan Huamei biotech, China), AMPK (Wuhan Huamei biotech, China), and SIRT1 (Cell Signaling Technology). Immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies, and visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and quantified by densitometry using a ChemiDoc XRS (Bio-Rad, Berkeley, California, USA). The data are expressed as the relative density of the protein normalized to β -actin. Triplicate experiments with triplicate samples were performed.

2.3.5 Laser scanning confocal microscopy analysis HUVECs (5×10^4 per well) were seeded in 12-well plates and then exposed to the various treatments with TNF- α or MO. The cells were washed twice with fresh medium and loaded with 100 nM LTG (Invitrogen) for 1 h in humidified air at 37°C in ECM culture medium. After three washes with PBS, the cells were examined using a Radiance 2000 laser scanning confocal microscope (Nikon, Japan).

2.3.6 siRNA transfection

HUVECs were transfected with 100 nM siRNAs for ATG5 or BECN1 (GenePharma, China) for 8 h according to the manufacturer's protocol. Cell culture was then switched to ECM medium for an additional 24 h before treatment of the cells with MO (10 μ M) for 4 h followed by exposure to 10 ng/mL of TNF- α for another 4 h. The cells were harvested and analyzed by Western blot.

2.3.7 Statistical analyses

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. Data was expressed as mean \pm SEM for three different determinations. For cell culture experiments, all results were repeated independently at least three times. Relative protein semi-quantification was performed using AlphaEaseFC software (AlphaInnotech, USA). Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests. $p < 0.05$ was considered as statistically significant. SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL).

3 Results

3.1 MO decreases serum lipid level and attenuates atherosclerotic plaque formation in ApoE $^{-/-}$ mice with high fat diet

After treatment with MO for 10 weeks, no differences of body weight of the ApoE $^{-/-}$ mice occurred between the treatment and vehicle mice. Elevation of TG, TC, and LDL levels was observed in mice with high fat diet. Serum lipid levels of MO-treated groups shown low levels of LDL, TC, TG, and HDL compared with the vehicle group (Table 1). And there were no differences of body weight and plasma lipids in C57 mice treated with MO for 10 weeks. The relative en face lesion area of the aorta was decreased in both MO-treated groups compared with the vehicle group, and treatment with 100 mg/kg MO showed larger reduction than treatment with 30 mg/kg MO (Fig. 1A and B). Moreover, there were no significant changes of body weight and plasma lipids in C57 mice treated with MO for 10 weeks in comparison with vehicle group (Supporting Information Table 1).

3.2 MO suppresses the production of inflammatory cytokines and increases the protein expression of cAMP-PKA-AMPK-SIRT1 pathway in MO-treated ApoE $^{-/-}$ mice

To observe the effects of MO on the production of inflammatory cytokines in serum of ApoE $^{-/-}$ mice, the levels of TNF- α and ICAM-1 were measured. Results showed that the levels of TNF- α and ICAM-1 in both MO-treated groups were lower than the corresponding measurements in the vehicle group (Fig. 1C and D). Moreover, MO increased the cAMP levels in aortas plaques accompanied with upregulation of PKA, p-AMPK, SIRT1 expression (Fig. 1E–G). In addition, MO increased the expression of microtubule associated protein 1 light chain 3 beta 2 (MAP1LC3B2), an autophagic biomarker (Fig. 1F and G).

3.3 MO suppresses production of inflammatory cytokines in TNF- α stimulated HUVECs

To determine the noncytotoxic concentrations of TNF- α and MO to be used in experiment, cell viability was determined after treatment with different concentrations of TNF- α and MO. Results showed that TNF- α at concentrations of up to 20 ng/mL was tolerated by HUVECs without significant change of cell viability (Fig. 2A). When the cells were exposed to increasing concentration of TNF- α (0, 5, 10, 20, 50 ng/mL), cell secretion of inflammatory cytokines including ICAM-1, MMP-9, and COX-2 significantly increased in a concentration-dependent manner (Fig. 2C). Thus, TNF- α (10 ng/mL) was used to induce inflammation on HUVECs with no effect on cell viability. Similarly, MO had no effect on cell viability at lower concentrations (less than 20 μ M) compared with 0.1% DMSO group (control group) (Fig. 2B, Supporting Information Fig. 1). Preincubation of the cells with MO (1, 10, and 20 μ M) for 4 h before exposure to TNF- α (10 ng/mL) for 4 h significantly suppressed the expressions of ICAM-1, MMP-9, and COX-2, as well as soluble ICAM-1 level, in a MO dose-dependent manner in comparison to TNF- α -treated cells (Fig. 2D–F).

3.4 MO induces autophagy in both normal HUVECs and TNF- α stimulated HUVECs

The increase of MAP1LC3B2 expression and the decrease of SQSTM1/p62 expression were regarded as autophagic biomarkers in HUVECs [27]. Chloroquine (CQ), which inhibits autophagosome-lysosome fusion, was used to monitor autophagic fluctuation through measuring the protein expression of MAP1LC3B2. Results showed that MO (1, 10, and 20 μ M) increased the expression of MAP1LC3B2 and decreased the expression of SQSTM1/p62 compared with control group in dose- and time-dependent manners. The presence of CQ showed to induce further accumulation of MAP1LC3B2 in HUVECs treated with MO (10 μ M) for 4 h compared with cells treated with CQ alone (Fig. 3A and C). These results revealed that MO promoted cellular autophagy in HUVECs, which was suppressed by CQ.

We found that TNF- α treatment had no effect on the expression of MAP1LC3B and SQSTM1/p62 compared with control group. Treatment of the cells with MO increased the expression of MAP1LC3B2 and decreased the expression of SQSTM1/p62 in dose- and time-dependent manners. In the presence of CQ, the autophagy induction in TNF- α -stimulated HUVECs of MO was also inhibited (Fig. 3B and D). These results suggested that MO induced autophagy in both normal HUVECs and TNF- α -stimulated HUVECs.

3.5 The anti-inflammatory effects of MO are dependent on autophagy

LysoTracker Green fluorescent dye (LTG), which stains acidic lysosomes, was used to monitor autophagy in MO- and TNF- α -treated endothelial cells. No significant difference of fluorescence intensity was found between TNF- α treated and untreated cells. After treatment with MO, however, fluorescence intensity was dramatically increased relative to cells treated with TNF- α alone, which was suppressed by the presence of 3-methyladenine (3-MA, an autophagic inhibitor) (Fig. 4A and C). When TNF- α -stimulated cells were exposed to either 5 mM 3-MA or 3 μ M CQ, no significant difference of the expression of inflammatory factors (ICAM-1, MMP-9, and COX-2) was seen in comparison to the cells treated with TNF- α alone. MO and TNF- α cotreated group decreased the expression of inflammatory factors compared with TNF- α -treated group, which were inhibited by 3-MA and CQ (Fig. 4B). In

addition, when autophagy was inhibited by siRNA suppression of ATG5 and BECN1 (the essential autophagy genes), the effect of MO on inhibition of the inflammatory factors was notably attenuated (Fig. 4D). siRNA ATG5 and BECN1 suppression had no effects on the expression of inflammatory factors compared with TNF- α treated group. These results manifested that autophagy was required for the anti-inflammatory effects of MO in HUVECs.

3.6 MO induces autophagy through activating cAMP-PKA-AMPK-SIRT1 signaling pathway in HUVECs

As shown in Fig. 5A, TNF- α stimulation had no effect on cAMP level in HUVECs. However, pretreatment of the cells with MO with or without TNF- α remarkably increased cAMP level compared with control group. Furthermore, introduction of an ADCY inhibitor KH7 to TNF- α -treated cells inhibited cAMP production compared with TNF- α treated alone. MO presence showed to increase cAMP expression compared with KH7 and TNF- α cotreated group. In addition, introduction of PDE4 inhibitor Rolipram to TNF- α -treated cells increased cAMP level compared with the TNF- α treated alone, and MO presence further enhanced cAMP level compared with Rolipram and TNF- α cotreated group. Expressions of PKA, p-AMPK (phosphorylated AMPK) and SIRT1 levels in HUVECs were increased in response to MO single-treatment in a dose- and time-dependent manner. Similar results were seen in MO and TNF- α cotreated cells. However, TNF- α treatment alone had no significant effect on the expression of PKA, p-AMPK, and SIRT1 (Fig. 5B and C). These results indicated that MO stimulates cAMP-PKA-AMPK-SIRT1 signaling pathway by activation of AC and inhibition of PDE4 in both unstimulated and TNF- α -stimulated HUVECs. To gain further insight into the contribution of cAMP-PKA-AMPK-SIRT1 signaling pathway to autophagy induction and anti-inflammatory effects of MO in HUVECs, SIRT1 inhibitors, including nicotinamide (VPP) (5 mM) and EX527 (2 mM), were also included in the study. Autophagy induction by MO on TNF- α -treated HUVECs was suppressed significantly by the presence of VPP (Fig 5D and E). Meanwhile, pretreatment of the cells with VPP or EX527 inhibited MO-induced increase of MAP1LC3B2 expression and expression of ICAM-1, COX-2, and MMP-9 (Fig. 5F). These results suggested that the anti-inflammatory effects of MO were mainly attributed to induction of autophagy through stimulating cAMP signaling pathway.

4 Discussion

Atherosclerosis is a chronic inflammatory disease [28]. With the development of atherosclerosis, the accumulation of lipid particles and cells of the immune system in subendothelial regions gradually form lesions corresponds with an inflammation process [3]. And the persistent inflammatory stimulation induced by interferon- γ , TNF- α , matrix metalloproteinase or monocyte chemoattractant protein-1, eventually lead to degeneration of atherosclerosis, including narrowing of arterial lumen and thrombosis [7]. MO has previously been reported to improve inflammation induced by inflammatory stimuli in several cell lines (epithelial cells, neuronal cells, lung carcinoma cells, and human chondrocytes) [21]. In this

study, to investigate the protective effects of MO on AS, a well-established atherosclerotic animal model, high fat diet ApoE^{-/-} mice were used in vivo [29]. Previous study had shown that high fat diets could significantly increase TNF- α concentration in vivo [30], which is a risk factor in AS. Therefore, a TNF- α -induced inflammatory model of endothelial cells was used to investigate the molecular mechanism of anti-inflammation of MO in AS in vitro. In vivo, two doses (30 mg/kg, 100 mg/kg) of morin were used to observe the effectiveness and toxicity in mice. Results showed that either low dose or high doses of MO attenuated the plaque formation and lipid accumulation in AS mice without significant decrease of body weight. This indicated that the two doses of morin had no significant toxicity on mice. Through dosage conversion of mice to human [31], the high and low doses in human are approximately 7.71 mg/kg and 2.31 mg/kg, respectively, which were modest in comparison with statins and nonstatins anti-atherosclerosis agents in clinic [32]. We found that MO administration could significantly decrease serum levels of low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C), four important independent factors in cardiovascular disease. The decrease of relative en face lesion area of the aortas was shown in the MO-treated group, which was consistent with the decrease of serum lipid levels. Accumulating evidence reveals that lipid molecules influence the regulatory mechanisms of inflammatory reaction that might be mediated by peroxisome proliferator-activated receptors (PPARs) [33]. In the present study, we found that MO significantly suppressed the expression of inflammatory cytokines, TNF- α and ICAM-1, in the serum of MO-treated mice. These results suggested that MO had potential anti-inflammation and anti-hyperlipidemia effects in vivo. However, the regulatory mechanisms of its anti-hyperlipidemia effects need further researches.

In vitro inflammation model, MO was seen to regulate TNF- α -induced inflammation by inducing autophagy. Autophagy, a dynamic process of subcellular degradation, is involved in the process various diseases such as inflammation, cancer, infection, and heart diseases [34]. There are strong evidences showing that autophagy protects cells from excessive, long-lasting inflammation by suppressing the formation of proinflammatory complexes directly and by allowing efficient clearance of damaged organelles indirectly [35]. Autophagy acts as a negative regulator of inflammasome formation through integrating a wide range of signals of innate immune receptors sensing PAMPs or DAMPs (TLRs and NLRs), cytokines (IL-1, IL-2, IL-6, TNF- α , TGF- β , and IFN- γ) [36], and ROS. Impaired autophagy in advanced stages of AS, which causes activation of the inflammasome, shows to inhibit AS [37]. Our results show that MO presence enhances autophagy to protect endothelial cells from the action of TNF- α -induced inflammatory cytokines (ICAM-1, COX-2, and MMP-9). This discovery indicates the possibility that MO might have therapeutic value for the treatment of inflammation-related cardiovascular diseases. We found that MO presence increased the cAMP levels and the expression of cAMP-dependent protein kinase (PKA) in endothelial cells. cAMP/PKA signaling pathway is known to play a critical role in controlling autophagy [38–41] and the presence of dominant-negative form of AMPK, a downstream mediator of PKA signaling, could inhibit autophagy [42]. AMPK is activated by high cAMP

level via a mechanism involving allosteric regulation, promotion of phosphorylation by AMPK kinase (AMPKK). cAMP/PKA signaling is thought to be involved in stress responses and maintenance of energy homeostasis by attenuation of SIRT1 expression [43]. In this study, MO shows to increase the expression of p-AMPK and SIRT1 in dose- and time-dependent manner in endothelial cells and introduction of SIRT1 inhibitors SIRT1, nicotinamide, or EX527, reduced autophagy induction and anti-inflammatory effects of MO. Consistently, MO increases the concentration of cAMP and the expression of PKA, p-AMPK, and SIRT1 in aortas plaques of ApoE^{-/-} mice. These results suggested that cAMP signaling plays a crucial role in MO-induced autophagy and its anti-inflammatory effect (Supporting Information Fig. 2). In conclusion, MO reduces serum lipid levels (LDL, HDL, TC, and TG), inflammatory cytokines (TNF- α , ICAM-1), and inhibits atherosclerotic plaque development in ApoE^{-/-} mice. MO reduces the expression of inflammatory cytokines (ICAM-1, COX-2, and MMP-9) in TNF- α -stimulated HU-VECs. The anti-inflammatory effect of MO is at least partly dependent on autophagy induction and is involved in cAMP-PKA-AMPK-SIRT1 signal transduction in vivo and in vitro. Thus, the anti-inflammatory effect of MO in atherosclerosis associated with autophagy induction through activation of the cAMP-PKA-AMPK-SIRT1 signaling. Y.Z. performed the research. Y.Z., and X.L.G. designed the research study. Z.Q.C., H.Y.W., Y.N.C., X.K.Z., and Y.S. contributed essential reagents or tools. Y.Z. analyzed the data. Y.Z., X.L.G., and L.G.Y. wrote the paper. This work was funded by the Major Project of Science and Technology of Shandong Province (2015ZDJS04001) and Shandong Province Science and Technology Key Project (2014GSF118032). The authors declare no conflict of interest.

5 References

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