The anti-inflammatory effects of Morin hydratein atherosclerosis is associated with autophagyinduction through cAMP signalling

Yue Zhou[1], Zhan-Qi Cao[1], Hong-Yuan Wang[1], Yan-Na Cheng[1], Lu-Gang Yu[2], Xin-Ke Zhang[1],Yan Sun[3] and Xiu-Li Guo1[1]

[1]Department of Pharmacology, Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, Jinan, China
[2]Department of Gastroenterology, Institute of Translational Medicine, University of Liverpool, Liverpool, UK
[3]Department of Anesthesiology, Qilu Hospital of Shandong University, Jinan, China

Abstract

Although the previous trials of inflammation have indicated that morin hydrate (MO)hold considerable promise, understanding the distinct mechanism of MO against inflammationremains 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 veinendothelial cells (HUVECs). Administration of MO significantly reduced serum lipid level,inflammatory cytokines (TNF-␣ and ICAM-1), and atherosclerotic plaque formation in vivo.MO presence attenuated the expression of TNF-␣-induced inflammatory cytokines (ICAM-1,COX-2, and MMP-9), and remarkably enhanced microtubule associated protein 1 light chain3 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 andBECN1. MO increased intracellular cAMP levels and activated cAMP-PKA-AMPK-SIRT1 sig-naling in vivo and in vitro. These changes resulted in increased expression of autophagy-relatedprotein MAP1LC3B2 and decreased secretion of inflammatory cytokines (ICAM-1, COX-2, andMMP-9).Conclusion: Our results suggest that anti-AS and anti-inflammatory effects of MO are largelyassociated with its induction of autophagy through stimulation of cAMP-PKA-AMPK-SIRT1signaling pathway.

Keywords:

Atherosclerosis / Autophagy / Inflammation / cAMP-PKA-AMPK-SIRT1signaling pathway / Morin hydrate

1. Introduction

Atherosclerosis is the most common pathological processassociated with serious cardiovascular diseases [1], suchas stroke, limb ischemia, and myocardial infarction [2]. Accumulating evidence reveal that an inflammation process,in which lipid particles and immune cells are gradually accu-mulated in subendothelial regions resulting in narrowing ofthe arterial lumen and thrombosis, is associated with develop-ment of atherosclerosis [1]. This inflammation process leadsto endothelial secretion of inflammation cytokines and otheracute-phase reactants such as IL-1, IL-2, IL-6, IL-8, IL-12, TNF-␣, interferon-□, and

platelet-derived growth factor [3, 4]. Andthese inflammation cytokines drive endothelial dysfunction,subclinical lesions, the late plaque formation, and disruptionof atherosclerotic plaque. Currently, agents directly acting onthe inflammatory cascade in atherosclerosis (AS) are now in-vestigated widely, raising hope for advances in the treatmentof atherosclerosis.

Various studies have shown that flavonoids such as morinhydrate (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 fromin a number of fruits, vegetables, and herbs of the Moraceaefamily [8]. MO has been reported to exert a variety of beneficialpharmacological effects including protection of cardiovascu-lar cells and hepatocytes against oxidative injury [5,6], inhibi-tion of xanthine oxidase activity [9], and apoptosis inductionhuman colon cancer cells (HCT-116) [10]. MO has also shownto improve inflammation in human epithelial cells by down-regulating nuclear factor-□B, mitogen-activated protein ki-nase signaling pathways, sphingosine kinase 1/sphingosine1-phosphate signaling pathway, etc. [11,12]. Recently, severalstudies have shown that autophagy acted as a negative reg-ulator of inflammation in macrophages [13, 14]. Autophagyis a degradation process that delivers cytoplasmic materialsto lysosomes via double-membraned organelles termed au-tophagosomes (an enclosed portion of the cytoplasm) [15].Autophagy, occurring in all cells to maintain mammalianhomeostasis [16], has been shown to protect against neu-rodegeneration, inflammation in cardiovascular disease andcancer [17]. Moreover, autophagy has been reported to con-tribute to the removal of misfolded proteins in endothelialcells and prevent the risk of atherosclerosis. The presenceof MO has recently been shown to increase the level of in-tracellular cAMP [18], one of the most important secondarymessengers and activates PKA in mammalian cells [19, 20].Activation of cAMP/PKA signaling pathway in endothelialcell is involved in induction of autophagy [21]. AMP-activatedprotein kinase (AMPK), a downstream kinase of PKA sig-naling pathway and an intracellular energy sensor kinase[22], has also been reported to regulate autophagy undernormal and stress conditions [23]. AMPK can initiate au-tophagy to exert neuroprotection against focal cerebral is-chemia and hence diminish ischemia related cell death [24].AMPK was reported to be required for activation of silent in-formation regulator of transcription 1 (SIRT1) [25] that couldattenuate endothelial inflammatory reactions by inducing au-tophagy through deacetylation of several autophagy-relatedgenes [26]. The aim of this study was to evaluate the anti-inflammatory effects and underlying mechanism of MO invitro in human umbilical vein endothelial cells and in vivo inmice.

2. Materials and methods

2.1 Animals

All animal care and experimental procedures complied withInstitutional Guidelines of Animal Care and Use Committeeat Shandong University (Shandong, China). This project wasconducted in the School of Pharmacy, Shandong Universityas per the approval granted by the Animal Ethics Committeeof Shandong University (NO. ECAESDUSM 201) A total of18 male 6-week old apolipoprotein E (ApoE)−/−mice and 18male 6-week old C57 mice were purchased from Peking Uni-versity Resources Centre (Permission number: SCXK 2011-0012).

The animals were maintained in constant temperature-controlled rooms (25 ± 2□C) with controlled lighting (12 hlight-dark cycle). C57 mice were received normal diet, andApoE−/−mice were received a high fat diet (0.25% choles-terol and 15% cocoa butter) for 16 weeks.

2.2 General procedures

Both the ApoE−/−mice and C57 mice were randomly di-vided into three groups (n = 6/group): vehicle (2% tween80) group, 30 mg/kg/d and 100 mg/kg/d MO groups. Malemice were treated with MO every day via gavage at the doseof 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 anesthetizedby 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-densitylipoprotein-cholesterol (HDL-C) were measured by biochem-istry automatic analyzer (Roche Cobas Integra 800, Basel,Switzerland). The concentrations of serum inflammatory cy-tokines (TNF-␣ and ICAM-1) were measured by ELISA kitsaccording to the manufacturer's instructions. Then, all micewere rapidly perfused through the left ventricle with PBS,followed by 4% paraformaldehyde. For en face analysis, theaortas from the ascending arch to the iliac bifurcation wereprepared and cleaned of peripheral tissue, opened longitudi-nally, pinned flat, stained with Oil-Red O and washed by 75%alcohol for examination of pathological changes and lipid de-position. Then the areas of the aorta and lesions were quan-tized by the software Image pro plus. The ratio of lesion area(R) was calculated as R = the areas of lesion aorta/the areas ofall 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 ␤-mercaptoethanol, 1 mM PMSF, and 1 g/mL apro-tinin) 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) wereisolated from umbilical cord veins (Qilu Hospital, Shan-dong University). HUVECs were maintained in endothelialcell medium (ECM, ScienCell) supplemented with 5% heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin(P/S), and endothelial cell growth supplement (ECGS) at 37□Cin a humid atmosphere (5% CO2–95% air) and were harvestedby brief incubation in 0.25% w/v EDTA-PBS. All experimentswere conducted with cells within 3–6 passages.

2.3.2 Cytotoxic assay

HUVECs (1 × 104per well) were seeded in 96-well plates andcultured till 80–90% confluence before introduction of vari-ous concentrations of TNF-␣ (Sigma-Aldrich) (5, 10, 20, 50,100, 200, and 400 ng/mL) for 4 h. Next, the cells were treatedwith various MO (Sigma-Aldrich) concentrations (1, 5, 10,20, 50, 100, 200, and 400 □M) for 24 h. The control groupwas treated with 0.1% DMSO. After removal of the culturemedium, the cells were washed with PBS. Ten percent ofCCK-8 solution (Dojindo, Japan) was then added to the

platesfor2hat37□C. Light absorbance was measured at 450 nm onThermo Multiskan GO microplate reader (Thermo, USA).Triplicate experiments with quintuplicate samples wereperformed.

2.3.3 ELISA assayHUVECs (1 × 104per well) were seeded in 96-well plates andcultured till 80–90% confluence before exposure to TNF-□ (5,10, 20, and 50 ng/mL) for 4 h. Some groups were pretreatedwith different concentrations of MO (1, 10, and 20 □M) for 4 hbefore exposure to 10 ng/mL TNF-□ for an additional 4 h. Thelevel 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 orwithout 10 □M MO for 4 h, followed by additional 4 h in-cubation with or without 10 ng/mL TNF-□. Cells were thentreated with 10 □M KH7 (Cayman) (an inhibitor of ADCY)for 1 h or 10 □M Rolipram (Selleckchem) (an inhibitor ofPDE4) for 1 h. Intracellular levels of cAMP in HUVECs andatherosclerotic plaques were measured also by an ELISA kit(R&D systems) according to the manufacturer's instructions.

2.3.4 Western blot

HUVECs (5 × 105per well) were seeded in 6-well platesand exposed to increasing concentrations of TNF-□ (5, 10, 20, and 50 ng/mL) or MO (1, 10, and 20 □M) for 4 hand then treated with 10 ng/mL TNF-□ for 4 h. The con-trol group was treated with 0.1% DMSO. The cells werethen treated with 3 □M CQ (Sigma-Aldrich), 5 mM 3-MA(Sigma-Aldrich), 5 mM Nicotinamide (Beyotime Biotechnol-ogy,China),2□M EX527 (Sigma-Aldrich), 10 □MKH7,or10 □M Rolipram for 2 h. The cells were then treated withor without TNF-□ (10 ng/mL) for another 4 h. Target pro-teins were detected using 1:1000-diluted primary antibodiesagainst ICAM-1 (ZhongshanJinqiao Biotechnology, China),COX-2 (Cell Signaling Technology), MMP-9 (Cell Signal-ing Technology), MAP1LC3B (microtubule associated pro-tein 1 light chain 3 beta 2) (Sigma-Aldrich), SQSTM1/p62 (se-questosome 1) (Cell Signaling Technology), BECN1 (Cell Sig-naling Technology), ATG5 (Epitomics, USA), PKA (WuhanHuamei biotech, China), p-AMPK (Wuhan Huamei biotech,China), AMPK (Wuhan Huamei biotech, China), and SIRT1(Cell Signaling Technology). Immunoblots were developedusing horseradish peroxidase-conjugated secondary antibod-ies, and visualized using an enhanced chemiluminescencereagent (Amersham Pharmacia Biotech) and quantified bydensitometry using a ChemiDoc XRS (Bio-Rad, Berkeley, Cal-ifornia, USA). The data are expressed as the relative densityof the protein normalized to □-actin. Triplicate experimentswith triplicate samples were performed.

2.3.5 Laser scanning confocal microscopy analysisHUVECs (5 × 104per well) were seeded in 12-well plates andthen exposed to the various treatments with TNF-□ or MO.The cells were washed twice with fresh medium and loadedwith 100 nM LTG (Invitrogen) for 1 h in humidified air at37□C in ECM culture medium. After three washes with PBS,the cells were examined using a Radiance 2000 laser scanningconfocal microscope (Nikon, Japan).

2.3.6 siRNA transfection

HUVECs were transfected with 100 nM siRNAs for ATG5or BECN1 (GenePharma, China) for 8 h according to themanufacturer's protocol. Cell culture was then switched toECM medium for an additional 24 h before treatment ofthe cells with MO (10 ?M) for 4 h followed by exposure to10 ng/mL of TNF-␣ for another 4 h. The cells were harvestedand analyzed by Western blot.

2.3.7 Statistical analyses

The data and statistical analysis comply with the recommen-dations on experimental design and analysis in pharmacol-ogy. Data was expressed as mean ± SEM for three differentdeterminations. For cell culture experiments, all results were repeated independently at least three times. Relative protein semi-quantification was performed using AlphaEaseFC soft-ware (AlphaInnotech, USA). Statistical significance was ana-lyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests. $p < 0.05$ was considered asstatistically significant. SPSS 13.0 for Windows (SPSS, Inc.,Chicago, IL).

3 Results

3.1 MO decreases serum lipid level and attenuatesatherosclerotic plaque formation in ApoE−/−mice with high fat dietA

fter treatment with MO for 10 weeks, no differences of bodyweight of the ApoE−/−mice occurred between the treatmentand vehicle mice. Elevation of TG, TC, and LDL levels wasobserved in mice with high fat diet. Serum lipid levels ofMO-treated groups shown low levels of LDL, TC, TG, andHDL compared with the vehicle group (Table 1). And therewere no differences of body weight and plasma lipids in C57mice treated with MO for 10 weeks. The relative en facelesion area of the aorta was decreased in both MO-treatedgroups compared with the vehicle group, and treatment with100 mg/kg MO showed larger reduction than treatment with30 mg/kg MO (Fig. 1A and B). Moreover, there were nosignificant changes of body weight and plasma lipids in C57mice treated with MO for 10 weeks in comparison with vehiclegroup (Supporting Information Table 1).

3.2 MO suppresses the production of inflammatorycytokines and increases the protein expressionof cAMP-PKA-AMPK-SIRT1 pathway inMO-treated ApoE−/−mice

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

3.3 MO suppresses production of inflammatorycytokines in TNF-␣ stimulated HUVECs

To determine the noncytotoxic concentrations of TNF-␣ andMO to be used in experiment, cell viability was determinedafter treatment with different concentrations of TNF-␣ andMO. Results showed that TNF-␣ at concentrations of upto 20 ng/mL was tolerated by HUVECs without significantchange of cell viability (Fig. 2A). When the cells were ex-posed to increasing concentration of TNF-␣ (0, 5, 10, 20,50 ng/mL), cell secretion of inflammatory cytokines includ-ing ICAM-1, MMP-9, and COX-2 significantly increased ina concentration-dependent manner (Fig. 2C). Thus, TNF-␣(10 ng/mL) was used to induce inflammation on HUVECswith no effect on cell viability. Similarly, MO had no effector lower inhibition on cell viability at lower concentrations(less than 20 □M) compared with 0.1% DMSO group (controlgroup) (Fig. 2B, Supporting Information Fig. 1). Preincuba-tion of the cells with MO (1, 10, and 20 □M) for 4 h before ex-posure to TNF-␣ (10 ng/mL) for 4 h significantly suppressedthe expressions of ICAM-1, MMP-9, and COX-2, as well assoluble ICAM-1 level, in a MO dose-dependent manner incomparison to TNF-␣-treated cells (Fig. 2D–F).

3.4 MO induces autophagy in both normal HUVECsand TNF-␣ stimulated HUVECs

The increase of MAP1LC3B2 expression and the decreaseof SQSTM1/P62 expression were regarded as autophagicbiomarkers in HUVECs [27]. Chloroquine (CQ), which in-hibits autophagosome-lysosome fusion, was used to monitorautophagic fluctuation through measuring the protein ex-pression of MAP1LC3B2. Results showed that MO (1, 10,and 20 □M) increased the expression of MAP1LC3B2 anddecreased the expression of SQSTM1/p62 compared withcontrol group in dose- and time-dependent manners. Thepresence of CQ showed to induce further accumulation ofMAP1LC3B2 in HUVECs treated with MO (10 □M) for 4 hcompared with cells treated with CQ alone (Fig. 3A and C).These results revealed that MO promoted cellular autophagyin HUVECs, which was suppressed by CQ.

We found that TNF-␣ treatment had no effect on theexpression of MAP1LC3B and SQSTM/p62 compared withcontrol group. Treatment of the cells with MO increasedthe expression of MAP1LC3B2 and decreased the expres-sion of SQSTM1/p62 in dose- and time-dependent manners.In the presence of CQ, the autophagy induction in TNF-␣-stimulated HUVECs of MO was also inhibited (Fig. 3B andD). These results suggested that MO induced autophagy inboth normal HUVECs and TNF-␣-stimulated HUVECs.

3.5 The anti-inflammatory effects of MO aredependent on autophagy

LysoTracker Green fluorescent dye (LTG), which stains acidiclysosomes, was used to monitor autophagy in MO- and TNF-␣-treated endothelial cells. No significant difference of fluo-rescence intensity was found between TNF-␣ treated and un-treated cells. After treatment with MO, however, fluorescenceintensity was dramatically increased relative to cells treatedwith TNF-␣ alone, which was suppressed by the presence of 3-methyladenine (3-MA, an autophagic inhibitor) (Fig. 4Aand C).When TNF-␣-stimulated cells were exposed to either5mM3-MAor3□M CQ, no significant difference of theexpression of inflammatory factors (ICAM-1, MMP-9, andCOX-2) was seen in comparison to the cells treated withTNF-␣ alone. MO and TNF-␣ cotreated group decreased theexpression of inflammatory factors compared with TNF-␣-treated group, which were inhibited by 3-MA and CQ (Fig.4B). In

addition, when autophagy was inhibited by siRNAsuppression of ATG5 and BECN1 (the essential autophagygenes), the effect of MO on inhibition of the inflamma-tory factors was notably attenuated (Fig. 4D). siRNA ATG5and BECN1 suppression had no effects on the expression ofinflammatory factors compared with TNF-␣ treated group.These results manifested that autophagy was required for theanti-inflammatory effects of MO in HUVECs.

3.6 MO induces autophagy through activatingcAMP-PKA-AMPK-SIRT1 signaling pathway inHUVECs

As shown in Fig. 5A, TNF-␣ stimulation had no effecton cAMP level in HUVECs. However, pretreatment of thecells with MO with or without TNF-␣ remarkably increasedcAMP level compared with control group. Furthermore, in-troduction of an ADCY inhibitor KH7 to TNF-␣-treated cells inhibited cAMP production compared with TNF-␣ treatedalone. MO presence showed to increase cAMP expressioncompared with KH7 and TNF-␣ cotreated group. In addition,introduction of PDE4 inhibitor Rolipram to TNF-␣-treatedcells increased cAMP level compared with the TNF-␣ treatedalone, and MO presence further enhanced cAMP level com-pared with Rolipram and TNF-␣ cotreated group. Expressionsof PKA, p-AMPK (phosphorylated AMPK) and SIRT1 levels inHUVECs were increased in response to MO single-treatmentin a dose- and time-dependent manner. Similar results wereseen in MO and TNF-␣ cotreated cells. However, TNF-␣ treat-ment alone had no significant effect on the expression ofPKA, p-AMPK, and SIRT1 (Fig. 5B and C). These resultsindicated that MO stimulates cAMP-PKA-AMPK-SIRT1 sig-naling pathway by activation of AC and inhibition of PDE4 inboth unstimulated and TNF-␣-stimulated HUVECs.To gain further insight into the contribution of cAMP-PKA-AMPK-SIRT1 signaling pathway to autophagy inductionand anti-inflammatory effects of MO in HUVECs, SIRT1 in-hibitors, including nicotinamide (VPP) (5 mM) and EX527(2 mM), were also included in the study. Autophagy in-ductionbyMOonTNF-␣-treated HUVECs was suppressedsignificantly by the presence of VPP (Fig 5D and E). Mean-while, pretreatment of the cells with VPP or EX527 inhib-ited MO-induced increase of MAP1LC3B2 expression andexpression of ICAM-1, COX-2, and MMP-9 (Fig. 5F). Theseresults suggested that the anti-inflammatory effects of MOwere mainly attributed to induction of autophagy throughstimulating cAMP signaling pathway.

4 Discussion

Atherosclerosis is a chronic inflammatory disease [28]. Withthe development of atherosclerosis, the accumulation of lipidparticles and cells of the immune system in subendothelialregions gradually form lesions corresponds with an inflam-mation process [3]. And the persistent inflammatory stim-ulation induced by interferon-?,TNF-␣, matrix metallopro-teinase or monocyte chemotactic protein-1, eventually leadto degeneration of atherosclerosis, including narrowing ofarterial lumen and thrombosis [7]. MO has previously beenreported to improve inflammation induced by inflammatorystimuli in several cell lines (epithelial cells, neuronal cells,lung carcinoma cells, and human chondrocytes) [21].In this

study, to investigate the protective effects of MOon AS, a well-established atherosclerotic animal model, highfatdietApoE−/−mice were used in vivo [29]. Previous studyhad shown that high fat diets could significantly increaseTNF-␣ concentration in vivo [30], which is a risk factor in AS. Therefore, a TNF-␣-induced inflammatory model ofendothelial cells was used to investigate the molecular mech-anism of anti-inflammation of MO in AS in vitro.In vivo, two doses (30 mg/kg, 100 mg/kg) of morin wereused to observe the effectiveness and toxicity in mice. Re-sults showed that either low dose or high doses of MOattenuated the plaque formation and lipid accumulation in ASmice without significant decrease of body weight. This indi-cated that the two doses of morin had no significant toxicity onmice. Through dosage conversion of mice to human [31], the high and low doses in human are approximately 7.71 mg/kgand 2.31 mg/kg, respectively, which were modest in compar-ison with statins and nonstatins anti-atherosclerosis agentsin clinic [32]. We found that MO administration could sig-nificantly decrease serum levels of low-density lipoproteincholesterol (LDL-C), total cholesterol (TC), triglycerides (TG)and high-density lipoprotein cholesterol (HDL-C), four im-portant independent factors in cardiovascular disease. Thedecrease of relative en face lesion area of the aortas was shownin the MO-treated group, which was consistent with the de-crease of serum lipid levels. Accumulating evidence revealsthat lipid molecules influence the regulatory mechanisms ofinflammatory reaction that might be mediated by peroxisomeproliferator-activated receptors (PPARs) [33]. In the presentstudy, we found that MO significantly suppressed the ex-pression of inflammatory cytokines, TNF-␣ and ICAM-1, inthe serum of MO-treated mice. These results suggested that MO had potential anti-inflammation and anti-hyperlipidemiaeffects in vivo. However, the regulatory mechanisms of itsanti-hyperlipidemia effects need further researches.

In vitro inflammation model, MO was seen to regulateTNF-␣-induced inflammation by inducing autophagy. Au-tophagy, a dynamic process of subcellular degradation, is in-volved in the process various diseases such as inflammation,cancer, infection, and heart diseases [34]. There are strongevidences showing that autophagy protects cells from exces-sive, long-lasting inflammation by suppressing the forma-tion of proinflammatory complexes directly and by allowingefficient clearance of damaged organells indirectly [35]. Au-tophagy acts as a negative regulator of inflammasome forma-tion through integrating a wide range of signals of innate im-mune receptors sensing PAMPs or DAMPs (TLRs and NLRs),cytokines (IL-1, IL-2, IL-6, TNF-␣,TGF-␤,andIFN-□) [36], andROS. Impaired autophagy in advanced stages of AS, which cases activation of the inflammasome, shows to inhibit AS[37]. Our results show that MO presence enhances autophagyto protect endothelial cells from the action of TNF-␣-inducedinflammatory cytokines (ICAM-1, COX-2, and MMP-9). Thisdiscovery indicates the possibility that MO might have ther-apeutic value for the treatment of inflammation-related car-diovascular diseases.We found that MO presence increased the cAMP levelsand the expression of cAMP-dependent protein kinase (PKA)in endothelial cells. cAMP/PKA signaling pathway is knownto play a critical role in controlling autophagy [38–41] andthe presence of dominant-negative form of AMPK, a down-stream mediator of PKA signaling, could inhibit autophagy[42]. AMPK is activated by high cAMP

level via a mecha-nism involving allosteric regulation, promotion of phospho-rylation by AMPK kinase (AMPKK). cAMP/PKA singaling isthought to be involved in stress responses and maintenanceof energy homeostasis by attenuation of SIRT1 expression[43]. In this study, MO shows to increase the expression ofp-AMPK and SIRT1 in dose- and time-dependent manner inendothelial cells and introduction of SIRT1 inhibitors SIRT1,nicotinamide, or EX527, reduced autophagy induction andanti-inflammatory effects of MO. Consistently, MO increasesthe concentration of cAMP and the expression of PKA, p-AMPK, and SIRT1 in aortas plaques of ApoE−/−mice. Theseresults suggested that cAMP signaling plays a crucial rolein 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-␣,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-␣-stimulated HU-VECs. The anti-inflammatory effect of MO is at least partlydependent 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 atherosclerosisis associated with autophagy induction through activation ofthe cAMP-PKA-AMPK-SIRT1 signaling.Y.Z. performed the research. Y.Z., and X.L.G. designed theresearch study. Z.Q.C., H.Y.W., Y.N.C., X.K.Z., and Y.S. con-tributed 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 Tech-nology of Shandong Province (2015ZDJS04001) and ShandongProvince Science and Technology Key Project (2014GSF118032).The authors declare no conflict of interest.

5 References

[1] Galkina, E., Ley, K., Immune and inflammatory mechanismsof atherosclerosis (*). Annu. Rev. Immunol. 2009, 27, 165–197.

2] Brichkina, A., Bulavin, D. V., WIP-ing out atherosclerosis withautophagy.Autophagy 2012, 8, 1545–1547.

[3] Hansson, G. K., Inflammation, atherosclerosis, and coronaryartery disease.N. Engl. J. Med. 2005, 352, 1685–1695.

[4] Kofler, S., Nickel, T., Weis, M., Role of cytokines in cardiovas-cular diseases: a focus on endothelial responses to inflam-mation.Clin. Sci. 2005, 108, 205–213.

[5] Tangney, C. C., Rasmussen, H. E., Polyphenols, inflamma-tion, and cardiovascular disease.Curr. Atheroscler. Rep.2013,15, 324.

[6] Visioli, F., Davalos, A., Polyphenols and cardiovascular dis-ease: a critical summary of the evidence.Mini Rev. Med.Chem. 2011, 11, 1186–1190.

[7] Perez-Vizcaino, F., Duarte, J., Andriantsitohaina, R., Endothe-lial function and cardiovascular disease: effects of quercetinand wine polyphenols.Free Radic. Res. 2006, 40, 1054–1065.

[8] Rizvi, F., Mathur, A., Krishna, S., Siddiqi, M. I. et al., Suppres-sion in PHLPP2 induction by morin promotes Nrf2-regulatedcellular defenses against oxidative injury to primary rat hep-atocytes.Redox Biol. 2015, 6, 587–598.

[9] Yu, Z., Fong, W. P., Cheng, C. H., The dual actionsof morin (3, 5, 7, 2',4'-pentahydroxyflavone) as a hy-pouricemic agent: uricosuric effect and xanthine oxidase in-hibitory activity.J. Pharmacol. Exp. Ther. 2006, 316, 169–175.

[10] Hyun, H. B., Lee, W., Go, S. I., Nagappan, A. et al., Theflavonoid morin fromMoraceae induces apoptosis by mod-ulation of Bcl-2 family members and Fas receptor in HCT 116cells.Int. J. Oncol. 2015, 46, 2670–2678.

[11] Manna, S. K., Aggarwal, R. S., Sethi, G., Aggarwal, B. B.et al., Morin (3,5,7,2',4'-Pentahydroxyflavone) abolishes nu-clear factor-kappaB activation induced by various carcino-gens and inflammatory stimuli, leading to suppression ofnuclear factor-kappaB-regulated gene expression and up-regulation of apoptosis.Clin. Cancer Res. 2007, 13, 2290–2297.

[12] Sivaramakrishnan, V., Devaraj, S. N., Morin fosters apop-tosis in experimental hepatocellular carcinogenesis model.Chem. Biol. Interact. 2010, 183, 284–292.

[13] Wang, X., Zhang, D. M., Gu, T. T., Ding, X. Q. et al., Morin re-duces hepatic inflammation-associated lipid accumulationin high fructose-fed rats via inhibiting sphingosine kinase1/sphingosine 1-phosphate signaling pathway.Biochem.Pharmacol. 2013, 86, 1791–1804.

[14] Saitoh, T., Fujita, N., Jang, M. H., Uematsu, S. Y. et al.,Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production.Nature 2008, 456, 264–268.

[15] Nakahira, K., Haspel, J. A., Rathinam, V. A., Lee, S. J. et al.,Autophagy proteins regulate innate immune responses byinhibiting the release of mitochondrial DNA mediated bythe NALP3 inflammasome.Nat. Immunol. 2011, 12, 222–230.

[16] Mizushima, N., Ohsumi, Y., Yoshimori, T., Autophagosomeformation in mammalian cells.Cell Struct. Funct. 2002, 27,421–429.

17] White, E., Karp, C., Strohecker, A. M., Guo, Y. et al., Role ofautophagy in suppression of inflammation and cancer.Curr.Opin. Cell Biol. 2010, 22, 212–217.

[18] Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K. et al., Au-tophagy promotes tumor cell survival and restricts necrosis,inflammation, and tumorigenesis.Cancer Cell 2006, 1, 51–64.

[19] Summanen, J., Vuorela, P., Rauha, J. P., Tammela, P. et al.,Effects of simple aromatic compounds and flavonoids onCa2+ fluxes in rat pituitary GH(4)C(1) cells.Eur. J. Pharmacol.2001,414, 125–133.

[20] Canalli, A. A., Franco-Penteado, C. F., Traina, F., Saad, S. T.et al., Role for cAMP-protein kinase A signalling in aug-mented neutrophil adhesion and chemotaxis in sickle celldisease.Eur. J. Haematol. 2007, 79, 330–337.

[21] Liang, Z., Liu, F., Grundke-Iqbal, I., Iqbal, K. et al., Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain.J. Neurochem.2007,103, 2462–2470.

[22] Chen, W. P., Wang, Y. L., Tang, J. L., Hu, P. F. et al.,Morin inhibits interleukin-1beta-induced nitric oxide andprostaglandin E2 production in human chondrocytes.Int.Immunopharmacol. 2012, 12, 447–452.

[23] Viollet, B., Guigas, B., Sanz Garcia, N., Leclerc, J. et al., Cellu-lar and molecular mechanisms of metformin: an overview.Clin. Sci. (Lond). 2012, 122, 253–270.

[24] Meijer, A. J., Codogno, P., AMP-activated protein kinase andautophagy.Autophagy. 2007, 3, 238–240.

[25] Jiang, T., Yu, J. T., Zhu, X. C., Wang, H. F. et al., Acute met-formin preconditioning confers neuroprotection against fo-cal cerebral ischaemia by pre-activation of AMPK-dependentautophagy.Br. J. Pharmacol. 2014, 171, 3146–3157.

[26] Chen, M. L., Yi, L., Jin, X., Liang, X. Y. et al., Resveratrolattenuates vascular endothelial inflammation by inducingautophagy through the cAMP signaling pathwa.Autophagy.2013,9, 2033–2045.

[27] Lee, I. H., Cao, L., Mostoslavsky, R., Lombard, D. B. et al.,A role for the NAD-dependent deacetylase Sirt1 in the reg-ulation of autophagy.Proc. Natl. Acad. Sci. USA 2008, 105,3374–3379.

[28] Mizushima, N., Yoshimori, T., Levine, B., Methods in mam-malian autophagy research.Cell 2010, 140, 313–326.

[29] Xiong, W., Wang, X., Dai, D., Zhang, B. et al., The anti-inflammatory vasostatin-2 attenuates atherosclerosis inApoE-/-mice and inhibits monocyte/macrophage recruit-ment.Thromb. Haemost. 2017.

[30] Nameni, G., Hajiluian, G., Shahabi, P., Farhangi, M. A. et al.,The impact of vitamin D supplementation on neurodegen-eration, TNF-␣ concentration in hypothalamus, and CSF-to-plasma ratio of insulin in high-fat-diet-induced obese rats[J].J. Mol. Neurosci. 2016, 1–9.

[31] Shin, J. W., Seol, I. C., Interpretation of animal dose andhuman equivalent dose for drug development.J. KoreanOrient.Med.2010, 31,1–7.

[32] Lloyd-Jones, D. M., Morris, P. B., Ballantyne, C. M., Birtcher,K. K. et al., 2016 ACC expert consensus decision path-way on the role of non-statin therapies for LDL-cholesterollowering in the management of atherosclerotic cardio-vascular disease risk[J].J. Am. Coll. Cardiol. 2016, 68,92.

[33] Pendse, A. A., Arbones-Mainar, J. M., Johnson, L. A., Al-tenburg, M. K. et al., Apolipoprotein E knock-out and knock-in mice: atherosclerosis, metabolic syndrome, and beyond.J. Lipid Res. 2009, 50, 178–182.

[34] Varga, T., Czimmerer, Z., Nagy, L., PPARs are a unique setof fatty acid regulated transcription factors controlling bothlipid metabolism and inflammation.Biochim. Biophys. Acta.2011,1812, 1007–1022.

[35] Mizushima, N., Levine, B., Cuervo, A. M., Klionsky, D. J., Au-tophagy fights disease through cellular self-digestion.Na-ture2008, 451, 1069–1075

[36] Lapaquette, P., Guzzo, J., Bretillon, L., Bringer, M. A., Cellularand molecular connections between autophagy and inflam-mation.Mediators Inflamm. 2015, 2015, 398483.

[37] Harris, J., Autophagy and cytokines.Cytokine 2011, 56, 140–144.

[38] Schrijvers, D. M., De Meyer, G. R., Martinet, W., Autophagyin atherosclerosis: a potential drug target for plaque sta-bilization.Arterioscler. Thromb. Vasc. Biol. 2011, 31, 2787–2791.

[39] Liu, Y., Su, Y., Wang, J., Sun, S. et al., Rapamycin de-creases tau phosphorylation at Ser214 through regulationof cAMP-dependent kinase.Neurochem. Int. 2013, 62, 458–467.

[40] Stephan, J. S., Yeh, Y. Y., Ramachandran, V., Deminoff, S.J., The Tor and cAMP-dependent protein kinase signalingpathways coordinately control autophagy in Saccharomycescerevisiae.Autophagy. 2010, 6, 294–295.

[41] Kimura, K., Kodama, A., Hayasaka, Y., Ohta, T., Acti-vation of the cAMP/PKA signaling pathway is requiredfor post-ecdysial cell death in wing epidermal cells ofDrosophila melanogaster.Development 2004, 131, 1597–1606.

[42] Meley, D., Bauvy, C., Houben-Weerts, J. H., Dubbelhuis, P.F. et al., AMP-activated protein kinase and the regulationof autophagic proteolysis.J. Biol. Chem. 2006, 281, 34870–34879.

[43] Gerhart-Hines, Z., Dominy, J. E., Jr., Bl¨attler, S. M.,Jedrychowski, M. P. et al., The cAMP/PKA pathway rapidlyactivates SIRT1 to promote fatty acid oxidation indepen-dently of changes in NAD (+).Mol. Cell. 2011, 44, 851–863