Aspirin suppresses TNF-α-induced MMP-9 expression via NF-κB and MAPK signaling pathways in RAW264.7 cells

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Abstract. Numerous studies have indicated that the expression of matrix metalloproteinase-9 (MMP-9) contributes to the atherosclerotic plaque hemorrhage and rupture. Aspirin, a non-steroidal anti-inflammation drug, has been known for its anti-platelet effect in the prevention of the vascular complications of atherosclerosis. The present study aimed to investigate the pharmacological effects of aspirin on tumor necrosis factor-α (TNF-α)-induced MMP-9 expression and the underlying molecular mechanisms in murine macrophage RAW264.7 cells. Western blot analysis indicated that the protein level of MMP-9 was reduced by aspirin in a dose-dependent manner. In addition, downregulation of MMP-9 mRNA and activity were detected in aspirin-treated cells using quantitative polymerase chain reaction and a gelatin zymography assay separately. It was also observed that aspirin has a suppressive effect on the activation of nuclear factor (NF)-κB and inhibits the phosphorylation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases 1/2, p38 and c-Jun N-terminal kinase. Furthermore, subsequent to inhibition of the MAPK pathway by specific inhibitors (PD98059, SB203580 and SP600125), the expression of MMP-9 was reduced, indicating that the inhibitory effect of aspirin on MMP-9 in TNF-α-treated RAW264.7 cells may be, at least in part, through suppression of NF-κB activation and the MAPK pathway. These findings support the notion that aspirin has therapeutic potential application in the prevention and treatment of atherosclerosis disease.

Introduction
Matrix metalloproteinases (MMPs) are a large family of zinc-dependent enzymes, which are able to collectively degrade collagen and the majority of the extracellular matrix (ECM) components (1). ECM provides structural support to the heart, and ECM quantity and quality are the major determinants of myocardial passive stiffness (2). Recent evidence demonstrated that excessive breakdown of the ECM by MMPs serves a pathogenic role in the atherosclerotic plaque, which is the major cause of mortality as a result of acute coronary syndrome and stroke (3). Therefore, MMPs are critical for vascular remodeling by regulating the degradation of the ECM in atherosclerotic plaques.

Atherosclerosis is a chronic inflammatory disease that is the basis of atherothrombosis and the development of acute coronary syndrome (4-6). Increasing evidence indicates that the concentrations of MMPs are elevated not only in certain patients with cancer, liver cirrhosis or rheumatoid arthritis (7,8), but is also involved in the pathogenesis and progression of atherosclerotic plaque formation and rupture (9). Among the MMP family, MMP-9 (also known as gelatinase B) is the predominant MMP and can degrade type IV collagen, which is the major component of the basement membrane (10). In addition, MMP-9 is indispensable for collagen-cleaving and degrading of the extracellular plaque matrix, leading to plaque instability and rupture (11). MMP-9 can be secreted by smooth muscle cells (SMCs) and macrophages (12). Furthermore, the macrophage-rich region of atherosclerotic plaque is the major source of MMP-9 (13). The secretion of specific proinflammatory cytokines can activate pro-MMP-9, therefore inducing the degradation of the ECM, which leads to the migration and proliferation of SMCs (14). In addition, activated endothelial cells express adhesion molecules that can promote the infiltration of monocytes and their adhesion to the endothelial cells, which enhances the MMP-9 production, consequently enhancing endothelial cell permeability and acceleration of plaque progression. A previous study has demonstrated that

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MMP-9 was highly expressed in humans with atherosclerotic lesions and animal models (15), and participated in mediating plaque instability, which is a major cause of acute coronary syndrome and stroke (12). MMP-9 appears to serve a central role in the loss of atherosclerotic plaque stability. Therefore, it is crucial to clarify the regulatory mechanism of MMP-9 in atherosclerosis and to determine whether inhibiting the MMP-9 activity may be an effective therapeutic strategy for the treatment of atherosclerosis.

The expression of MMP-9 appears to be regulated by a range of different signaling pathways. Several studies have demonstrated that mitogen-activated protein kinases (MAPKs) are involved in the regulation of MMPs by various cell types (16,17). The MAPK pathway is one of the important mediators of signal transduction, which participates in multiple fundamental cellular processes, including cell growth, proliferation, differentiation and death (18). However, tumor necrosis factor-α (TNF-α) can activate three MAPK cascades, including the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinase (JNK)/stress-activated protein kinases and p38 (19,20). The ERK pathway has been linked to cell proliferation, cell growth and differentiation, whereas JNK and p38 MAPK pathways have been linked to apoptosis, cell survival, transformation, development, cell migration and immune activation (21). Holvoet et al also proved that increased expression of MMP-9 induced by TNF-α was reduced by the specific inhibitors of MAPK signaling pathway in human keratinocytes (22). Nuclear factor-xB (NF-xB) binds to the proximal promoter region of the MMP-9 gene and regulates MMP-9 transcription in response to distinct extracellular stimulation of TNF-α (23,24), which is one of the strongest physiological inducers of MMP-9 expression (25).

Aspirin, a conventional nonselective non-steroidal anti-inflammation drug, is widely used in the primary prevention against cardiac-cerebral vascular diseases, such as myocardial infarction and stroke, and 20-25% of patients with various vascular diseases who were treated with aspirin presented decreased development of vascular events (26). The anti-platelet function of aspirin is known to contribute to the therapy of atherosclerotic cardiovascular disease. However, the anti-inflammatory effect of aspirin in atherosclerosis is not widely reported. Previous studies (3-5) have demonstrated that atherosclerosis is a complex vascular inflammation disease. A clinical study has shown that patients receiving treatment with aspirin exhibited lower macrophage density of the carotid atherosclerotic plaque, suggesting that aspirin is involved in the suppression of the vascular inflammation process (27). Hua et al (28) also reported that aspirin prevented against atherosclerotic plaque rupture by inhibiting MMP-9 expression by upregulating peroxisome proliferator-activated receptor α/γ (PPARα/γ) expression in oxidized low-density lipoprotein-stimulated macrophages and by inducing TIMP metallopeptidase inhibitor 1 (TIMP1) and TIMP2 expression. However, whether aspirin inhibits the expression of MMP-9 via the MAPK and NF-xB signaling pathways in TNF-α-stimulated RAW264.7 cells remains unknown. Therefore, the present study investigated the effects and mechanisms of aspirin on MMP-9 expression in TNF-α-stimulated RAW264.7 cells.

Materials and methods

Materials. Antibodies against JNK (1:500 dilution, BS6448), p38 (1:500 dilution, BS3566), ERK (1:1000 dilution, AP0485), phospho-JNK (1:500 dilution, BS4763), phospho-p38 (1:500 dilution, BS4635) and phospho-ERK (1:1000 dilution, BS4759) were purchased from Bioworld Technology (Beijing, China). SB203580 (p38MAPK inhibitor, 5633S), SP600125 (JNK inhibitor, 8177S) and PD98059 (ERK1/2 inhibitor, 9900S) and PDTC (NF-xB inhibitor) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). An antibody against the p65 subunit of NF-xB was also purchased from Cell Signaling Technology, Inc. (1:500 dilution, 8242). An antibody against MMP-9 was purchased from EMD Millipore (Chemicon; Billerica, MA, USA, 1:500 dilution, AB19016). Recombinant murine TNF-α was purchased from Thermo Fisher Scientific, Inc. (Biosource; MA, USA), and aspirin was purchased from Langtze Biomedical Technology (Nanjing, China).

Cell cultures. Murine macrophage RAW264.7 cells, purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were cultured in plastic dishes containing Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO₂. For all experiments, cells were grown to 60-80% confluence in culture flasks. Then, the medium was replaced with fresh DMEM and cells were transferred into multiple flasks for further expansion. The control groups were treated with medium only. In order to study the expression of MMP-9, TNF-α (10 ng/ml) was added in the presence or absence of aspirin (75, 150, 300 and 600 µM) for 24 h. For the inhibitory study, PDTC, an inhibitor of NF-xB, can significantly inhibit NF-xB activity, and further reduce the production of inflammatory cytokines, alleviating the systemic inflammatory response (29). In order to determine the effect of PDTC on TNF-α-induced expression of MMP-9 in RAW264.7 cells, the cells were divided into six groups and incubated with either TNF-α or TNF-α plus PDTC, PDTC and aspirin, aspirin or PDTC only group, respectively. The cells were treated with or without aspirin and PDTC for 1 h, then stimulated with TNF-α for 24 h. And for the MAPK inhibitors, the cells were divided into six groups and incubated with TNF-α or TNF-α plus PD98059, SB203580, SP600125 or aspirin. Cells were pre-incubated with or without 10 µM PD98059 (p-ERK inhibitor) (30), 10 µM SB203580 (p-p38 inhibitor) (30), SP600125 (p-JNK inhibitor) (31) and aspirin (600 µM) for 1 h with TNF-α (10 ng/ml). These cultured cells and supernatants were then collected for measurement of the following design parameters after treatment with TNF-α for 24 h.

Cell viability. MTT was used to evaluate the cytotoxicity of aspirin in RAW264.7 cells. Briefly, the cells were seeded at a density of 4x10⁴ cells/ml in a 96-well plate. The cells were pretreated with various concentrations of aspirin (75, 150, 300 and 600 µM) for 1 h, and then stimulated with or without TNF-α (10 ng/ml) for 24 h at 37°C in an atmosphere with 5% CO₂. Subsequently, MTT solution (5 mg/ml in a phosphate-buffered saline) was added to each well and the cells were incubated for a further 4 h. The medium was then discarded and 100 ml...
dimethyl sulfoxide was added. The absorbance was recorded at 490 nm with a microplate reader in order to determine the cell viability.

Determination of MMP-9 mRNA levels by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was abstracted from the TNF-α-stimulated RAW264.7 cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Equal amounts of RNA (1 µg) were reverse transcribed using a First-Strand cDNA synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China, 639504). qPCR was then performed using SYBR-Green (Takara Biotechnology Co., Ltd., 639655) on a Real-Time Quantitative Thermal Block (Biometra, Göttingen, Germany). The following specific primers were used: MMP-9 forward, 5′-TTCACCCGGTTGTGGAACCT-3′, and reverse, 5′-AAATGGGTGTACACAGGC-3′; GAPDH forward, 5′-TGGAATCTCTGTGGCATCCATGAAA-3′; and reverse, 5′-TAACAGCGACCTCGAAAGTTCCG-3′. The entire amplification course was initiated at 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec, and a final step at 60°C for 30 sec. The specificity of the amplified products was analyzed through dissociation curves generated by the equipment yielding single peaks. GAPDH was used as an internal control to normalize samples. PCR reactions of each sample were conducted in triplicate. Data were analyzed through the comparative cycle threshold (Cq) method, obtained from the iQ5 Optical System software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Gelatin zymography assay. To analyze MMP-9 enzyme expression, RAW264.7 cells were seeded in 6-well culture plates (2×10^4 cells/well). After the medium was changed with serum-free medium, the cells were pretreated with aspirin for 1 h and then stimulated with TNF-α for 24 h. Next, the samples were collected and separating by passing through 10% zymography gels. Following electrophoresis, sodium dodecyl sulfate (SDS) was removed by washing the gels three times with buffer (50 mM Tris/HC1, pH 7.6, 150 mM NaCl, 5 mM CaCl₂, 2 mM ZnCl₂, and 0.1% Triton X-100) for 30 min at room temperature with gentle agitation to renature enzymes. The gels were subsequently incubated in zymogen development buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 1 mM ZnCl₂] at 37°C for 24-48 h. After briefly washing in water, gels were stained with Coomassie blue R-250 (Bio-Rad Laboratories, Inc.) for 1 h. Gels were destained with 40% methanol and 5% acetic acid until clear white bands against a blue background were visible.

Western blot analysis. Total cells were washed with ice-cold phosphate buffer saline and then harvested using RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride. In order to obtain the nuclear extracts of NF-κB, the nuclear proteins were prepared using a nuclear protein extraction kit (Beyotime Institute of Institute of Biotechnology, Haimen, China, p0027) according to the manufacturer's protocol. Protein concentrations were then measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, po012s). Following incubation on ice for 30 min, the supernatant was collected by centrifugation at 12,000 x g for 10 min at 4°C, and the amount of protein was measured using a Bradford assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA, 5000202EDU). Subsequently, proteins were denatured in sample buffer containing 2-mercaptoethanol and bromophenol blue for 10 min at 100°C. Equal quantities of total cell lysates were size fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes using the Hoefer electrottransfer system (GE Healthcare, Chicago, IL, USA). Subsequent to blocking, the membrane was incubated with primary antibodies against NF-κB p65, β-actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:1,000 dilution, sc-7210), phospho-ERK, ERK, phospho-p38, p38, phospho-JNK, JNK and MMP-9 overnight at 4°C. The membrane was then washed with Tris-buffered saline/Tween 20 and incubated with anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated immunoglobulin G secondary antibodies (Santa Cruz Biotechnology, Inc.; 1:10,000 dilution, sc-2030) for 1 h at room temperature. The specific proteins were detected using enhanced chemiluminescence, and images were captured with a Fluorochrom Gel Image Analyzer (ProteinSimple, San Jose, CA, USA).

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). The results are expressed as the mean ± standard deviation, and differences between the means of two groups were determined by unpaired Student's t-test. The minimum significance level was set at P<0.05 for all analyses. All experiments were performed at least three times.

Results

Cytotoxicity of aspirin on RAW264.7 cells. The cytotoxic effect of aspirin on RAW264.7 cells was evaluated using MTT assay (Fig. 1A). Treatment of aspirin for 24 h did not have a significant cytotoxic effect on the cells at the concentrations of 75, 150, 300 and 600 µM, when compared with the untreated cells. Next, the cytotoxic effects of aspirin on TNF-α-treated RAW264.7 cells were determined. Cells were incubated in the presence of aspirin (0-600 µM) in serum-depleted medium for 1 h and then stimulated with TNF-α (10 ng/ml) for 24 h. The results indicated that aspirin had no evident cytotoxic effect on TNF-α-stimulated RAW264.7 cells at a concentration of up to 600 µM (Fig. 1B). Therefore, an aspirin dose of up to 600 µM was used in subsequent experiments.

Inhibitory effects of aspirin on MMP-9 expression in TNF-α-treated RAW264.7 cells. The next experiment attempted to determine the effect of aspirin on TNF-α-induced MMP-9 expression. RAW264.7 cells were pretreated without or with aspirin (75-600 µM/l) for 1 h and stimulated with TNF-α (10 ng/ml) for 24 h. Thereafter, the cultured medium was harvested for analysis of MMP-9 enzymatic activity, mRNA levels and protein expression by gelatin zymography, RT-qPCR and western blot analysis, respectively. As shown in Fig. 2A, MMP-9 secretion was significantly induced by TNF-α. However, the induction of MMP-9 activity by TNF-α was significantly inhibited by aspirin pre-treatment in a dose-dependent manner. Similarly, aspirin exhibited an inhibitory effect on TNF-α-induced MMP-9 expression at the mRNA and protein levels in a dose-dependent manner.
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Effect of aspirin on TNF-α-induced activation of NF-κB in RAW264.7 cells. NF-κB, which has a binding site located in the MMP-9 promoter region, has been implicated in the TNF-α-induced expression of MMP-9 in several cell lines (32). In order to determine whether the inhibitory effect of aspirin on the TNF-α-induced expression of MMP-9 is mediated by NF-κB, the effect of the nuclear translocation of p65 was investigated, which is a major subunit of NF-κB that has been shown to be induced by TNF-α. The cells were treated with aspirin in the presence of TNF-α for 1 h and then assessed by western blotting. As shown in Fig. 3A, the nuclear translocation of p65 as a result of TNF-α stimulation was strongly inhibited in the presence of aspirin at a concentration of 600 µM. This inhibitory effect was increased in a dose-dependent manner.

The association between NF-κB activation and MMP-9 expression was then further examined by exposure of cells to a specific inhibitor of NF-κB, pyrrolidine dithiocarbamate (PDTC), prior to TNF-α stimulation. PDTC can inhibit NF-κB activity and further reduce the production of inflammatory cytokines, alleviating the systemic inflammatory response (29). The results demonstrated that the combination of aspirin and PDTC also reduced TNF-α-induced MMP-9 expression (P<0.01; Fig. 3B and C). Therefore, these results indicated that the inhibitory effect of aspirin on MMP-9 expression and activity are associated with the suppression of NF-κB activation in TNF-α-stimulated RAW264.7 cells.

Effect of aspirin on the inhibition of ERK1/2, JNK and p38 phosphorylation. Several studies have indicated that MAPK pathways are involved in the expression of MMP-9 (33,34). To explore whether the inhibitory effect of aspirin on the expression of MMP-9 was mediated through the MAPK pathway, the phosphorylated protein levels of ERK1/2, JNK and p38 were examined by western blot in RAW264.7 cells pre-treated with aspirin and then with TNF-α for various times (0, 10, 20, 30 and 60 min). As shown in Fig. 4A, the protein expression levels of non-phosphorylated ERK, JNK and p38 were not evidently altered in the TNF-α alone and the TNF-α plus aspirin stimulation groups. By contrast, the expression of...
phosphorylated (p)-p38, p-ERK1/2 and p-JNK significantly increased by TNF-α stimulation, whereas aspirin inhibited the increase of p-p38, p-ERK1/2 and p-JNK induced by TNF-α at each time point.

The present study also examined whether MAPK pathways are involved in the MMP-9 expression in the TNF-α-stimulated RAW264.7 cells using inhibitors of ERK1/2 inhibitor (PD98059), p38 MAPK and JNK (SP600125). As shown in Fig. 4B and C, the mRNA and protein levels of TNF-α-induced MMP-9 expression levels were significantly downregulated in the presence of each of the MAPK inhibitors. These results suggest that the specific inhibition of the MAPK signaling pathway may be involved in the regulation of TNF-α-induced MMP-9 expression by aspirin in RAW264.7 cells.

**Discussion**

Cardiovascular disease, in particular atherosclerosis, is regarded as a type of inflammatory disease (35). Macrophages...
serve a major role during atherosclerotic lesion development in atherosclerotic plaque at various stages of development, partially through participation in the inflammatory response (4). Therefore, understanding the regulatory mechanisms of inflammation and finding pharmacological agents that can inhibit the inflammatory disease may have a potential effect in the prevention and treatment of atherosclerosis.

Aspirin, a platelet-inhibitory drug, is used to prevent complications of atherosclerotic cardiovascular disease, such as myocardial infarction and stroke (36). A previous study has demonstrated that aspirin, together with its anti-platelet activity, suppressed vascular inflammation and increased the stability of atherosclerotic plaques in murine atherosclerosis, thus exhibiting an anti-atherogenic effect (37). Aspirin can inhibit the expression and release of MMP-2/9 by upregulation of PPARα/γ gene expression, and also inhibit the activity of MMP-2/9 by induction of TIMP1 expression, which may be beneficial for the stabilization of atherosclerotic plaques (38). However, to the best of our knowledge, no studies have reported the potential effects of aspirin on MMP-9 expression in TNF-α-treated RAW264.7 cells. In the present study, the mechanism underlying the MMP-9 inhibition by aspirin treatment in TNF-α-stimulated RAW264.7 cells was investigated. It was demonstrated that aspirin has potent inhibitory effects on MMP-9 expression, possibly together with an anti-inflammatory and anti-platelet function.

MMPs are a major family of endopeptidases that can selectively degrade various components of the ECM and serve crucial roles in various physiological and pathological processes, including wound healing, vascular remodeling, rheumatoid arthritis, angiogenesis and invasion (39-41). MMP-9 is a member of the MMPs family and a marker for coronary atherosclerosis. Plasma MMP-9 concentration has been identified as a predictor of cardiovascular mortality in patients with coronary artery disease (42), and the atherosclerotic lesion development is initiated by infiltrated macrophages that mainly produce MMP-9. In turn, the expression and activity of MMP-9 were increased in advanced atherosclerotic lesions, followed by macrophage infiltration (43). Additionally, accumulating evidence demonstrates that the activity of MMP-9 is induced by TNF-α in a variety of cell types (44,45). The results of the present study consistently identified that TNF-α enhanced MMP-9 expression and activity in cultured RAW264.7 cells. Notably, these data indicated that the elevated mRNA expression, protein level and activity of MMP-9 by TNF-α stimulation were inhibited by aspirin pre-treatment in a dose-dependent manner in RAW264.7 cells. Therefore, the present results suggested that the inhibition of MMP-9 expression and activity may be responsible for the inhibitory effects of aspirin on TNF-α-treated RAW264.7 cells.

The binding site for NF-κB in the promoter region of MMP-9 serves a key function in the upregulation of MMP-9 expression by TNF-α induction. (46,47). NF-κB serves an important role in regulating the inflammatory and immune responses to extracellular stimuli. NF-κB is normally sequestered in the cytoplasm by inhibitory IκB proteins. Once activated, the NF-κB subunit p65 dissociates from its inhibitory protein IκB and translocates from the cytoplasm to the nucleus. Western blot analysis in the current study revealed that aspirin inhibited the nuclear translocation of NF-κB p65 in TNF-α-stimulated RAW264.7 cells in a dose-dependent manner. Furthermore, the NF-κB signaling specific inhibitor PDTC was used to determine whether NF-κB signaling is involved in the regulation of MMP-9 expression. The present data demonstrated that PDTC suppressed the MMP-9 expression, which agrees with a previous study (48) showing that aspirin inhibited MMP-9 mRNA expression and the nuclear translocation of NF-κB p65 subunit, thus suppressing the activity of this inflammatory molecule and maintaining the plaque stability. Collectively, the results of the present study imply that the inhibitory effects of aspirin on TNF-α-induced MMP-9 expression were mediated, at least partially, by suppression of the NF-κB transcription factor.

MMP-9 has been demonstrated to be stimulated by TNF-α via activating MAPKs (49). Considerable evidence indicated that numerous natural products inhibit the expression of pro-inflammatory genes by modulating the activation of MAPK pathways (50,51). Therefore, the present study aimed to reveal the inhibitory mechanism of aspirin on MMP-9 transcription through MAPK pathways. The data demonstrated that aspirin downregulated TNF-α-stimulated the phosphorylation of ERK1/2, p38 and JNK upon treatment for different time points. In addition, exposure to PD98059 (an inhibitor of ERK1/2 phosphorylation), SB203580 (an inhibitor of p38 phosphorylation) and SP600125 (an inhibitor JNK phosphorylation) also suppressed TNF-α-induced MMP-9 expression. According to the aforementioned results, it is strongly suggested that aspirin inhibits TNF-α-induced MMP-9 expression possibly by blocking the NF-κB and MAPK signaling pathways in RAW264.7 cells. However, the MMP-9 promoter has several transcription factor-binding motifs, including the AP-1, Sp-1 and NF-κB binding sites (52). Therefore, other possible transcription factors and signaling pathways may be involved in the regulation of MMP-9 expression, which requires further investigation.

In conclusion, the results of the present study suggested that aspirin effectively inhibited the expression of MMP-9 in TNF-α-stimulated RAW264.7 cells, possibly by inhibiting the activation of NF-κB and MAPK pathways. It was, thus, demonstrated that aspirin may contribute to the stabilization of atherosclerotic plaque and prevention of atherosclerosis.

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