

P2X7 receptor regulates EMMPRIN and MMP-9 expression through AMPK/MAPK signaling in PMA-induced macrophages

LU LIN, SHANJUN HUANG, ZHOUYANG ZHU, JIBO HAN,
ZHENGXIAN WANG, WEIJIAN HUANG and ZHOUQING HUANG

The Key Laboratory of Cardiovascular Disease of Wenzhou, Department of Cardiology, Cardiac Center,
The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, P.R. China

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Abstract. The rupture of atherosclerotic plaques may result in the formation of thrombi, which may induce subsequent cardiac events such as acute myocardial infarction. Overproduction of matrix metalloproteinases (MMPs) and extracellular matrix metalloproteinase inducers (EMMPRINs) by monocytes and macrophages may lead to rupture of atherosclerotic plaques as a result of the degradation of the extracellular matrix. The purinergic 2X7 receptor (P2X7R) is expressed in macrophages that are assembled in atherosclerotic lesions of human carotid arteries. P2X7R may serve a crucial role in the development of atherosclerosis; therefore, the present study aimed to determine whether P2X7R regulated the expression of EMMPRIN and MMP-9 in phorbol 12-myristate 13-acetate (PMA)-induced macrophages. In addition, the potential molecular mechanisms involved in this process were investigated. THP-1 human monocytic cells were pretreated with A-438079 (a specific inhibitor of P2X7R) for 1 h and subsequently incubated with or without PMA for 48 h. Exposure to A-438079 significantly decreased the expression of MMP-9 and EMMPRIN in the PMA-induced macrophages and attenuated the activation (phosphorylation) of mitogen-activated protein kinase (MAPK) signaling, including c-Jun N-terminal kinase, p38 and extracellular signal-regulated kinase. The present study also demonstrated that 5'-AMP-activated protein kinase (AMPK) was activated by PMA exposure during differentiation from monocytes to macrophages. This activation was reversed by A-438079 treatment through the inhibition of P2X7R

expression. These results suggested that the inhibition of P2X7R may be able to suppress the AMPK/MAPK signaling pathway and consequently downregulate both EMMPRIN and MMP-9 expression in PMA-induced macrophages.

Introduction

Atherosclerosis may lead to ischemia of the heart, brain or extremities and may further lead to infarction, which is the primary cause of mortality in the United States, Europe and much of Asia (1). The stability of plaques in coronary arteries is of great importance, as the rupture of plaques may lead to fatal complications such as myocardial infarction.

Purinergic 2X7 receptor (P2X7R) is a ligand-gated cation channel that is expressed by most immune cells such as macrophages, monocytes and lymphocytes (2). P2X7R is a member of the purinergic receptor family that is involved in the production and activation of the inflammatory cytokine interleukin (IL)-1 β and modulates the inflammatory response (3). A previous study demonstrated that P2X7R was highly expressed in endothelial cells and macrophages that infiltrate atherosclerotic plaques of human carotid arteries (4); in addition, P2X7R serves a crucial role in the development of atherosclerosis by regulating the activation of the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome (5).

Matrix metalloproteinase (MMP)-9 is a 92 kDa protein that belongs to a family of zinc- and calcium-dependent proteases (6). MMP-9 is considered to have various pathological functions. A number of studies have demonstrated the key role of MMP-9 in atherosclerosis was in the rupture of plaques through the degradation of the extracellular matrix (7,8). Extracellular matrix metalloproteinase inducer (EMMPRIN; also known as CD147 or basigin) is a highly glycosylated transmembrane protein that was first described in tumor cells (9). Previous studies have demonstrated that EMMPRIN, as an upregulator of local MMP-9 expression (10), was involved in numerous physiological and pathological processes, including tumor invasion (9) and atherosclerosis (11). It has also been indicated that during differentiation from monocytes into macrophages, the expression of EMMPRIN and MMP-9 is significantly increased (12), thereby accelerating the transition of stable plaques into unstable plaques through atherogenic

Correspondence to: Dr Weijian Huang or Dr Zhouqing Huang, The Key Laboratory of Cardiovascular Disease of Wenzhou, Department of Cardiology, Cardiac Center, The First Affiliated Hospital of Wenzhou Medical University, 2 Fuxue Road, Wenzhou, Zhejiang 325000, P.R. China
E-mail: weijianhuang69@126.com
E-mail: susiehzq@126.com

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cells (13). Consequently, downregulation of EMMPRIN and MMP-9 expression may ameliorate the development of atherosclerosis. Notably, several studies have revealed that P2X7R regulates the expression of MMP-9 and that P2X7R is involved in fibrosis progression in the lungs (14) and liver (15). Upon ATP stimulation, P2X7R in human peripheral blood mononuclear cells was reported to mediate MMP-9 activities by rapidly increasing the release of MMP-9 and decreasing the release of tissue inhibitor of metalloproteinases 1 (TIMP-1) (16). However, whether P2X7R expressed in phorbol 12-myristate 13-acetate (PMA)-induced THP-1 cells is able to regulate EMMPRIN and MMP-9 expression remains unexplored.

AMPK is a cellular energy sensor that acts as a kinase to maintain various processes of energy homeostasis, such as fatty acid oxidation, protein synthesis and glucose uptake (17-19). Our previous study demonstrated that the inhibition of AMPK α with compound C (a specific AMPK inhibitor) reduced MMP-9 and EMMPRIN expression levels in PMA-induced THP-1 cell differentiation, which suggested that activation of the AMPK α pathway may be involved in the regulation of EMMPRIN and MMP-9 expression in PMA-induced macrophages (20). Moreover, a number of previous studies have reported that MAPK signaling pathways are special regulators for EMMPRIN and MMP-9 (21,22). On the basis of these results, the present study hypothesized that AMPK α and MAPK signaling pathways may regulate the levels of EMMPRIN and MMP-9 expression. However, whether P2X7R regulate the activation of AMPK α and MAPK signaling pathways is still unknown.

Therefore, the present study aimed at exploring the role of P2X7R in mediating the expression of EMMPRIN and MMP-9 in PMA-induced THP-1 cells and to further reveal its mechanisms.

Materials and methods

Cell culture and treatment. The human monocytic cell line THP-1 was obtained from American Type Culture Collection (Manassas, VA, USA) and maintained at a density of 10^6 cells/ml as the control group in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1% penicillin/streptomycin solution at 37°C in a 5% CO₂ incubator. Cells were cultured in six-well at a density of 10^6 cells/ml for 48 h at 37°C in the presence of 100 nM PMA (20), which allowed them to differentiate into adherent macrophages. Cells were pretreated with 100 μ M A-438079 (Selleck Chemicals, Houston, TX, USA) for 1 h at 37°C (23), and then stimulated with 100 nM PMA for another 48 h at 37°C, which was added directly to the medium.

Protein isolation and western blot analysis. Following treatment, cells were washed with cold PBS (pH 7.4) and cell pellets were lysed for 30 min with a lysis buffer (0.5% Nonidet P-40; 50 mmol/l Tris-HCl, pH 7.5; 1 mmol/l EDTA; 1 mmol/l EGTA and 150 mmol/l NaCl; containing 10% glycerol; 50 mmol/l sodium fluoride; 10 mmol/l sodium

pyrophosphate; 1 mmol/l sodium orthovanadate; 80 μ mol/l β -glycerophosphate; 1 mmol/l phenylmethylsulfonyl fluoride; 10 μ g/ml aprotinin; 100 μ g/ml soybean trypsin inhibitor and 10 μ g/ml leupeptin), followed by centrifugation at 4°C for 10 min at 12,000 \times g. Protein concentrations were measured by BCA protein assay (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The protein extracts were denatured and the solubilized proteins (20 μ g) subjected to electrophoresis by 10% SDS-PAGE. Proteins were subsequently transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with TBS containing 0.05% Tween-20 (TBST) and 5% skimmed milk for 1 h at room temperature, followed by probing with primary antibodies against GAPDH (cat. no. 5174), P2X7R (cat. no. 13809), matrix metalloproteinase (MMP)-9 (cat. no. 13667), 5'-AMP-activated protein kinase (AMPK) α (cat. no. 5832), phosphorylated (p)-AMPK α (cat. no. 50081), p38 (cat. no. 8690), p-p38 (cat. no. 9215), c-Jun N-terminal kinase (JNK) (cat. no. 9252), p-JNK (cat. no. 9255) (all 1:1,000 in TBST; Cell Signaling Technology, Inc., Danvers, MA, USA), EMMPRIN (1:1,000 in TBST; cat. no. ab666, Abcam, Cambridge, MA, USA), extracellular signal-regulated kinase (ERK)1/2 (cat. no. sc81457) or p-ERK1/2 (cat. no. sc81492) (both 1:300 in TBST; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. Following primary antibody incubations, the membranes were incubated with goat anti-rabbit or goat anti-mouse secondary antibody (1:1,000; cat. no. A0239 or cat. no. A0216, respectively, Beyotime Institute of Technology, Haimen, China) for 1 h. Protein bands were visualized by Enhanced Chemiluminescence Detection Reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results were analyzed with Quantity One software 4.62 (Bio-Rad Laboratories, Inc.) and data were normalized based on GAPDH.

RNA isolation, cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from 10^6 cells treated with indicated conditions was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized using the Reverse Transcription Reagent, according to the manufacturer's protocol (cat. no. N8080234, Thermo Fisher Scientific, Inc.) and RT-qPCR was performed with the SYBR Premix Ex Taq kit (cat. no. DRR041; Takara Biotechnology Co., Ltd., Dalian, China) according to the following PCR conditions: initial denaturation at 95°C for 30 sec followed by 50 cycles of amplification at 95°C for 5 sec and 60°C for 34 sec. The amplified fluorescent signal was detected by the ABI-7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used in the study were as follows: MMP-9 (NCBI accession no. NM_004994.2), forward 5'-TGACGC CGCTCACCTTCACT-3', reverse 5'-CGCGCCATCTGCGTT TCCAA-3'; EMMPRIN (NCBI accession no. NM_001728.3), forward 5'-TTGGAGGTTGTAGGACCGGCGA-3', reverse 5'-TGGGACCCTGCCCTTCAAACCA-3'; and GAPDH (NCBI accession no. NM_001256799.2), forward 5'-CCG CATCTTCTTTTTCGCTCGCC-3', reverse 5'-TCTCAGCCT TGACGGTGCCA-3'. The expression compared was using the 2^{- $\Delta\Delta$ C_q} method (24). All results were normalized with GAPDH.

Gelatin zymography. Cells (1×10^6 cells/well) in the logarithmic phase were seeded in 6-well plates and incubated in serum-free medium with or without $100 \mu\text{M}$ A-438079 for 1 h at 37°C in a $5\% \text{CO}_2$ incubator, followed by incubation with 100 nM PMA for an additional 48 h at 37°C . Culture supernatants were collected and $20 \mu\text{l}$ aliquots were loaded onto a 10% polyacrylamide gel containing 1 mg/ml gelatin. Following electrophoresis, gels were washed twice with 2.5% Triton X-100 (37°C ; 30 min each) and incubated at 37°C for 18 h in developing buffer comprising 10 mM Tris base, 40 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl_2 , 0.02% Brij-35. Gels were subsequently stained with 0.5% (w/v) Coomassie Brilliant Blue R-250 for 2 h at room temperature, followed by destaining with a solution containing 50% methanol, 10% glacial acetic acid and 40% water. MMP-9-digested regions were visualized as light bands against a dark background. An image of each gel was captured by an Odyssey Imaging System and analyzed by Image Studio 5.2.5 (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. Statistical analyses were performed using SPSS v18 software (SPSS Inc., Chicago, IL, USA). Three or more groups were compared using one-way analysis of variance (ANOVA) with Student-Newman-Keuls and Dunnett methods as post-hoc analysis if the result of ANOVA was significant. Data were presented as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least three times.

Results

PMA treatment stimulated the expression of P2X7R in a time-dependent manner. The use of PMA to induce THP-1 cells to differentiate into macrophages is a classical cell model that is widely used to explore the inflammatory function of macrophages *in vitro* (25,26). THP-1 cells were treated with 100 nM PMA for different incubation periods ranging between 6 and 48 h. The protein expression level of P2X7R was measured by western blot analysis, which indicated that the level of PMA-stimulated P2X7R expression was in a time-dependent manner (Fig. 1). Therefore, the cells were treated with 100 nM PMA for 48 h in the subsequent experiments.

Inhibition of P2X7R reduces MMP-9 and EMMPRIN expression and MMP-9 activity in PMA-induced macrophages. THP-1 cells were pretreated with A-438079, a specific antagonist of P2X7R, for 1 h, followed by incubation with 100 nM PMA for 48 h to determine the effects of P2X7R inhibition on MMP-9 and EMMPRIN expression in PMA-induced macrophages. MMP-9 and EMMPRIN protein (Fig. 2A and B) and mRNA (Fig. 2C and D, respectively) expression levels were significantly increased in the PMA-induced macrophages, and the suppression of P2X7R expression by A-438079 treatment significantly inhibited the PMA-upregulated expression level of MMP-9 (Fig. 2). EMMPRIN, which is the most well-characterized and major cell surface regulator of MMP-9 (27), exhibited a similar reduction in expression as MMP-9 following suppression of P2X7R expression (Fig. 2). These results indicated that P2X7R inhibition affected the expression of EMMPRIN and MMP-9 in PMA-induced macrophages at both the protein and mRNA levels.

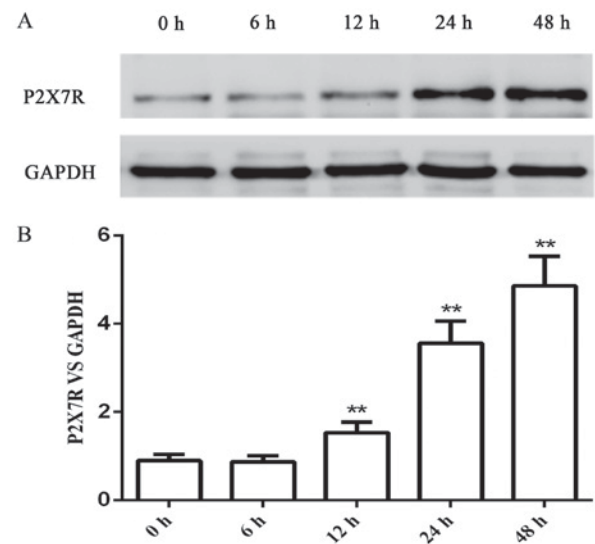


Figure 1. PMA-induced P2X7R expression at different time points in THP-1 cells. Following 0-48 h PMA induction, whole cell lysates were collected for western blot analysis of P2X7R and GAPDH protein expression. (A) Representative blot of P2X7R and GAPDH expression at the various time points. (B) Densitometric analysis of P2X7R protein expression. For densitometry, the protein expression level of control (0 h) group was arbitrarily set as '1' in each blot, and P2X7R expression levels were normalized to the GAPDH. Data are expressed as the mean \pm standard deviation; $n=3$; ** $P < 0.01$ vs. 0 h control group. P2X7R, purinergic $2\text{X}7$ receptor; PMA, phorbol 12-myristate 13-acetate.

The effects of P2X7R on the enzymatic activities of MMP-9 were examined by gelatin zymography. As previously reported (20), following staining with Coomassie Blue, an unstained transparent band was observed at $\sim 92 \text{ kDa}$; this band represented the theoretical size of the gelatin digested by MMP-9. In the THP-1-derived macrophages, MMP-9 activity was significantly increased in cells treated with PMA, compared to untreated control cells (Fig. 3A and B), and A-438079-inhibited expression of P2X7R significantly reduced MMP-9 activity.

P2X7R mediates AMPK activation induced by PMA. The potential mechanism associated with P2X7R regulation of MMP-9 and EMMPRIN expression in PMA-induced macrophages was determined by examining the potential involvement of the AMPK pathway. Cells were pretreated with A-438079 for 1 h and induced with PMA for another 48 h. The protein expression levels of p-AMPK α and total AMPK α were examined by western blot analysis (Fig. 3C and D). PMA treatment induced the activation of AMPK α in THP-1 cells, and the phosphorylation of AMPK α was significantly reduced by A-438079 co-treatment. This result suggested that the inhibition of P2X7R inhibited AMPK α activation.

P2X7R inhibition suppresses mitogen-activated protein kinase (MAPK) pathway in PMA-induced THP-1 cells. Previous studies indicated that PMA treatment promoted the expression of EMMPRIN and MMP-9 by activating the MAPK signaling pathway (20). Therefore, whether P2X7R regulated the expression of EMMPRIN and MMP-9 through the MAPK pathway was examined. To verify this hypothesis, THP-1 cells were pretreated with A-438079 for 1 h prior to incubation with PMA

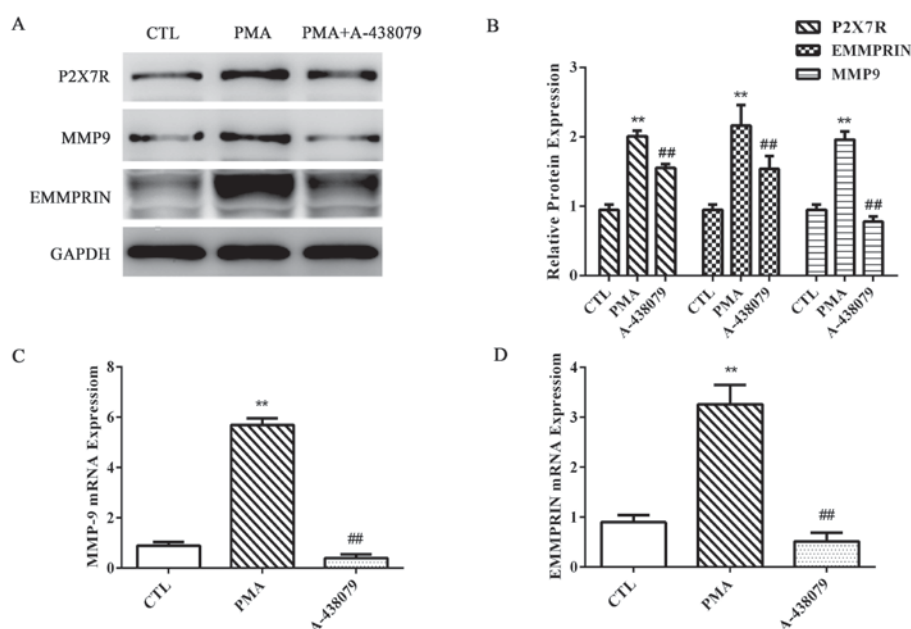


Figure 2. Inhibition of P2X7R by A-438079 decreases the expression level of EMMPRIN and MMP9 in PMA-induced THP-1 cells. (A and B) Protein expression levels of P2X7R, MMP-9, EMMPRIN and GAPDH were examined by (A) western blotting and (B) densitometric analysis. (C and D) mRNA expression levels of (C) MMP-9 and (D) EMMPRIN were measured by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean \pm standard deviation; $n=3$; ** $P<0.01$ vs. CTL group; ## $P<0.01$ vs. PMA group. CTL indicates that cells incubated in a medium without PMA. CTL, untreated control group; EMMPRIN, extracellular matrix metalloproteinase inducer; MMP-9, matrix metalloproteinase 9; P2X7R, purinergic 2X7 receptor; PMA, phorbol 12-myristate 13-acetate.

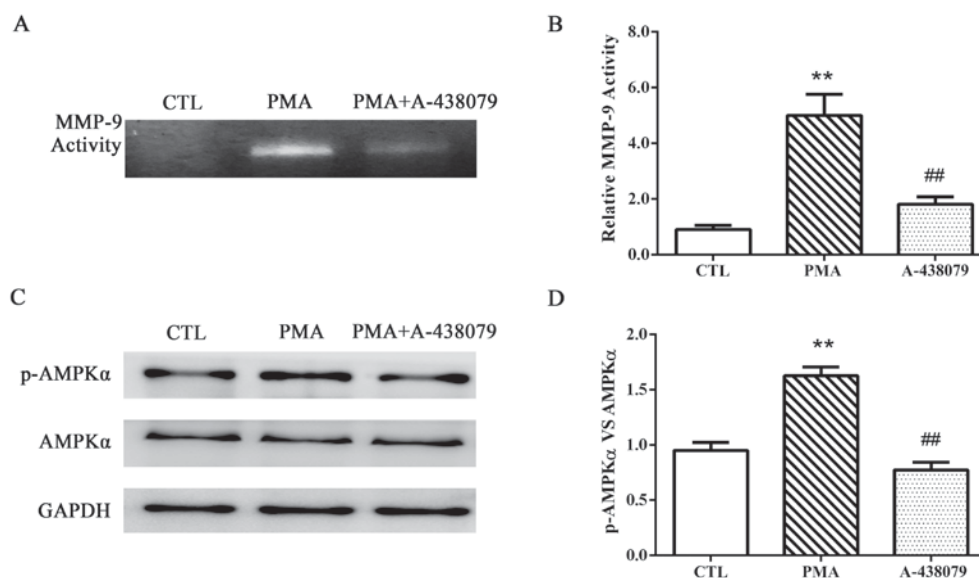


Figure 3. Inhibition of P2X7R by A-438079 suppresses the activation of MMP-9 and AMPK activation. (A and B) Effects of P2X7R inhibition on the activity of MMP-9 from cultured supernatant. THP-1 cells were pretreated with A-438079 prior stimulating with PMA (100 nM) for an additional 48 h; MMP-9 activities were detected by (A) gelatin zymography assay and (B) densitometric analysis. (C and D) Effects of P2X7R inhibition on total AMPK and p-AMPK α expression. THP-1 cells were treated with PMA or PMA + A-438079 and the protein level of total AMPK α and p-AMPK α were examined by (C) western blotting and (D) densitometric analysis. Data are expressed as the mean \pm standard deviation; $n=3$; ** $P<0.01$ vs. CTL; ## $P<0.01$ vs. PMA group. AMPK, 5'-AMP-activated protein kinase; CTL, untreated control group; MMP-9, matrix metalloproteinase 9; p, phosphorylated; P2X7R, purinergic 2X7 receptor; PMA, phorbol 12-myristate 13-acetate.

for 48 h. The inhibition of P2X7R by A-438079 co-treatment significantly decreased the PMA-induced phosphorylation of ERK1/2, p38 MAPK and JNK (Fig. 4A-D). These results suggested that the MAPK pathway may be involved in the regulation of EMMPRIN and MMP-9 expression by P2X7R in PMA-induced THP-1 cells.

Discussion

Regulation of plaque stability is vital to patients with atherosclerosis, particularly in cases of thrombosis and fatal complications. Previous studies have suggested that the involvement of P2X7R in atherosclerotic regulation was

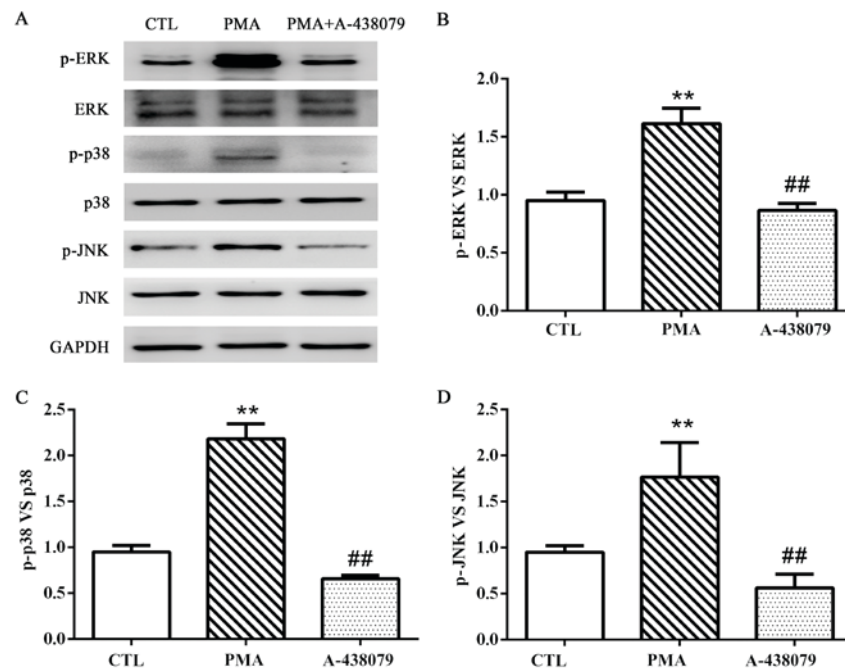


Figure 4. P2X7R regulates the phosphorylation of ERK1/2, p38 and JNK. (A) Protein expression levels of ERK, p-ERK, p38, p-p38, JNK, p-JNK and GAPDH were examined by western blot analysis. (B-D) Protein quantification was carried out by densitometric analysis. Proteins were normalized to the internal control GAPDH. Data are expressed as the mean \pm standard deviation; n=3; **P<0.01 vs. CTL group; ##P<0.01 vs. PMA group. CTL, untreated control group; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p, phosphorylated; P2X7R, purinergic 2X7 receptor; PMA, phorbol 12-myristate 13-acetate.

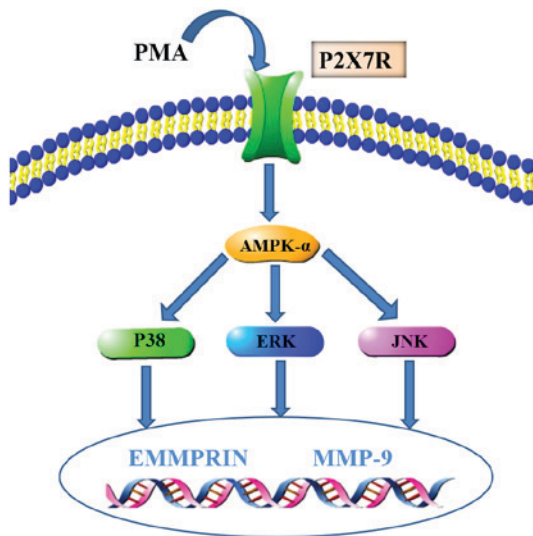


Figure 5. Model for the regulation of MMP-9 and EMMPRIN by P2X7R. PMA may induce EMMPRIN and MMP-9 expression in macrophages through the AMPK/MAPK pathway. P2X7R inhibition may attenuate the expression of MMP-9 and EMMPRIN by inhibiting the activation of AMPK-MAPK pathway. AMPK, 5'-AMP-activated protein kinase; EMMPRIN, extracellular matrix metalloproteinase inducer; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinase 9; P2X7R, purinergic 2X7 receptor; PMA, phorbol 12-myristate 13-acetate.

through different targets, such as the NLRP3 inflammasome (4,28). However, despite the general proinflammatory effects of P2X7R, the mechanism by which it mediates atheromatous progression has been poorly investigated. Additionally, the elevated expression levels of EMMPRIN and MMP-9 have been correlated with advanced atherosclerotic lesions,

followed by plaque rupture and myocardial infarction (13,29). The present study demonstrated that P2X7R inhibition by A-438079 significantly downregulated the expression of EMMPRIN and MMP-9 at the protein and mRNA levels, probably by suppressing the AMPK and MAPK pathways in PMA-induced THP-1 cells. Therefore, P2X7R may be a potential therapeutic target for ameliorating the development of atherosclerotic plaques.

P2X7R is considered to be only activated in circumstances in which the local concentration of ATP increases, such as infection and injury, or in tumor microenvironments (30). However, currently unknown allosteric modulators may serve a role in P2X7R activity *in vivo* by decreasing its K_m for ATP so that P2X7R may be activated even at low nucleotide concentrations (2). Similar to this hypothesis, our previous research demonstrated that the expression of P2X7R is highly elevated when stimulated by PMA in monocytes-derived macrophages (31). Therefore, the present study this cell model was used to explore the underlying biological mechanism. Although there is no explicit association between PMA and ATP, it was speculated that the stimulation of PMA may influence the extracellular concentration of ATP, but this needs to be investigated further. The present results suggested the possibility of P2X7R serving a role in the differentiation of THP-1 cells from monocytes to macrophages, but this needs to be studied further.

Furthermore, the regulation between P2X7R and MMP-9 in different physiologic and pathologic processes has also been reported. For example, P2X7 receptor activated by ATP stimulation in human peripheral blood mononuclear cells was revealed to rapidly increase MMP-9 release and thus enhanced extracellular MMP-9 activity (16). P2X7R

was also demonstrated to be involved in the regulation of the blood-brain barrier by mediating MMP-9 activities and the degradation of the extracellular matrix (32,33). The present results revealed that the inhibition of P2X7R expression may significantly inhibit the PMA-induced upregulation of MMP-9 expression and activity. In addition, EMMPRIN, as the major cell surface regulator of MMP-9, was also regulated by P2X7R in PMA-induced THP-1 cells.

To determine the molecular mechanisms by which P2X7R may regulate the expression of EMMPRIN and MMP-9 in differentiated macrophages, the level of phosphorylated AMPK α was investigated. A previous study revealed that the activation of AMPK α may be induced by PMA treatment (20). In addition, another study reported that P2X7R was able to mediate the activation of AMPK during autophagy induced by LL-37 in macrophages (34). On the basis of these results, the present study hypothesized that P2X7R may regulate the activation of AMPK α to adjust the levels of EMMPRIN and MMP-9 expression in PMA-induced macrophages. As expected, the inhibition of P2X7R expression in the present study led to the reduced activation of the AMPK α pathway, and the downregulation of EMMPRIN and MMP-9 expression. Consequently, AMPK α activation may be necessary for P2X7R to regulate the expression of MMP-9 and EMMPRIN in PMA-induced macrophages. Notably, our previous data indicated that compound C also suppressed the phosphorylation of MAPK signaling, including the ERK, JNK and p38 pathways in PMA-induced macrophages (20). Therefore, the activation of the AMPK pathway is the upstream of MAPK in THP-1 cells stimulated with PMA.

Furthermore, P2X7R serves an essential role in the regulation of MAPK pathways during physiological and pathological processes, such as sympathoexcitatory response in myocardial infarction (35) and the differentiation of bone marrow stem cells into osteoblasts (36-38). In the present study, the activation of the MAPK signaling pathway was examined in PMA-induced THP-1 cells and it was revealed that the inhibition of P2X7R significantly decreased the phosphorylation of ERK1/2, p38 MAPK and JNK in these macrophages. These results indicated that MAPK pathways may serve an essential role in the regulation of EMMPRIN and MMP-9 expression by P2X7R.

In conclusion, P2X7R expression was significantly increased in the PMA-induced macrophages, and the inhibition of P2X7R expression was followed by the downregulation of EMMPRIN and MMP-9 expression, which probably occurred through the suppression of AMPK and MAPK signaling pathway activation. AMPK, as an upstream activator of MAPK signaling, may be involved in the regulation of EMMPRIN and MMP-9 expression in PMA-induced macrophages (20). Therefore, the present study suggested that P2X7R may regulate EMMPRIN and MMP-9 expression through AMPK/MAPK signaling in PMA-induced macrophages and the schematic model is illustrated in Fig. 5. These data provided novel insights into the regulatory mechanisms of EMMPRIN and MMP-9 and suggested that P2X7R may be a potential strategy for combating plaque ruptures.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LL, ZH and WH conceived and designed the study. LL, SH, ZW, ZZ and JH performed the experiments. ZZ and ZH analyzed and integrated the results. LL wrote the paper. ZZ, JH and ZH reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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