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Lysophosphatidic Acid Promotes Expression and Activation of Matrix Metalloproteinase 9 (MMP9) in THP-1 Cells via Toll-Like Receptor 4/Nuclear Factor- κ B (TLR4/NF- κ B) Signaling Pathway

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCDE 1 **Zhi-bin Zhou***

AEG 2 **Bo Yang***

CDF 3 **Xiaohao Li***

BCD 1 **Hao Liu**

CDF 1 **Gang Lei**

1 Department of Neurology, Tianyou Hospital Affiliated to Wuhan University of Science and Technology, Wuhan, Hubei, P.R. China

2 Department of Cardiology, The Third People's Hospital of Hubei, Wuhan, Hubei, P.R. China

3 Department of Internal Medicine, Wuhan Institute of Technology Hospital, Wuhan, Hubei, P.R. China

Corresponding Authors:

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* Zhi-bin Zhou, Bo Yang, and Xiaohao Li contributed to the work equally and should be regarded as co-first authors

Bo Yang, e-mail: yangbo6120@163.com, Xiaohao Li, e-mail: 464229823@qq.com

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Background:

Lysophosphatidic acid (LPA) is an active compound of oxidized low-density lipoprotein that serves as an endogenous TLR4 ligand. Ligand activation of TLR4 activates nuclear factor-kappaB (NF- κ B) and the transcription of NF- κ B-regulated inflammatory cytokines, which are involved in the development of atherosclerosis. MMP9 is a member of the MMP family and can affect plaque stability. However, the mechanism responsible for the effect of LPA on the expression and activation of MMP9 has not been fully elucidated. In the present study we examined the effect of LPA on MMP9 expression and activity in THP-1 cells and the involvement of Toll-like receptor 4/nuclear factor- κ B (TLR4/NF- κ B) signaling pathway in this effect.

Material/Methods:

Human THP-1 cells were treated with 0–10 μ M LPA for 4 h, or treated with 1 μ M LPA for 0–8 h, and were then transfected with TLR4-specific siRNA or treated with 20 μ g/ml cafestol acid phenethyl ester (CAPE, an NF- κ B inhibitor). MMP9 mRNA and protein levels were measured by quantitative RT-PCR and Western blot analysis, respectively, and MMP9 activity was measured by zymography.

Results:

LPA upregulated MMP9 mRNA and protein levels and MMP9 activity in THP-1 cells in both concentration- and time-dependent manners. Transfection of cells with TLR4-siRNA-2 or treatment with CAPE significantly inhibited the upregulated MMP9 expression and activation. This inhibition was further enhanced by combining the TLR4-siRNA-2 transfection and CAPE pretreatment.

Conclusions:

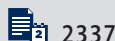
LPA can promote MMP9 expression and enhance MMP9 activity in THP-1 cells, in part via the TLR4/NF- κ B signaling pathway.

MeSH Keywords:

Matrix Metalloproteinases • Receptors, Lysophosphatidic Acid • Toll-Like Receptor 4

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Background

The development of atherosclerosis involves a persistent inflammatory immune response that plays an important role in the processes of unstable plaque formation, development, and rupture. Toll-like receptors (TLRs) play a key role in these processes, as does the endogenous TLR ligand, lysophosphatidic acid (LPA), a phospholipid derivative. Ligand activation of TLR4, activates nuclear factor- κ B (NF- κ B) and the transcription of NF- κ B-regulated inflammatory cytokines [1,2], which are involved in the pathophysiological process of atherosclerosis.

Matrix metalloproteinases (MMPs) are key enzymes in extracellular matrix metabolism, and are closely involved in atherosclerotic plaque formation and rupture [3] by degrading type IV collagen (a major structural component of the vascular basement membrane) [4]. MMP9 is a member of the MMP family and can degrade extracellular matrix (ECM) components and the basement membrane of the vessel wall. This can cause breakdown of the fibrous cap and influence plaque stability. MMP9 transcription is regulated by NF- κ B and activator protein 1 (AP-1) transcription factors [5].

However, the effect of LPA on the expression and activation of MMP9 and the involvement of TLR4/NF- κ B signaling pathway in this effect are unclear. In this study, we tested the effect of LPA on MMP9 mRNA and protein expression and MMP9 activity in the human monocytic cell line THP-1 and investigated the role of TLR4/NF- κ B signaling in this process.

Material and Methods

Materials

We used the following materials: Trizol reagent (Invitrogen, Carlsbad, CA, USA); RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas); Deoxyribonuclease I (DNase I, Fermentas); RiboLock™ Ribonuclease Inhibitor (Fermentas); SYBR™ Green PCR Master Mix (ABI, Foster City, CA, USA); Lipofectamine™ 2000 (Invitrogen); Opti-MEM® I Reduced-Serum Medium (Invitrogen, Carlsbad, CA, USA); MMP Zymography assay kit (for MMP-2 and MMP-9) (Applygen Technologies Inc., Beijing, China); Nuclear and cytoplasmic protein extraction kit (Santa Cruz, CA, USA); THP-1 cells (Biochain, Newark, CA, USA); and Lysophosphatidic acid (LPA, St Louis, MO, USA), purity (TLC) \geq 98%. LPA was dissolved in phosphate-buffered saline (PBS) containing 0.1% fatty acid-free bovine serum albumin (BSA).

Methods

Cell culture and treatment

THP-1 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in 5% CO₂. Medium was changed every 2 to 3 days and cells were passaged when the cell density reached (4~6)×10⁶ cells/mL. For LPA treatment, THP-1 cells were cultured in serum-free medium overnight and seeded at 10⁶ cells/ml in a 24-well culture plate, then cells were treated with 0, 0.1, 0.5, 1, 5, or 10 μ M LPA for 4 h, or treated with 1 μ M LPA for 0, 1, 2, 4, or 8 h. Cells were then harvested.

The MMP9 mRNA was detected by fluorescence quantitative RT-PCR. Proteins were extracted and the MMP9 protein was detected by Western blot analysis. MMP9 activity was determined by zymography.

THP-1 cells were divided into 6 groups and transfected with TLR4-specific siRNA or treated with 20 μ g/ml CAPE [6] for 1 h as follows: negative control siRNA, TLR4-siRNA transfection, 20 μ g/ml CAPE treatment, 20 μ g/ml CAPE pretreatment, TLR4-siRNA transfection, and blank control.

Quantitative RT-PCR

Total RNA was extracted from cells using one-step Trizol reagent. Reverse transcription (RT) was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Real-time quantitative PCR was performed using SYBR Green fluorescent dye. β -Actin was used as an internal reference gene. TLR4 primer sequences were: Upstream, 5'-ACATCAAATGCCCTACTCA-3'; Downstream, 5'-CTAAAC CAGCCAGACCTGA-3'; for β -actin: Upstream, 5'-CATTAAAGGAGA AGCTGTGCT-3'; Downstream, 5'-GTTGAAGGTAGTTTCTGTGGA-3'; for MMP9: Upstream, 5'-TTCTCCAGAAGCAACTGTCC-3'; Downstream, 5'-TAGGTGATGTTGTGGTGGTG-3'. The reaction system included 1 μ l cDNA, 12.5 μ l 2×SYBR Green PCR Master mix, and 100 nM of each upstream and downstream primer, and ddH₂O was added to bring the final volume to 25 μ l. The reaction conditions were: 95°C for 5 min, 40 cycles of 94°C for 20 s, 56°C for 20 s, 72°C for 20 s, 72°C for 5 min, and 55°C for 10 s. Melting curve analysis was performed in the range 60°C to 95°C with 0.5°C at 5-s increments after the amplification cycles were completed. Data were collected automatically using a Real-Time PCR System (ABI 7500) and Ct values were analyzed on a computer. The relative level of MMP9 mRNA to that of β -actin was calculated using Δ Ct values: Δ Ct TLR4=CtMMP9-Ct β -actin. The data were processed using the 2^{- $\Delta\Delta$ Ct} (RQ) method. Three wells were run in parallel 3 times for each specimen.

Design of siRNAs

TLR4-siRNA and negative control siRNA sequences were designed based on *TLR4* sequences:

TLR4-siRNA-1 Sense 5'-UCUAGAGCACUUGGACCTTTT-3'; Antisense 3'-TTAGAUCUCGUGAACCUGGUU-5'; TLR4-siRNA-2 Sense 5'-GUUGAUCUACCAAGCCUUGTT-3'; Antisense 3'-TTCAACUAG AUGGUUCGGAAC-5'; TLR4-siRNA-3 Sense 5'-CGAAUGGAU GUGCAACTT-3'; Antisense 3'-TTGCUUACCUACACGUUGUG-5' Negative control siRNA Sense 5'-UUCUCCGAACGUGUCACGUTT-3'; Antisense 5'-ACGUGACACGUUCGGAGAATT-3'

siRNA transfection

The day before transfection, 5×10^4 cells were seeded in 6-well plates with 2 mL basal medium containing fetal bovine serum. The next day, when cells reached 70% confluence, the culture medium was replaced with medium without serum and antibiotics. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 5 μ l of Lipofectamine™ 2000 was diluted in 250 μ l Opti-MEM® I, mixed gently, and incubated at room temperature for 5 min. Then, 7.5 μ l siRNA was diluted in 250 μ l of Opti-MEM® I, mixed gently, and incubated at room temperature for 5 min. Diluted siRNA and Lipofectamine™ 2000 were combined, mixed gently, and incubated at room temperature for 20 min to allow for complex formation. siRNA-Lipofectamine™ 2000 complexes were added to each well containing cells and medium and gently mixed by rocking the plate back and forth. The plates were incubated at 37°C in a CO₂ incubator for 24–72 h. The transfected cells were observed under a fluorescence microscope. TLR4 mRNA and protein were detected using quantitative RT-PCR and Western blot analysis, respectively, to determine the optimal siRNA. Cells were observed at 24 h after transfection under fluorescence and light microscopy, and transfection efficiency was determined by comparing cells in bright-field and fluorescent views. The transfection efficiency was more than 80%, which was suitable for use in the following experiments.

Detection of MMP9 activity

MMP9 activity was detected by zymography. Total protein was extracted from cells using a total protein extraction kit (ProMab Biotechnologies, Richmond, CA, USA) following the manufacturer's instructions. We mixed 5 microliters of extracted protein with an equal volume of 2×SDS-PAGE non-reducing buffer (P1700), and we mixed 5 μ l of a 1:1 dilution of extracted protein with 5 μ l 2×SDS-PAGE non-reducing buffer and loaded it directly onto gels without heat-denaturation. Electrophoresis was performed at a constant current of 30 mA, and was terminated when the bromophenol blue front ran out of the gel. Then, the stacking gel was carefully removed, placed in a clean dish,

and rinsed with distilled water. We then added 10 ml 1×buffer A and the gel was rinsed at room temperature for 30 min, during which the buffer was changed 2–3 times. The gel was then incubated at room temperature for 4 h in 10 ml 1×buffer B. Gels were then placed in Coomassie brilliant blue solution and stained gels were scanned on a scanner. All scanned images were converted to grayscale and the integrated optical density (IOD) was determined. The band with a molecular weight of 92 kDa represents MMP9, and the 97 kDa band represents pro-MMP9. The OD value of the band in the control group was defined as 1 and served as a reference value. The OD value of the same band in the remaining groups was compared to the OD value in the control group. The relative value reflects the enzyme activity.

Western blot assay

Cells were harvested and cell lysates prepared as previously described [7]. Protein concentration was determined by the BCA method. Ten micrograms of protein were separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with non-fat milk and membranes were then incubated with a mouse anti-TLR4 (1: 500, Santa Cruz, GAPDH 1: 80 000, Santa Cruz) or a rabbit anti-MMP9 (1: 400 Santa Cruz, histone H2 1: 40 000, Santa Cruz) antibody at room temperature for 2 h. After washing 3 times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1: 80 000, Santa Cruz) or goat anti-rabbit secondary antibody (1: 40 000 Santa Cruz) at room temperature for 2 h. Protein bands were visualized by color development with 4-chloro-1-naphthol and H₂O₂ solution. Positive bands were analyzed using Gelpro 4.0 software and the IOD values were measured. Relative protein levels are represented as the ratio of target band to GAPDH band IOD values.

Statistical analysis

All data were collected from 3 independent experiments. Results were analyzed using SPSS 16.0 software and are expressed as the mean \pm SD. Means among groups were analyzed using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Effects of LPA on levels of MMP9 mRNA and protein and on MMP9 activity in THP-1 cells

The levels of MMP9 mRNA and protein and MMP9 activity in THP-1 cells increased after treatment with increasing concentrations of LPA and reached a peak at 1 μ M LPA. At higher LPA

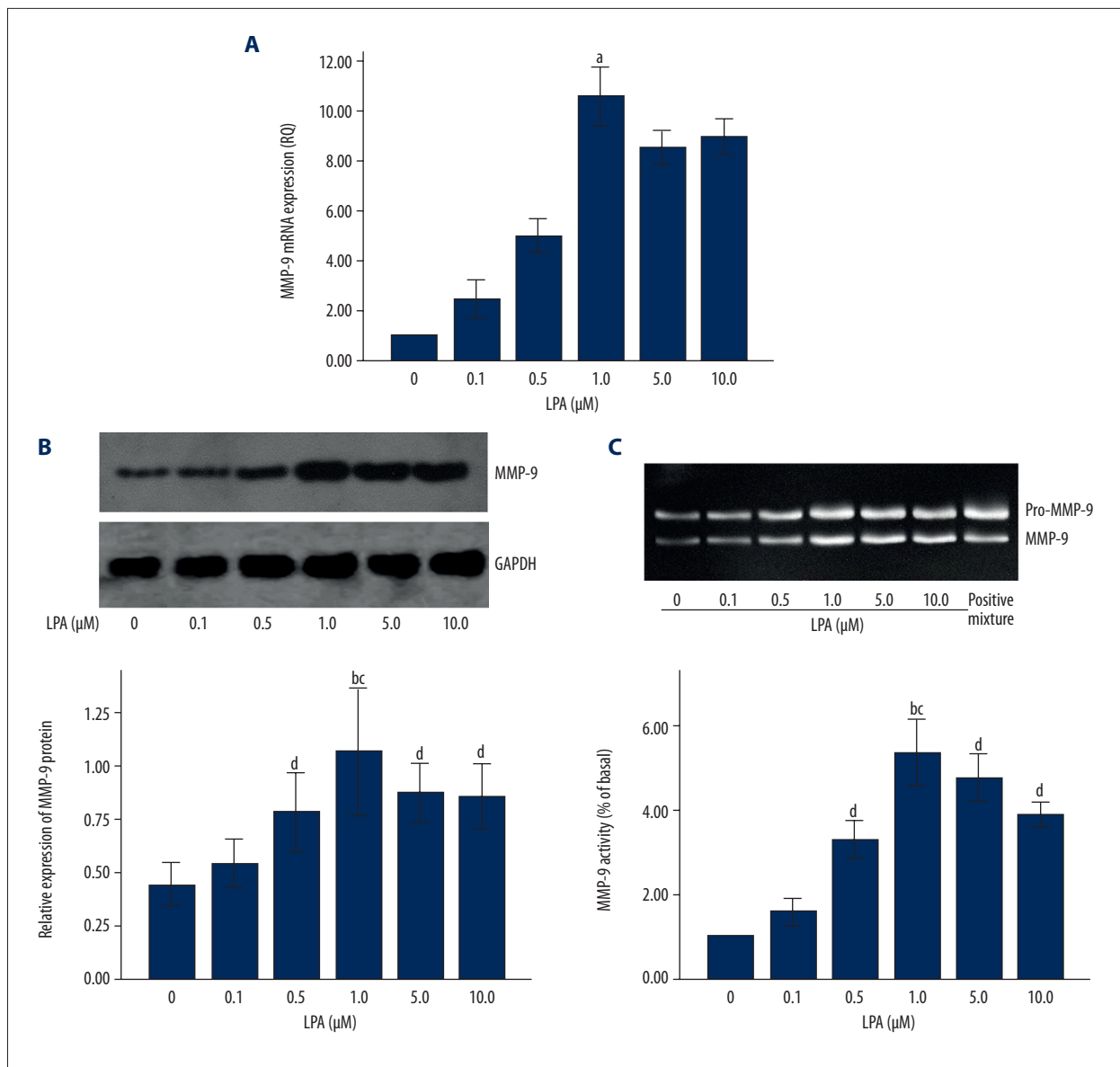


Figure 1. Effects of different LPA concentrations on MMP9 mRNA and protein levels and MMP9 activity in THP-1 cells. Compared with 0, 0.1, 0.5, 5.0, and 10.0 μM LPA-treated groups, MMP9 mRNA (**A**) and protein (**B**) expression and MMP9 activity (**C**) in THP-1 cells in 1.0 μM LPA-treated groups were significantly increased. The levels of MMP9 mRNA and protein and MMP9 activity were then decreased in 5.0 and 10.0 μM LPA-treated groups. ^a $P < 0.01$, vs. 0, 0.1, 0.5, 5.0, and 10.0 μM LPA-treated groups, ^b $P < 0.01$, vs. 0, 0.1, 0.5, and 10.0 μM LPA-treated groups; ^c $P < 0.05$, vs. 5.0 μM LPA-treated groups; ^d $P < 0.01$, ^e $P < 0.05$ vs. 0 μM LPA-treated group; $n = 3$.

concentrations, the levels of MMP9 mRNA and protein and MMP9 activity then decreased (Figure 1A–1C). After treatment of THP-1 cells with 1 μM LPA for 0, 1, 2, 4, or 8 h, MMP9 mRNA and protein levels and MMP9 activity increased with time up to 4 h and then decreased (Figure 2A–2C). These results indicate that LPA can increase the mRNA and protein levels and activity of MMP9 in THP-1 cells in concentration- and time-dependent manners.

Screening of optimal siRNA Sequences

TLR4 mRNA levels in TLR4-siRNA-1, TLR4-siRNA-2, and TLR4-siRNA-3 transfection groups were significantly lower than those in the control and negative control siRNA groups ($P < 0.05$), and the lowest *TLR4* mRNA level was observed in the TLR4-siRNA-2 transfection group. There was no significant difference in *TLR4* mRNA level between the control and negative control siRNA

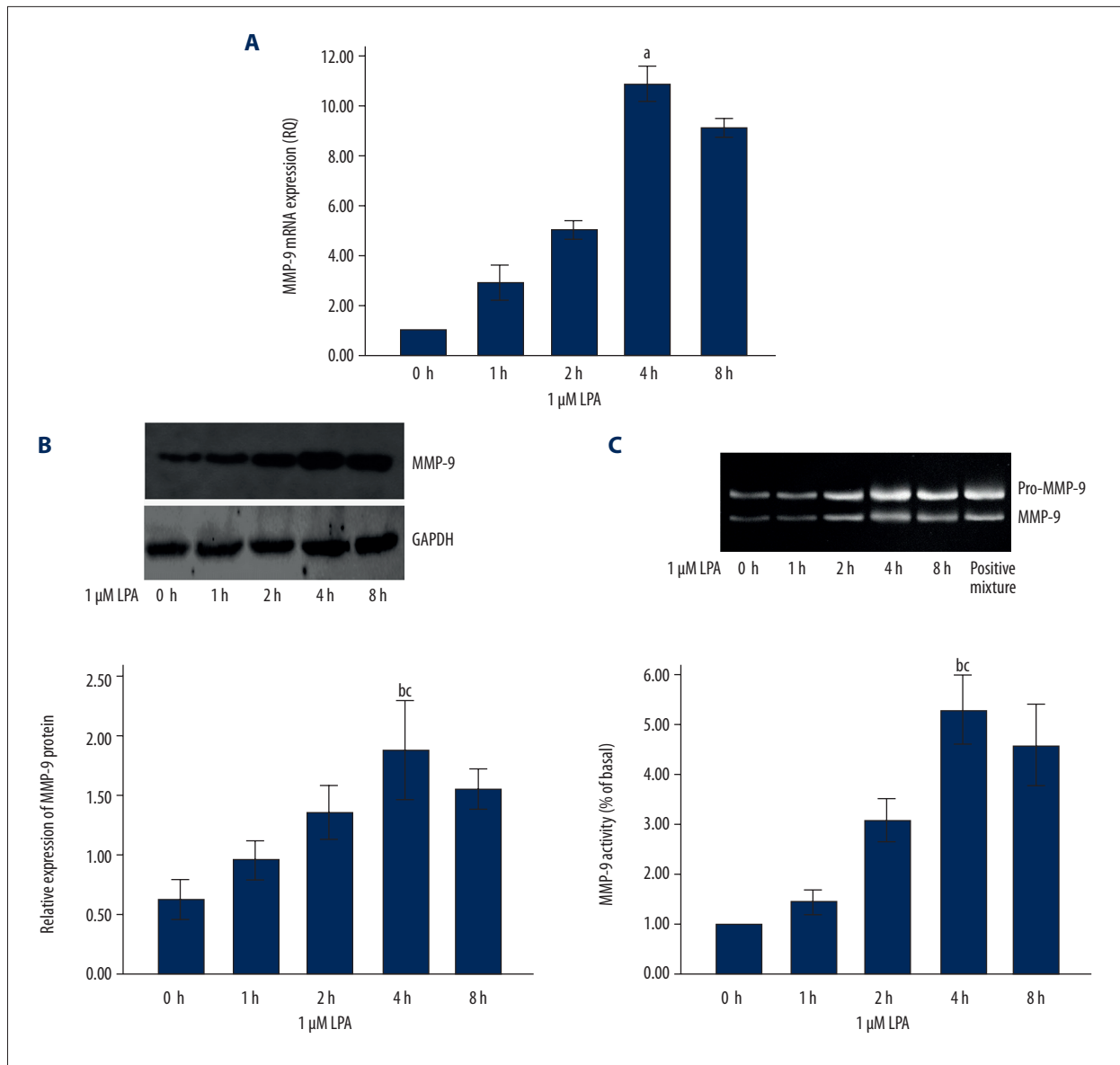


Figure 2. MMP9 mRNA and protein levels and MMP9 activity in THP-1 cells after treatment with 1 μ M LPA for different times. The MMP9 mRNA (A) and protein levels (B) and MMP9 activity (C) were significantly increased after treatment of THP-1 cells with 1 μ M LPA for 4 hours. ^a $P < 0.01$, vs. 0 h, 1 h, 2 h and 8 h groups, ^b $P < 0.01$ vs. 0 h, 1 h, and 2 h groups, ^c $P < 0.05$ vs. 8 h group. $n = 3$.

groups (Figure 3A), which confirmed the specificity of the siRNA for the target gene.

In comparison with the control and negative control siRNA groups, TLR4 protein levels were significantly decreased in the TLR4-siRNA-1, TLR4-siRNA-2, and TLR4-siRNA-3 groups. TLR4-siRNA-2 exhibited the strongest inhibition effect (Figure 3B); therefore, siRNA-2 was used in subsequent experiments.

Effect of TLR4-siRNA-2 transfection and CAPE treatment on MMP9 mRNA and protein levels and on MMP9 activity in THP-1 cells treated with LPA

TLR4-siRNA-2 transfection alone and CAPE treatment alone significantly inhibited the increased MMP9 mRNA and protein levels and MMP9 activity in LPA-induced THP-1 cells. The inhibition was further enhanced by combining TLR4-siRNA-2 transfection and CAPE pretreatment (Figure 4).

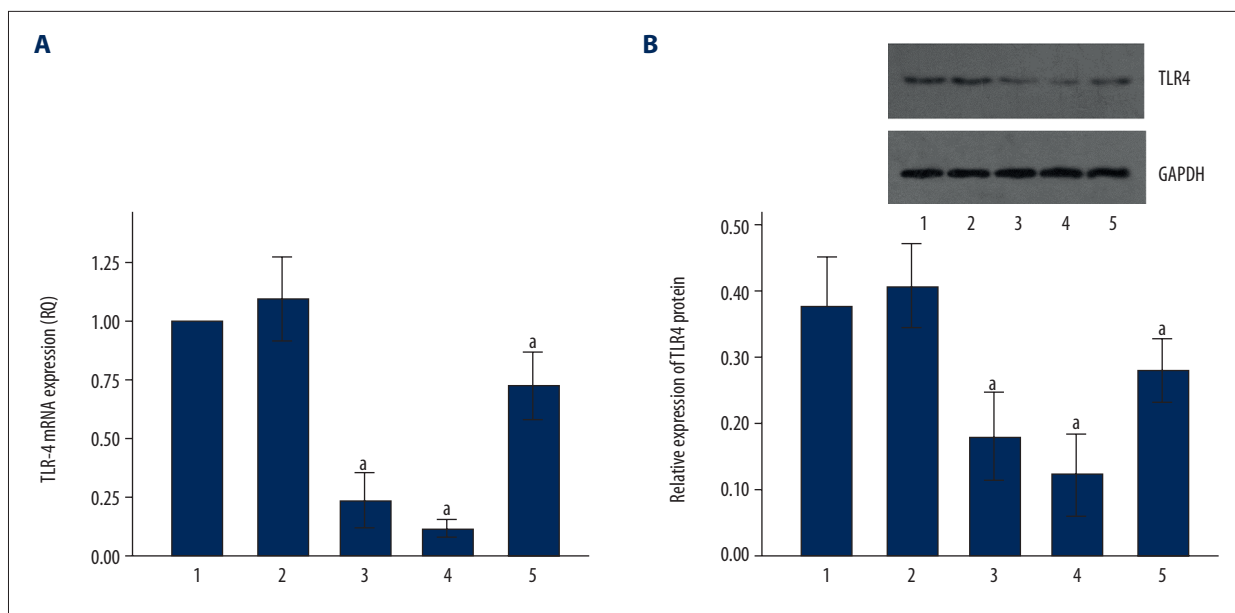


Figure 3. Effect of TLR4 siRNA transfection on TLR4 mRNA and protein levels in THP-1 cells. Compared with control and negative control siRNA groups, the TLR4 mRNA (A) and protein levels (B) were significantly decreased in the TLR4-siRNA-1, TLR4-siRNA-2, and TLR4-siRNA-3 groups, and the lowest TLR4 mRNA level was observed in the TLR4-siRNA-2 group. No significant difference was found between the control and negative control siRNA groups. 1: control group; 2: negative control siRNA group; 3: TLR4-siRNA-1 transfection group; 4: TLR4-siRNA-2 transfection group; 5: TLR4-siRNA-3 transfection group. ^a $P < 0.01$, vs. control and negative control siRNA groups.

Discussion

LPA can cause platelet activation and endothelial cell dysfunction, and can trigger an inflammatory response in the vascular wall, all of which can induce plaque instability, rupture, and local embolus formation. This can lead to stroke and ischemic events, such as myocardial infarction [8,9]. LPA is therefore a pathogenic factor in promoting arteriosclerosis and thrombosis, but can also be used as an early warning factor to reflect different degrees of platelet activation [10].

Increased expression of MMP9 is associated with many diseases, including cancer [11] and atherosclerosis [12,13]. MMP9 can promote the development of atherosclerosis and is recognized as a major factor in causing plaque instability [12,13]. Mononuclear macrophages are frequently observed during the formation of unstable plaques [14]. LPA can activate monocytes and T lymphocytes and promote the expression and secretion of MMPs, leading to plaque instability and rupture. LPA and MMP9 levels were significantly increased in a rat atherosclerotic plaque model, with a significant positive correlation between LPA and MMP9 levels [15]. Wu et al. [16] found that LPA can promote MMP2 expression and activity in human endothelial cells. In the present study, we found that LPA can upregulate MMP9 mRNA and protein levels and MMP9 and activity in THP-1 cells in concentration- and time-dependent manners, the MMP9 mRNA and protein levels and MMP9 and activity were

clearly increased after treatment of THP-1 cells with 1 μ M LPA for 4 h. The upregulated MMP9 expression and activation induced by LPA may be one of the mechanisms contributing to the development of atherosclerosis and the formation of unstable plaques, as well as the occurrence of vascular events.

MMP9 is mainly regulated at 3 levels: gene transcription, activation of zymogen, and interaction with TIMP metalloproteinase inhibitor 1 (TIMP1) [17]. The transcriptional regulation of *MMP9* is mediated by transcription factor activator protein 1 (AP1) and the NF- κ B pathway [5]. NF- κ B comprises a group of transcription factors. NF- κ B dimers are normally present in the cytoplasm complexed with the inhibitory protein I κ B. When cells are stimulated by various factors, I κ B is activated by phosphorylation and is translocated from the cytoplasm into the nucleus. After binding with NF- κ B-specific sites (operators), gene transcription is activated [18–20]. NF- κ B activation can induce the expression of chemokines, adhesion factors, MMPs, and chemokines. Adhesion factors and MMPs play a direct role in target cell activation, proliferation, infiltration, chemotaxis, and secretion. The MMP9 promoter sequence contains NF- κ B binding sites, termed C-terminal activation region (CTAR)-1 and CTAR-2. The Epstein-Barr virus oncoprotein, LMP1, can activate NF- κ B and upregulate MMP9 [21].

In a previous study by our group [22], we found that LPA can upregulate TLR4 mRNA and protein levels in THP-1 cells, as

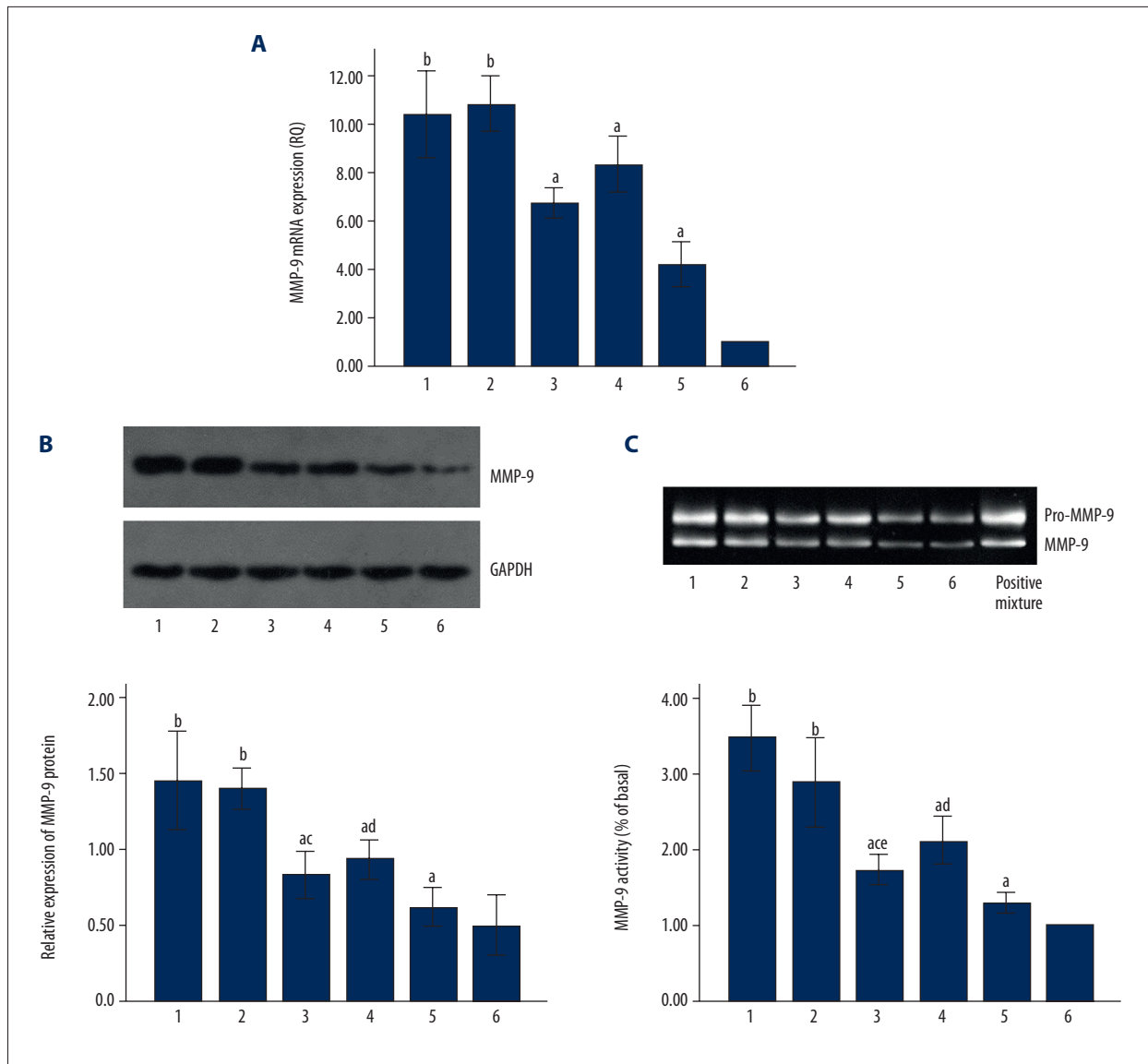


Figure 4. Effect of TLR4-siRNA-2 transfection on MMP9 mRNA and protein levels and MMP9 activity in LPA-induced THP-1 cells. In comparison with the 1 μ M LPA-treated 4 h group, negative control siRNA group, and blank control group, the MMP9 mRNA (A) and protein (B) levels and MMP9 activity (C) in LPA-induced THP-1 cells were significantly decreased in the TLR4-siRNA transfection group, 20 μ g/ml CAPE pretreated and negative control siRNA group, and the 20 μ g/ml CAPE pretreated and TLR4-siRNA transfection group. The most obvious decrease was observed in the 20 μ g/ml CAPE pretreated and TLR4-siRNA transfection group. 1: 1 μ M LPA-treated 4 h group; 2: negative control siRNA group; 3: TLR4-siRNA transfection group; 4: 20 μ g/ml CAPE pretreated and negative control siRNA group; 5: 20 μ g/ml CAPE pretreated and TLR4-siRNA transfection group; 6: Blank control group. ^a $P < 0.01$, vs. 1 μ M LPA-treated 4 h group, negative control siRNA group and blank control group, ^b $P < 0.01$ vs. Blank control group, ^c $P < 0.05$, ^d $P < 0.01$ vs. 20 μ g/ml CAPE pretreated and TLR4-siRNA transfection group, ^e $P < 0.05$ vs. 20 μ g/ml CAPE pretreated and negative control siRNA group.

well as promoting NF- κ B activation and increasing NF- κ B p65 expression in THP-1 cells, and the NF- κ B p65 expression was consistent with the TLR4 expression. In this study, we found that after the LPA-induced THP-1 cells were transfected with TLR4-siRNA-1, TLR4-siRNA-2, and TLR4-siRNA-3, the TLR4 mRNA and protein levels were significantly decreased and the inhibition effect was more obvious in cells transfected with

TLR4-siRNA-2. We also found that TLR4-siRNA-2 transfection alone and CAPE treatment alone can significantly inhibit the increased levels of MMP9 mRNA and protein and MMP9 activity in LPA-induced THP-1 cells. This inhibition effect was further enhanced by combining TLR4-siRNA-2 transfection and CAPE pretreatment.

Conclusions

LPA can promote MMP9 expression and activation in THP-1 cells, partly through the TLR4/NF- κ B signaling pathway, thereby leading to the development of AS, the formation of unstable plaques, and the occurrence of vascular events. Therefore, therapeutic interventions targeting the LPA-TLR4/NF- κ B-MMP9

signaling pathway may provide a new approach for anti-atherosclerosis treatment.

Conflicts of interest

None.

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