Beta-adrenoceptor Activation by Norepinephrine Enhances Lipopolysaccharide-induced Matrix Metalloproteinase-9 Expression Through the ERK/JNK-c-Fos Pathway in Human THP-1 Cells

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**Aim:** Atherosclerosis is a chronic inflammatory disease, which leads to thrombosis and acute coronary syndrome. Matrix metalloproteinase-9 (MMP-9) is involved in the stability of the extracellular matrix (ECM) and atherosclerosis plaque. Until now, it is established that lipopolysaccharide (LPS) and norepinephrine (NE) are associated with the pathological process of atherosclerosis. However, the combined effect of LPS and NE on MMP-9 is unclear. We investigated the combined effect of LPS and NE on MMP-9 expression in human monocytes and the mechanism involved in the process.

**Methods:** THP-1 cells were cultured and treated with LPS and/or NE. MMP-9 and TIMP-1 gene and protein expression were detected by real time PCR and ELISA, respectively. MMP-9 activity was detected by gelatin zymography. Adrenoceptor antagonists and MAPKs inhibitors were used to clarify the mechanism. Pathway-related proteins were detected by Western blot.

**Results:** We found that NE enhances LPS-induced MMP-9 and TIMP-1 expression as well as MMP-9 activity in THP-1 cells. This effect is reversed by the beta (β)-adrenoceptor antagonist propranolol, extracellular signal-regulated kinases (ERK) inhibitor U0126, and c-Jun N-terminal kinase (JNK) inhibitor SP600125. NE enhances LPS-induced ERK/JNK phosphorylation. NE up-regulates LPS-induced c-Fos expression, which is counteracted by propranolol, U0126, and SP600125. Furthermore, c-Fos silence reverses the effect of NE on MMP-9 activity.

**Conclusions:** Our results suggest that NE enhances LPS-induced MMP-9 expression through β-adrenergic receptor and downstream ERK/JNK-c-Fos pathway. This study may help us to understand the combined effect and mechanism of NE/LPS on MMP-9 expression.

**Key words:** Norepinephrine, Lipopolysaccharide, Matrix metalloproteinase-9, Atherosclerosis

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**Introduction**

Atherosclerosis is recognized as a chronic inflammatory disease. Considerable evidence supports that immune cells, such as monocytes, are involved in this process. Monocytes and macrophages accumulate in the growing lesion, ingest lipids, and contribute to the development of atherosclerosis. Rupture of atherosclerosis plaques, which frequently occurs in regions that contain many monocytes, leads to coronary thrombosis and acute coronary syndrome.² Rupture of atherosclerosis plaques always occurs when there is severe inflammation response and stress stimulation, which leads to the structure of the thin fibrous cap being destroyed. Matrix metalloproteinase-9 (MMP-9) is involved in this process. MMP-9 and its natural inhibitor, which is called the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), play an important role in the regulation of extracellular matrix (ECM) metabolism. Studies have shown that MMP-9 is related to plaque instability.² High expression of MMP-9 is observed in atherosclerotic plaques, particularly in the shoulder regions, which are more prone to instability and rupture.³,⁴
Cell Culture

Primary monocytes from healthy donors were isolated using a lymphocyte separation kit and the density gradient separation method. Informed consent was obtained from all participants. The studies were approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. The monocytic cell line THP-1 was obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). Cells were maintained in RPMI-1640 containing 10% FCS as well as 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Experiments were performed after a 24 h cell starvation in RPMI-1640 with 1% FBS. These cells were centrifuged and resuspended in fresh RPMI-1640 with 1% FBS and seeded into 24-well plates at a density of 1 × 10⁶ cells/ml for treatment.

ELISA

The levels of MMP-9 and TIMP-1 in the cell supernatants were determined using MMP-9 and TIMP-1 ELISA kits (eBioscience, USA) according to the manufacturer’s instructions.

Real-time PCR (RT PCR)

Total RNA was isolated from primary monocytes and THP-1 cells using Trizol reagent (Life Technology) and was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). RT PCR was performed with the FastStart Universal SYBR Green Master (Roche), and the reactions were conducted in ABI 7500 FAST RT PCR system. The nucleotide sequences of primers used are shown in Table 1.

Western Blot Analysis

THP-1 cells were harvested in RIPA lysis buffer (Biotek Co, Beijing, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and then centrifuged at 12,000 g for 15 min at 4°C. Whole cell lysates were used for analysis. Equal amounts of protein were sepa-
assess gelatinolytic activity, the gels were incubated for 24 h at 37°C in a developing buffer (LC2671, Life Technology) and stained with Coomassie Brilliant Blue solution.

**RNA Interference**

c-Fos siRNA (catalog number sc-29221) and negative control siRNA (catalog number sc-37007) were purchased from Santa Cruz Biotechnology, Inc. For the transfection procedure, cells were grown to 70%–80% confluence, and c-Fos siRNA and control siRNA were transfected using Lipofetamine™2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. At 24 h post-transfection, the cells received different treatments. Then, cells were harvested for Western blot analyses and gelatin zymography.

**Assay of MMP-9 by Gelatin Zymography**

MMP-9 activity was assessed by gelatin zymography. In brief, we cultured cells (1 × 10⁶ cells/ml) for different treatments, collected culture supernatants, added buffer without reducing the agent to the supernatants, and subjected the supernatants to electrophoresis on 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, we incubated the gels in 1× Zymogram Renaturing Buffer (LC2670, Life Technology) for 30 min with gentle agitation. To

Fig. 1. LPS upregulates MMPs and TIMP-1 expression in human monocyte cells

Primary human monocytes and THP-1 cells (1 × 10⁶ cells/ml) were dispensed on 24-well plates until 70%–80% confluent and then treated with LPS (1 µg/ml). The MMPs mRNA level was detected by RT-PCR 3 h after stimulation in (A) primary human monocytes (B) THP-1 cells. TIMP-1 mRNA and protein levels were detected for the indicated time using RT-PCR and ELISA KIT in THP-1 cells (C and D). The cell-free supernatants were assayed for MMP-9 activity by gelatin zymography (E). Data are expressed as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001.
LPS Upregulates MMPs and Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) Expression in Human Monocytes

As shown in Fig. 1A and Fig. 1B, LPS at 1 µg/ml significantly increased the gene expression of several main matrix metalloproteinases (MMP-1, MMP-3, MMP-9, and MMP-12) ($P<0.001$) in primary isolated human monocytes and THP-1 cells. LPS could also upregulate TIMP-1 gene and protein expression, which is more obvious at the time point of 12 h and 24 h, respectively (Fig. 1C and Fig. 1D). We also assessed gelatinolytic activity of MMP-9 in the culture supernatants after LPS stimulation. As shown in Fig. 1E, LPS-treated cells had strong gelatinolytic activity 24 h and 48 h post-LPS treatment. In contrast, the activity of MMP-2 was unchanged by LPS treatment.

NE Enhances LPS-induced MMP-9 and TIMP-1 Expression

MMP-9 plays an important role in the stability of atherosclerotic plaque. To investigate whether NE could affect LPS-induced TIMP-1 and MMP-9 expres-
ergic receptor antagonist phentolamine (Pht) for 30 min, and then with NE and LPS. Gene and protein expression of MMP-9 were measured in THP-1 cells and cell supernatants, respectively. As described in Fig. 3A and Fig. 3B, NE significantly increased LPS-induced MMP-9 mRNA \((P < 0.001)\) and protein expression \((P < 0.01)\), which were reversed by pre-treatment with propranolol. Furthermore, gelatinolytic activity of MMP-9 enhanced by NE in LPS-challenged THP-1 cells was reversed by propranolol, but not by phentolamine (Fig. 3C).

**The Expression of MMP-9 Induced by NE and LPS is Dependent on ERK/JNK**

It is well recognized that MAPKs activation is involved in the regulation of LPS-induced MMPs expression. Thus, we investigated the effect of extracellular regulated protein kinases (ERK) inhibitor U0126, c-Jun N-terminal kinase (JNK) inhibitor SP600125, and P38 MAPK inhibitor SB203580 on MMP-9 expression after NE and LPS stimulation. As shown in Fig. 4A, U0126 and SP600125 not only reversed the...
NE Enhances LPS-induced c-Fos Expression, which can be Reversed by Propranolol, U0126, and SP600125

Much evidence indicates that activator protein 1 (AP-1) plays an important role in the expression of MMP-9. We further investigated the effect of LPS and NE on c-Fos expression, which forms the AP-1 transcription factor. c-Fos expression increased immediately after LPS-stimulation (Fig. 6A). NE increased c-Fos expression in LPS-challenged THP-1 cells (Fig. 6B). Then, we investigated the role of propranolol, SP600125, and U0126 on c-Fos expression after NE and LPS stimulation. As shown in Fig. 6C and Fig. 6D, Pro, U0126, and SP600125 pretreatment almost completely inhibited c-Fos expression induced by NE and LPS.

NE Enhances LPS-induced c-Jun Phosphorylation

c-Jun is another component of AP-1. We investigated the effect of NE/LPS on c-Jun and P-c-Jun expression. LPS increased c-Jun expression. However, different concentrations of NE (0.01 µmol, 0.1 µmol, and 1.0 µmol) failed to increase LPS-induced c-Jun expression (Fig. 6E). Next, phosphorylation of c-Jun was measured by Western blot in NE/LPS treated cells. We found that NE could increase P-c-Jun expression in LPS-challenged THP-1 cells (Fig. 6F).

c-Fos Silencing Reverses the Effect of NE on MMP-9 Activity in LPS-challenged THP-1 Cells

To investigate the role of c-Fos in NE and LPS-induced MMP-9 expression, we used c-Fos siRNA. As
endothelial cells, fibroblasts, and monocytes/macrophages). Monocytes and macrophages, which are capable of forming foam cells, are protagonists in the process of atherosclerosis. We intended to clarify the effect of NE and LPS on MMP-9 expression in monocyte cells. We found that LPS upregulated MMPs expression in both primary isolated human monocytes and THP-1 cells and also increased MMP-9 activity, which is in accordance with previous reports. We also found that TIMP-1, a natural inhibitor of the MMPs, was upregulated by LPS-stimulation in THP-1 cells, which may be a concomitant response of MMP-9.

Although many previous reports have investigated the interaction between NE and LPS, they mainly focused on the effect of the cytokines release by NE and LPS. Speidl et al. have demonstrated that catecholamines could potentiate LPS-induced expression of MMP-9 expression in U937 cells; however, little is known about the mechanism and pathway involved in this process. In the present study, we found that NE increased LPS-induced MMP-9 gene expression as well as the protein level and gelatinolytic activity in THP-1 cells.

**Discussion**

The results of the present study suggest that NE enhances LPS-induced MMP-9 expression through β-adrenergic receptor and downstream ERK/JNK-c-Fos pathway. It is well established that the expression of MMPs plays an important role in the pathology of atherosclerosis diseases. Recent study has shown that MMP-9, also known as gelatinase, is strongly correlated with plaque instability. MMP-9 is significantly higher in patients with coronary artery disease than in those in the control group. Over-expressed MMP-9 is accumulated in the shoulder region of the plaque where it is vulnerable to rupture; inhibition of MMP-9 stabilizes the arteries by increasing their collagen content.

MMP-9 can be secreted by various cells, such as endothelial cells, fibroblasts, and monocytes/macrophages. Monocytes and macrophages, which are capable of forming foam cells, are protagonists in the process of atherosclerosis. We intended to clarify the effect of NE and LPS on MMP-9 expression in monocyte cells. We found that LPS upregulated MMPs expression in both primary isolated human monocytes and THP-1 cells and also increased MMP-9 activity, which is in accordance with previous reports. We also found that TIMP-1, a natural inhibitor of the MMPs, was upregulated by LPS-stimulation in THP-1 cells, which may be a concomitant response of MMP-9.

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Fig. 6. NE enhances LPS-induced c-Fos and P-c-Jun expression. NE/LPS induced c-Fos expression can be reversed by propranolol, U0126, and SP600125.

THP-1 cells were incubated with LPS for 0, 5, 10, 20, 30, 40, 50, and 60 min. c-Fos expression was measured by Western blot (A). After being exposed to different concentrations of NE for 40 min, THP-1 cells were stimulated with LPS for another 30 min. c-Fos expression was determined by Western blot (B). Cells were pre-treated with Pro, U0126, SP600125, or a vehicle for 30 min, NE for 40 min, and then with LPS for another 30 min. c-Fos levels were detected by Western blot (C-D). After being exposed to NE for 40 min, THP-1 cells were stimulated with LPS for another 30 min. c-Jun and P-c-Jun were determined by Western blot (E-F). NS indicates no significant difference. 

*P < 0.05, **P < 0.01, ***P < 0.001.
MMP-9 production process. We found that the $\beta$-adrenergic receptor inhibitor propranolol nearly completely inhibited NE-induced MMP-9 (1) gene expression, (2) the protein level, and (3) gelatinolytic activity, whereas phentolamine, an $\alpha$-adrenergic receptor inhibitor, had no effect on MMP-9 activity. Hori et al. proved that administration of isoprenaline ($\beta$-adrenoceptor agonist) could increase MMP-9 expression 22). Another study showed that atenolol ($\beta$-1 adrenoceptor antagonist) attenuates the MMP-9 increment by Angiotensin $\alpha$23). All these studies indicate that $\beta$-adrenergic receptors play an important role in MMP-9 expression.

Accumulating evidence indicates that LPS increases MMP-9 production by activating MAPKs in various cells24, 25). We employed MAPKs inhibitors to clarify the pathway involved in NE- and LPS-challenged THP-1 cells. We found that NE/LPS-induced AP-1 activation also upregulates TIMP-1 expression. We believe this effect is very important. Meanwhile, extracellular stimulus increases MMP-9 and TIMP-1 expression to maintain ECM balance.

NE could activate $\alpha$- and $\beta$-adrenergic receptors. Previous studies have shown that different receptor stimulations exert different effects23). Thus, we intended to clarify which type of receptor was involved in the MMP-9 production process. We found that the $\beta$-adrenergic receptor inhibitor propranolol nearly completely inhibited NE-induced MMP-9 (1) gene expression, (2) the protein level, and (3) gelatinolytic activity, whereas phentolamine, an $\alpha$-adrenergic receptor inhibitor, had no effect on MMP-9 activity. Hori et al. proved that administration of isoprenaline ($\beta$-adrenoceptor agonist) could increase MMP-9 expression 22). Another study showed that atenolol ($\beta$-1 adrenoceptor antagonist) attenuates the MMP-9 increment by Angiotensin $\alpha$23). All these studies indicate that $\beta$-adrenergic receptors play an important role in MMP-9 expression.

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MMP-9 expression. On the contrary, P38 inhibitor SB203580 increased MMP-9 expression by LPS and NE/LPS. Furthermore, NE inhibits P38 phosphorylation evoked by LPS. It seems that inhibition of P38 activity may contribute to MMP-9 expression in our study. However, other studies have shown P38 kinase phosphorylation is essential in the process of LPS-induced MMP-9 expression. The inconsistent results may be due to the different cell types used. Previous studies also showed that there is a crosstalk between P38 and the ERK pathway; inhibition of P38 phosphorylation enhances ERK phosphorylation. It is reasonable to speculate that inhibition of P38 in LPS and NE challenged THP-1 cells enhanced the ERK signaling pathway, which resulted in the overexpression of MMP-9.

The most prominent transcription factors implicated in MMP-9 gene activation are AP-1 and NF-κB. We detected NE/LPS induced NF-κB P-P65 and P65 expression (Supplemental Fig. 2). However, we found that NE could not enhance LPS-induced P65 activation. It seems that the NF-κB pathway is not involved in the mechanism. c-Fos and c-Jun are two important components of the AP-1 transcription factor. In our study, we found that LPS increased c-Fos expression, and NE enhanced this effect. We also employed c-Fos siRNA to clarify its role in MMP-9 expression. As is shown, c-Fos knockdown partly abolished MMP-9 gelatinolytic activity which is evoked by NE and LPS. Interestingly, when we pre-treated THP-1 cells with ERK and JNK inhibitors, the augmentation of c-Fos expression is alleviated. When pre-treated with propranolol, as we expected, the NE-induced augmentation of c-Fos expression in LPS-challenged cells is also abolished. However, NE could not affect LPS-induced c-Jun expression at all. But we found that NE could also increase P-c-Jun expression in LPS-challenged THP-1 cells. It seems that c-Jun activation is also involved in NE/LPS induced MMP-9 expression. This result could also explain why c-Fos knockdown did not completely abolish MMP-9 gelatinolytic activity in Fig. 7B. However, more studies are needed to clarify the role and mechanism of P-c-Jun in NE/LPS induced MMP-9 expression.

Conclusions

In conclusion, we demonstrated that LPS and NE, two important factors in the pathologic process of atherosclerosis, have a synergistic effect on the expression of MMP-9. This effect is associated with the β-adrenergic receptor, and the ERK/JNK-c-Fos pathway.

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Conflict of Interest (COI)

All authors disclose no conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, their work.

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Supplemental Fig. 1. NE enhances LPS-induced MMP-9 activity in the THP-1 derived macrophage

THP-1 cells were incubated with PMA (100 ng/ml) for 72 h. A CD11b-FITC antibody was used to detect macrophages (A). After being exposed to different concentrations of NE or a vehicle for 40 min, and then to LPS for another 48 h, MMP-9 activity was measured by gelatin zymography (B).
Supplemental Fig. 2. NE did not enhance LPS-induced NF-κB P65 phosphorylation. After being exposed to NE for 40 min, THP-1 cells were stimulated with LPS for another 30 min. P-P65 and P-65 expression were determined by Western blot.