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Original Article

Activation of β1 adrenoceptors promotes LPS-induced NGF secretion in macrophages via Notch signaling pathways

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Abstract: *Background*: Macrophages are the main sources of NGF, playing a decisive role in the process of sympathetic nerve remodeling after myocardial infarction (MI). Adrenoceptors present on macrophage membranes and catecholamines act on adrenergic receptors to promote the release of inflammatory cytokines. However, whether epinephrine promotes lipopolysaccharide (LPS)-mediated macrophages to secrete NGF remains unclear. Notch signaling plays an important role in LPS-induced macrophage activation. However, the relationship between Notch pathways and the effects of epinephrine on LPS-mediated macrophages has not been studied. Thus, the present study aimed to explore the effects of epinephrine on LPS-induced NGF expression by macrophages, as well as the underlying molecular mechanisms. *Materials and methods*: Dose- and time-dependent effects of epinephrine on NGF expression in RAW264.7 macrophages were evaluated after LPS activation. Additionally, different adrenergic blockers were administered to explore the receptor subtypes that promote NGF expression. The γ-secretase inhibitor DAPT, which acts by inhibiting the cleavage of transmembrane proteins, including the Notch receptor, was used to investigate whether Notch signaling pathways participate in the effects exerted by epinephrine on LPS-induced macrophage response. *Results*: Present research reveals that epinephrine (0.01 nmol/mL) preconditioning for 2 hours significantly promoted expression of NGF by LPS-induced macrophages, while both metoprolol, a β1 receptor blocker, and DAPT, a γ-secretase inhibitor, significantly inhibited this process. *Conclusion*: Results suggest that β1 adrenoceptors enhance the secretion of NGF by LPS-induced RAW264.7 macrophages and the underlying mechanisms may involve Notch pathways.

Keywords: Macrophages, NGF, Notch, β1 adrenoceptor

Introduction

Previous studies have shown that rapid and sustained upregulation of nerve growth factor (NGF) in infarcted myocardium is the key to cardiac regeneration and sympathetic nerve remodeling [1-4]. Inflammatory cells (macrophages, cardiac fibroblasts, and T-cells) infiltrate after myocardial infarction (MI) and abundantly express NGF in a manner that is consistent in time and space with sympathetic hyperplasia [5-7]. Additional studies have shown that macrophages are the main sources of NGF, playing a decisive role in the process of sympathetic nerve remodeling after MI [5]. However, the underlying molecular mechanisms of peripheral macrophage-induced NGF expression after MI remain poorly understood.

In recent years, the relationship between autonomic nervous system regulation and innate immune system activation has been highlighted [8]. Flierl et al. [9] and Nguyen et al. [10] showed that macrophages synthesize catecholamines and adrenoceptors, which present on macrophage membranes, and autocrine or

paracrine catecholamines, which act on adrenergic receptors to promote the release of inflammatory cytokines. Adrenergic blockers suppress the production of a variety of macrophage-derived inflammatory cytokines. During the process of sympathetic nerve remodeling after MI, increased NGF is almost always accompanied by increased expression of inflammatory factors [11-14]. However, whether epinephrine, a catecholamine, promotes LPS-mediated macrophages to secrete NGF remains unclear.

The Notch signaling pathway is a highly conserved cell signaling system present in most multicellular organisms. Growing evidence has suggested that Notch signaling plays a very important role in lipopolysaccharide (LPS)-induced macrophage activation [15, 16]. Notch signaling promotes the polarization of M1 macrophages and regulates the release of proinflammatory cytokines [16]. In a rat model of MI, the Notch pathway was shown to be involved in the release of NGF from M1 macrophages in the infarct zone [15]. Inhibiting Notch signaling pathways with the specific γ-secretase inhibitor DAPT (N-[N-(3, 5-difluorophenacetyl)-1-alanyl]-S-phenylglycine tert-butyl ester) reduced NGF release from M1 macrophages. However, the relationship between Notch pathways and the effects of epinephrine on LPS-mediated macrophages has not been studied.

Thus, the present study aimed to explore the effects of epinephrine on LPS-induced NGF expression by macrophages, as well as the underlying molecular mechanisms. Dose- and time-dependent effects of epinephrine on NGF expression in RAW264.7 macrophages were evaluated after LPS activation. Additionally, different adrenergic blockers were administered to explore receptor subtypes that promote NGF expression. The γ-secretase inhibitor DAPT, which acts by inhibiting the cleavage of transmembrane proteins, including the Notch receptor, was used to investigate whether the Notch signaling pathways participate in the effects exerted by epinephrine on LPS-induced macrophage response.

The present study identifies new mechanisms that regulate NGF expression, providing alternative strategies to target sympathetic remodeling resulting from NGF overexpression following MI.

Materials and methods

Cell culture

Murine macrophage RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in high-glucose DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Gibco, Wien, Austria) under a humidified atmosphere with 5% CO_2 at 37°C.

Exploring the relationship between LPS processing time and NGF expression

Macrophage RAW264.7 cells (1×10^6 cells/well) were cultured overnight in DMEM containing 10% FBS in two 6-well plates at 37°C and 5% CO_2. High concentrations of LPS may cause the rapid apoptosis of macrophages [17]. Therefore, a lower LPS concentration (10 ng/mL) was chosen to stimulate macrophages. After the cells were washed with PBS, 10 ng/mL LPS (*Escherichia coli* 026:B6, Sigma, St Louis, MO, USA) was added to one plate, while PBS alone was added to the other plate. After incubation for 0, 12, 16, 20, 24, and 36 hours, the supernatants of the two plates were collected. Concentrations of NGF were analyzed by an enzyme-linked immunosorbent assay (ELISA) kit (Cusabio, Wuhan, Hubei, China), according to manufacturer instructions.

Exploring the relationship between epinephrine preconditioning time and NGF expression

Animal studies have shown that concentrations of catecholamines around the infarcted myocardium peak at 1 hour after MI [18], while the number of macrophages in peripheral zones peaks at 24 hours [19]. These findings indicate that macrophages are exposed to catecholamines before they are stimulated by cytokines in the surrounding infarcted area after MI. In early post-MI, macrophages in the myocardium are mainly of the M1 type. Studies have shown that the phenotypes of LPS-induced macrophages and M1-type macrophages are almost the same. They can express TNF-α, IL-1β, IL-6, and other proinflammatory factors, while upregulating expression of costimulatory molecules, such as CD80 and CD86 [20]. Accordingly, in the present study, macrophages were preconditioned with epinephrine and LPS was added later.

Macrophages were plated and the medium was replaced, as previously described. Next, 0.01 nmol/mL epinephrine (MCE, Monmouth Junction, NJ, USA) was added to each well and the cells were incubated for varying periods of time (0, 1, 2, 4, and 6 hours). Afterward, 10 ng/mL LPS was added to each well for 24 hours. Concentrations of NGF in the supernatant were detected by ELISA, determining the optimal epinephrine preconditioning time.

Exploring the relationship between epinephrine preconditioning concentration and NGF expression

Epinephrine was added at various concentrations (0, 0.001, 0.01, 0.1, 1 nmol/mL) into individual wells and incubated for 2 hours at 37°C and 5% CO_2. Macrophages were then stimulated with 10 ng/mL LPS for 24 hours under the same conditions. Supernatants were collected and NGF expression was detected by ELISA, determining the optimal concentration of epinephrine.

Exploring the effects of different blockers on epinephrine-promoted NGF expression by macrophages

Studies have shown that macrophages induced by LPS produce large amounts of pro-inflammatory cytokines IL-1β and TNF-α, which promote NGF expression [21, 22]. To verify the correlation between inflammatory cytokines and NGF expression, this study simultaneously examined changes in concentrations of IL-1β, TNF-α, and NGF.

Macrophage RAW 264.7 cells were preconditioned with various receptor blockers (prazosin, yohimbine, metoprolol, and ICI 118551, all obtained from MCE) (0.1 nmol/mL) [23] for 1 hour, followed by preconditioning with 0.01 nmol/mL epinephrine for 2 hours and stimulation with 10 ng/mL LPS for 24 hours. Expression of NGF and inflammatory cytokines IL-1β and TNF- α was detected by ELISA, RT-PCR, and Western blotting.

Exploring the effects of metoprolol on Notch 1/Jagged 1 expression by macrophages

Macrophages were divided into the following four groups: Group A: PBS, Group B: LPS, Group C: epinephrine + LPS, and Group D: metoprolol + epinephrine + LPS. Macrophages were first preconditioned with metoprolol (0.1 nmol/mL) for 0.5 hours in Group D, then 0.01 nmol/mL epinephrine was exposed to Groups C and D for 2 hours. Finally, 10 ng/mL LPS was added to Groups B, C, and D simultaneously. After 24 hours, cells were collected to perform RT-PCR and Western blotting.

Exploring the effects of DAPT on epinephrine-promoted NGF expression by macrophages

Macrophages were divided into the following six groups: Group A: PBS, Group B: LPS alone, Group C: epinephrine + LPS, Group D: metoprolol + epinephrine + LPS, Group E: DAPT + epinephrine + LPS, and Group F: DAPT + LPS. First, macrophages were preconditioned with metoprolol (0.1 nmol/mL) or the γ-secretase inhibitor DAPT (Sigma, St. Louis, MO, USA) (10 μg/mL) [24] for 1 hour in Groups D, E, and F, simultaneously. Groups C, D, and E were then exposed to 0.01 nmol/mL epinephrine for 2 hours. Finally, Groups B-F were simultaneously stimulated with 10 ng/mL LPS for 24 hours. Supernatants were then assayed, determining their NGF concentrations by ELISA.

Quantitative real-time PCR

RAW264.7 macrophages were collected and dissolved in TRIzol Reagent (Sigma, St Louis, MO, USA). Total RNA was extracted, according to manufacturer instructions. Potential DNA contamination was eliminated using DNase. For RT-PCR, cDNA was synthesized from 2 μg of total RNA using Reverse Transcription Kit 036A (TaKaRa, Dalian, Liaoning, China). The housekeeping gene β-actin was amplified as a quality and positioning control. The following forward and reverse primers were used for PCR amplification [21, 25, 26]: NGF, forward (5'-ATGGTGGAGTTTTGGCCTGT-3') and reverse (5'-GTACGCCGATCAAAAACGCA-3'); Notch 1, forward (5'-CTGTGTGGATGAGGGAGATAA-3' and reverse (5'-GGCATAGACAGCGGTAGAAA-3'); Jagged 1, forward (5'-CCTCCAGCCTCCAGCCAGTG-3') and reverse (5'-TGTTTGTCCAGTTCGGGTGTTTTG-3'); IL-1β, forward (5'-TGCAGAGTTCCCCAACTGGTACA-3') and reverse (5'-GTGCTGCCTAATGTCCCCTT-G-3'); TNF-α, foward (5'-TCAGCCTCTTCTCATTCCTG-3') and reverse (5'-TGAAGAGAACCTGGGAGTAG-3'); and β-actin, forward (5'-GATCCGTGAAGATCAAGATCATTGCT-3') and reverse (5'-TGATCTTCATTTTTTACGCGTGAATT-3'). After obtaining threshold cycle (CT) values, RNA concentrations of each gene relative to β-actin

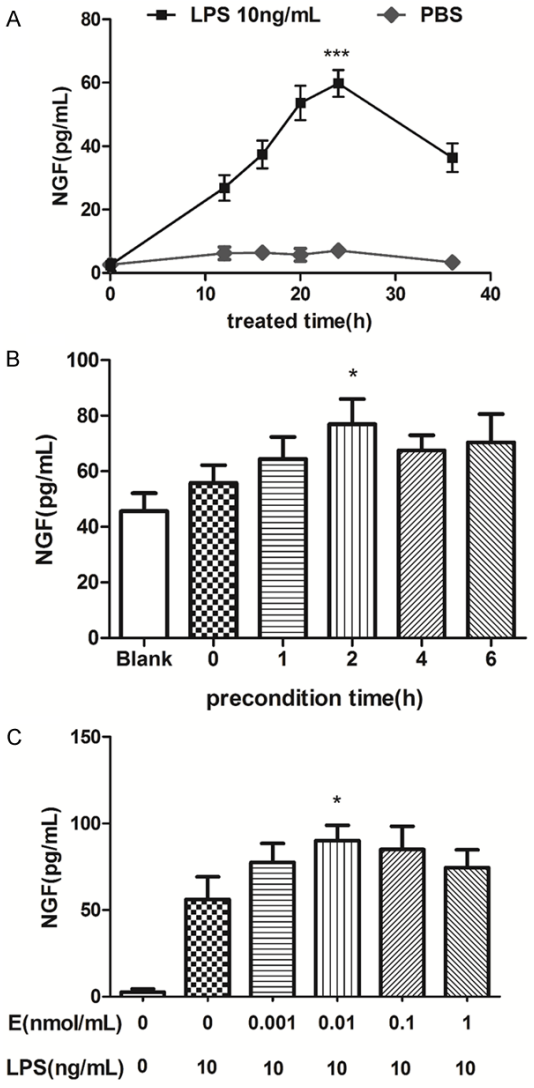

Figure 1. Epinephrine (E) preconditioning promotes LPS-induced macrophage expression of NGF. A. Macrophage RAW 264.7 cells were stimulated with 10 ng/mL LPS or PBS for different time. ***P<0.001 compared to the PBS group. B. Macrophages were preconditioned with 0.01 nmol/mL epinephrine for different times before being stimulated with 10 ng/mL LPS for 24 hours. (Blank group received LPS only). *P<0.05, compared with the 0-hour group. C. Macrophages were preconditioned with different concentrations of epinephrine for 2 hours before stimulation with 10 ng/mL LPS for 24 hours. *P<0.05, **P<0.01, compared to the group stimulated with 10 ng/mL LPS alone without epinephrine. Concentrations of NGF in the supernatant were measured by ELISA. All data are presented as the mean ± SD; n = 3 per group.

mRNA were determined using the following equation: 2-ΔCT, where ΔCT = (CT mRNA - CTβ-actin mRNA).

Western blotting

Western blotting analysis of NGF, Notch 1, Jagged I, and β-actin was performed, as previously described [27]. First, 50 μg of total cell lysates was separated on a 10% SDS-polyacrylamide gel and transferred to PVDF membranes (BioTrace, Millipore, USA). Each membrane was blocked with 5% skim milk in Tris-buffered saline (150 mM NaCl, 10 mM Tris-HCl, pH 7.5) with 0.01% Tween 20 (TBS-T) buffer. To detect target proteins, the membrane was incubated with primary antibodies (1:1000, Cell Signaling, Beverly, MA, USA) specific for NGF, Notch 1, Jagged 1, and β-actin overnight at 4°C. The membrane was then washed with TBS-T buffer and incubated with horseradish peroxidase (HRP)-linked anti-mouse IgG secondary antibodies (Abcam, Cambridge, MA, USA). Detection was performed using enhanced chemiluminescence (ECL, Millipore, USA). Detected images were captured using a Western imaging system ChemiDOC (Bio-Rad, USA) and quantified using Image Lab (Bio-Rad, USA).

Statistics

Statistical analysis was performed with Graph Prism 6 (Graph Pad, La Jolla, CA). Results are expressed as the mean ± SD. Unpaired Student's t-test and one-way ANOVA were used to compare normality, homogeneity of variance, and differences among groups. P<0.05 indicates statistical significance.

Results

Macrophages pretreated with 0.01 nmol/mL epinephrine for 2 hours significantly increased LPS-induced NGF expression

After incubation with LPS, concentrations of NGF in macrophage supernatants gradually increased and peaked at 24 hours (**Figure 1A**). Thus, 24 hours was chosen as the time point for supernatant extraction after LPS stimulation.

Macrophages were pretreated with 0.01 nmol/mL epinephrine for different periods of time. Results showed that increases in NGF concentrations were most obvious in the 2-hour preconditioning group (**Figure 1B**). Therefore, macrophages were pretreated with different con-

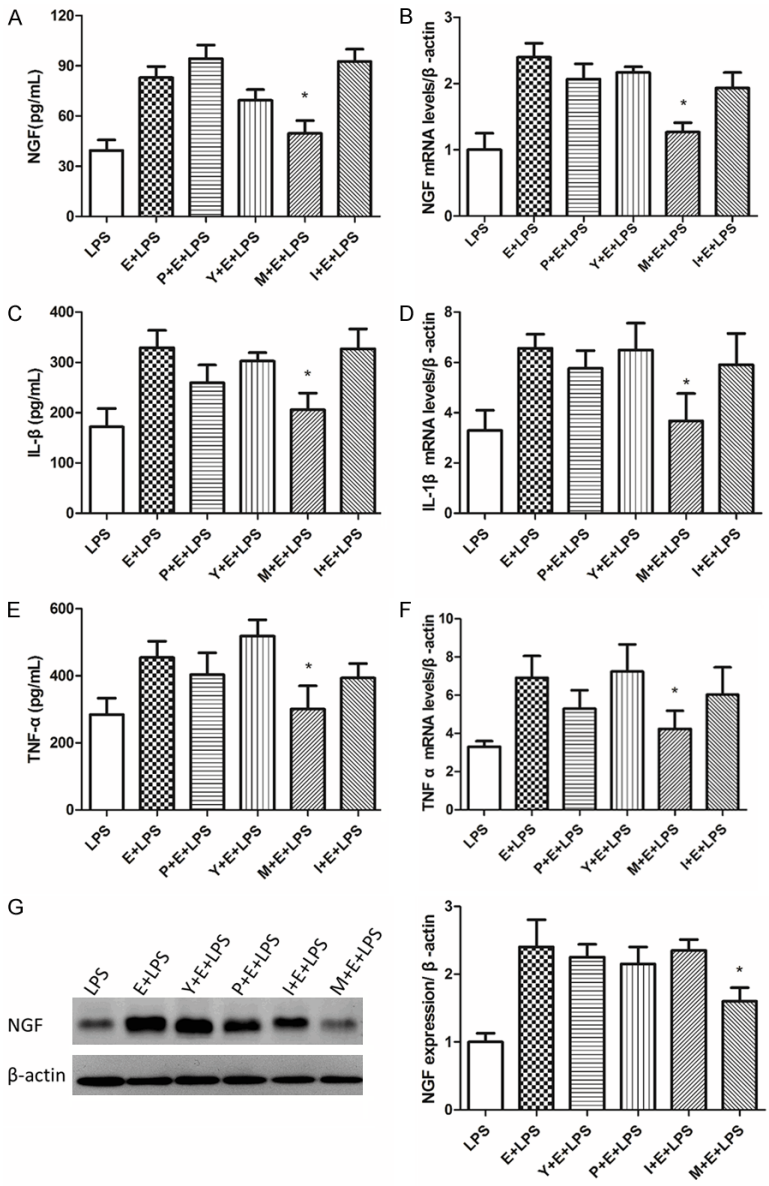

Figure 2. Metoprolol (0.1 nmol/mL) inhibited the effects of epinephrine on expression of NGF, IL-1β, and TNF-α in macrophages. Macrophage RAW 264.7 cells were preconditioned with various receptor blockers (P: prazosin; Y: yohimbine; M: metoprolol; I: ICI 118551) (0.1 nmol/mL) for 1 hour, followed by preconditioning with 0.01 nmol/mL epinephrine for 2 hours and finally 10 ng/mL LPS stimulation for 24 hours. Concentrations of NGF, IL-1β, and TNF-α in the supernatant were measured by ELISA (A-C), mRNA expression was detected by RT-PCR (D-F) (compared with β-actin), and NGF translation levels were detected by Western blotting (G shows electrophoresis results and semi-quantitative analysis). *P<0.05, **P<0.01 compared to E + LPS control group. All data are presented as the mean ± SD; n = 3 per group.

centrations of epinephrine for 2 hours prior to the addition of LPS. After 24 hours, concentrations of NGF in the 0.01 nmol/mL epinephrine preconditioning group increased most obviously (**Figure 1C**).

Metoprolol, the main receptor for epinephrine, promotes LPS-induced NGF and inflammatory cytokine expression

Secretion (**Figure 2A**), transcription (**Figure 2D**), and translation (**Figure 2G**) of NGF in the metoprolol group were significantly inhibited, compared with levels in groups preconditioned with other receptor blockers. Metoprolol also inhibited the effects of epinephrine on secretion and transcription of IL-1β (**Figure 2B** and **2E**) and TNF-α (**Figure 2C** and **2F**).

Metoprolol inhibits the effects of epinephrine on Notch 1/Jagged 1 expression

As shown in **Figure 3**, Notch 1 and Jagged 1 expression was markedly increased after LPS treatment, while transcription (**Figure 3A**) and translation (**Figure 3B**) levels were further increased after epinephrine preconditioning. However, metoprolol inhibited the effects of epinephrine on Notch 1/Jagged 1 transcription and translation levels.

DAPT inhibited the effects of epinephrine on NGF expression

As shown in **Figure 4**, the Notch receptor inhibitor DAPT, as well as metoprolol, significantly inhibited epinephrine-induced NGF expression. NGF expression in the DAPT + LPS group also decreased significantly, compared with the LPS group.

Discussion

Results of the present study suggest that preconditioning with low concentrations of epinephrine for 2 hours promotes expression of large

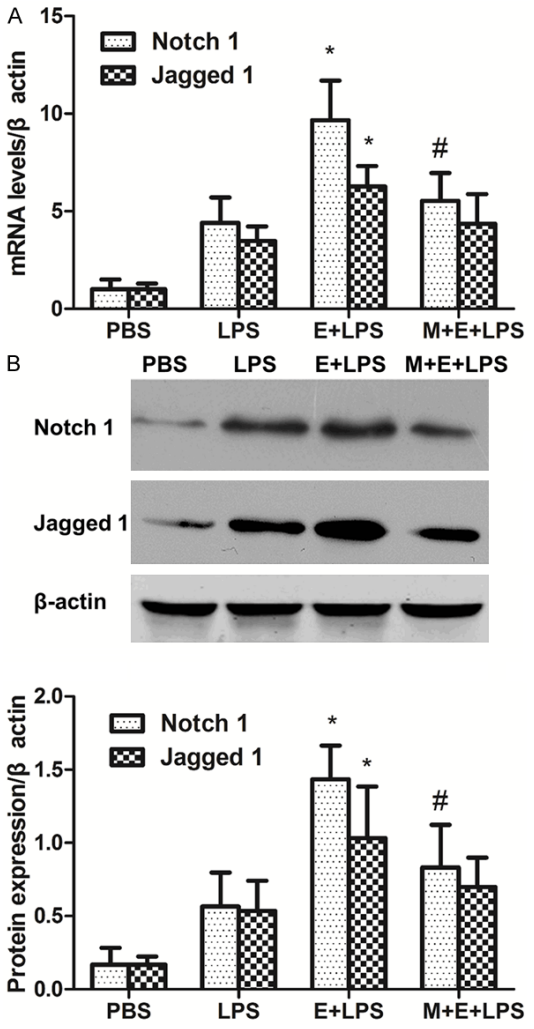

Figure 3. β1 receptor activation promotes LPS-mediated macrophage expression of Notch 1 and Jagged 1. Macrophage RAW264.7 cells were divided into the following four groups: Group A: PBS, Group B: LPS, Group C: epinephrine (E) + LPS, and Group D: metoprolol (M) + epinephrine + LPS. Macrophages were first preconditioned with metoprolol (0.1 nmol/mL) for 1 hour in group D and 0.01 nmol/mL epinephrine was then exposed to Groups C and D for 2 hours. Finally, 10 ng/mL LPS was simultaneously added to Groups B, C, and D. After 24 hours, cells were collected to perform RT-PCR and Western blotting. (A) shows RT-PCR results (mRNA expression levels compared with β-actin) and (B) shows Western blotting electrophoresis result and semi-quantitative analysis results. *P<0.05, **P<0.01 compared to Group C. All data are presented as the mean ± SD; n = 3 per group.

amounts of NGF by LPS-mediated macrophages. This process may be achieved by activating β1 adrenergic receptors, thereby promoting activation of Notch signaling pathways.

The conclusion that macrophages express large amounts of NGF after LPS stimulation has been confirmed [28]. Present results showed that preconditioning with epinephrine for 2 hours promoted expression of NGF, but concentrations of NGF did not increase significantly when the preconditioning time was too short or too long. This may be because macrophages can synthesize, secrete, and metabolize catecholamines [9]. Macrophages may be able to regulate catecholamine concentrations in the surrounding environment. Lower concentrations of epinephrine (0.01 nmol/mL) significantly promoted expression of NGF. This is in accord with concentrations of epinephrine in myocardial tissue after MI [18]. However, the effects of high concentrations of epinephrine on NGF expression are not obvious. It was previously reported [29, 30] that lower concentrations of norepinephrine may affect the α2 adrenergic receptor, causing the release of inflammatory cytokines. However, higher concentrations of norepinephrine may act through the β2 adrenergic receptor, thereby inhibiting phagocytosis and producing anti-inflammatory effects [31]. Results indicate that different concentrations of norepinephrine act on different types of receptors. Like norepinephrine, epinephrine also acts on a variety of receptors with varying affinities for each receptor. Therefore, it was hypothesized that different concentrations of epinephrine produce different results by acting on different receptors.

The present study reveals that epinephrine significantly promoted expression of NGF, IL-1β, and TNF-α and expression of NGF was correlated with IL-1β and TNF-α expression. The β1 blocker metoprolol significantly inhibited expression of these cytokines. Although previous studies [32, 33] have shown that β1 blockers inhibit expression of IL-1β and TNF-α, these results were obtained in animal experiments. However, the present study demonstrates that β1 blockers inhibit the increase in inflammatory cytokines induced by epinephrine preconditioning at the cellular level, directly confirming that β1 blockers inhibit the effects of epinephrine on macrophages expressing NGF.

Previous studies have shown that LPS stimulates macrophages to express large amounts of Notch 1 and Jagged 1 [24, 34]. Expression of Notch 1 and Jagged 1 promotes the activation

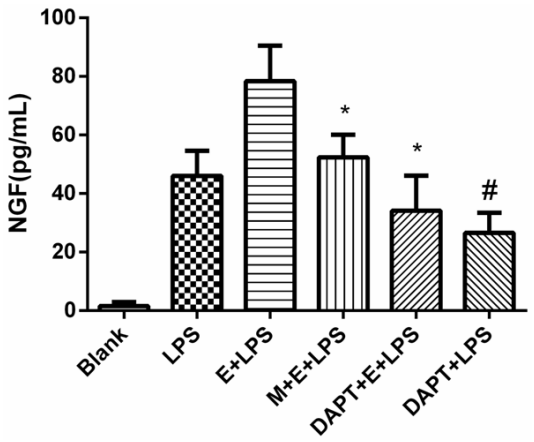

Figure 4. DAPT inhibits the effects of epinephrine on expression of NGF. Macrophages were divided into the following six groups: Group A: PBS, Group B: LPS alone, Group C: epinephrine + LPS, Group D: metoprolol + epinephrine + LPS, Group E: DAPT + epinephrine + LPS, and Group F: DAPT + LPS. First, macrophages were preconditioned with metoprolol (0.1 nmol/mL) or the γ-secretase inhibitor DAPT (Sigma, St. Louis, MO, USA) (10 μg/mL) for 1 hours in Groups D, E, and F simultaneously. Groups C, D, and E were then exposed to 0.01 nmol/mL epinephrine for 2 hours. Finally, Groups B-F were simultaneously stimulated with 10 ng/mL LPS for 24 hours. Supernatants were assayed for their NGF concentrations by ELISA. *P<0.05, **P<0.01 compared with Group B. #P<0.05 compared with group A. All data are presented as the mean ± SD; n = 3 per group.

of M1 macrophages and expression of related inflammatory factors [35]. The present study also confirmed that LPS-stimulated macrophages increased the transcription and translation of Notch 1 and Jagged 1. Expression of Notch 1 and Jagged 1 increased significantly after epinephrine preconditioning, compared with LPS treatment alone, indicating that epinephrine enhanced the effects of LPS. After the addition of metoprolol, the adrenergic-promoting effects were significantly inhibited. Results suggest that β1 receptor activation promotes the activation of Notch pathways. Studies [36] have shown that Notch pathway activation is involved in regulating macrophage activation by LPS. When the Notch pathway is inhibited, LPS cannot induce mouse macrophages to become M1 macrophages. However, mouse macrophages are like M2 macrophages. While M1 macrophages are rich in NGF [28], M2 macrophages are not. Therefore, it was hypothesized that activation of β1 receptors promotes expression of Notch signaling and enhances the ability of LPS to induce M1 macrophages. More macrophages are eventually activated as M1 macrophages. Regrettably, due to experimental limitations, flow cytometry to observe changes in cell types was not conducted.

Using DAPT to inhibit Notch pathways directly explains the effects of the Notch pathways on NGF expression. Present results demonstrate that DAPT inhibits LPS-induced NGF expression in macrophages by inhibiting Notch pathways. DAPT also significantly inhibited expression of NGF in the adrenergic preconditioning group. DAPT demonstrated stronger inhibitory effects on epinephrine-induced NGF expression than metoprolol. One possible explanation for this phenomenon is that DAPT inhibited most Notch receptors and LPS induction, but metoprolol only inhibited the effects of β1 receptors on Notch receptors and not LPS itself. Therefore, the above studies show that Notch pathways induce NGF expression. When the Notch pathway is inhibited, epinephrine cannot promote LPS-mediated NGF expression by macrophages. However, more definitive conclusions require genetic methods, such as knockout or overexpression, to improve understanding. Previous studies [37] have shown that norepinephrine controls the intercellular conduction of Notch signaling by activating β2-AR-PKA-mTOR pathways in endothelial cells co-cultured with breast cancer cells. However, in macrophages, the mechanisms by which β1 adrenergic receptors affect Notch pathway expression remain unclear.

In summary, the present study mimicked environmental changes experienced by macrophages after MI, revealing that preconditioning with low concentrations of epinephrine for 2 hours promoted expression of large amounts of NGF by LPS-mediated macrophages. A possible reason is that β1 receptor activation enhances expression of Notch pathway receptors, thus enhancing the ability of LPS to activate macrophages, ultimately promoting NGF expression. Present results lay a preliminary foundation for the clinical prevention of severe arrhythmia caused by sympathetic nerve remodeling after MI. Experimental data were collected only from a cell line model. The next step requires more comprehensive animal experiments to confirm present conclusions.

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Disclosure of conflict of interest

None.

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