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Norepinephrine induces the expression of interleukin-6 via β-adrenoreceptor-NAD(P)H oxidase system -NF-κB dependent signal pathway in U937 macrophages

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ABSTRACT

Atherosclerosis is an inflammatory disease in the vessel wall. IL-6 is a mediator of inflammation and a major alarm hormone signaling tissue damage and infection to the body's host defense system, in particular to the liver, where it induces the synthesis of acute phase plasma proteins. Although Norepinephrine (NE) can stimulate the vascular cells to produce IL-6, it is unknown whether NE induces IL-6 expression in macrophages. The present study was to observe effect of NE on IL-6 production and the related signal pathway in U937 macrophages so as to provide more evidence for the proinflammatory action of NE. The results showed that NE significantly increased mRNA and protein expression of IL-6 in U937 macrophages in time- and concentration-dependent manners. β-adrenoreceptor inhibitor propranolol blocked NE-induced IL-6 expression in mRNA and protein levels in U937 macrophages. Propranolol and DPI [NAD(P)H oxidase inhibitor] decreased NE-stimulated reactive oxygen species (ROS) generation, and antioxidant NAC completely abolished NE-induced IL-6 expression in U937 macrophages. The further study indicated that NAD(P)H oxidase inhibitor DPI and NF-κB inhibitor PDTC reduced NE-induced mRNA and protein expression of IL-6 in U937 macrophages. These demonstrate that NE is capable of inducing IL-6 generation in macrophages via β-ADR-ROS-NF-κB signal pathway, which contributes to better understanding of the proinflammatory and proatherosclerotic actions of NE.

1. Introduction

Numerous clinical and experimental studies have suggested that systemic inflammation or local inflammation in the vascular wall is connected with atherogenesis. Much data have also demonstrated that the elevated levels of circulating inflammatory markers, like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), C-reactive protein (CRP), matrix metalloproteinases-9 (MMP-9) and interleukin-6 (IL-6) are associated with the increased risk of cardiovascular diseases [1–3]. However, among the inflammatory cytokines, IL-6 shows strong association with increased risk of cardiovascular events. It is not only an inflammatory predictor, but also an active player in the formation and development of atherosclerosis. It is well known that IL-6 acts as a mediator for the induction of proliferation by various agents in several cell types. It was reported that intracellular IL-6 is involved in the platelet derived growth factor-induced proliferation of human fibroblasts, vascular smooth muscle cells, mesangial cells and in the proliferation of synovial fibroblastic cells [4,5]. In addition, a latest study showed that IL-6 exerts direct proinflammatory and proatherogenic effects on several cell types through increasing expressions of adhesion molecules, cytokines, chemokines and growth factors in vitro.

Norepinephrine (NE), the principal neurotransmitter released from postganglionic sympathetic nerves, acts on adrenergic receptors (ARs) on postjunctional effector cells, including VSMC,

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endothelial cells and macrophages. Evidence has suggested the important roles of NE in vascular cell growth and tissue remodeling following atherosclerosis, hypertension and vascular injury. Meanwhile, NE is also known to be a potential proinflammatory factor, since NE induces TNF-α, Matrix Metalloproteinase (MMP)-2, MMP-9, reactive oxygen species (ROS) and toll-like receptor (TLR4) release from cells [6–8]. Furthermore, much data suggested that NE stimulated on the phosphorylation of mitogen-activated protein kinases (MAPK) and ROS synthesis leading to cell proliferation in vitro [8]. Previous study demonstrated that NE induces inflammatory factors in cells mainly through activation of α and (or) β-adrenoreceptor (ADR) [9,10].

Although NE is recognized to evoke the inflammatory responses in macrophages, little information concerning the underlying mechanism of catecholamine-induced IL-6 release is available. Therefore, our present study was to offer a novel molecular mechanism underlying immunomodulatory actions of exogenous NE at the level of IL-6 expression, particularly focusing β-ADR- ROS-NF-κB signal pathway.

2. Materials and methods

2.1. Chemicals and antibodies

NE, Phentolamine (Pht), Propranolol (Pro), pyrrolidine dithiocarbamate (PDTC), thenoyltrifluoroacetone (TIFA), *N*-acetylcysteine (NAC) and diphenyleneiodonium (DPI) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Human IL-6 ELISA kit was provided by Alpha Diagnostics (San Antonio, TX, USA). ROS assay kits were ordered from the Beyotime Institute of Biotechnology (Jiangsu, China). BCA protein assay kit was supplied by Pierce (Rockford, IL, USA).

2.2. Cell cultures

U937 histiocyte (American Type Culture Collection) is also called resident macrophage [18]. U937 macrophages were cultured in a 5% CO_2 atmosphere in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml). The medium was changed every 2 or 3 days. When the cells were seeded into a 6-well plate at 1×10^6 cells/ml, the medium was changed to the serum free medium for an additional 24 h incubation to render the cells quiescent before adding the stimulus. In the inhibitor experiments, U937 macrophages were exposed to NE (10^{-7} M) for 12 h after pretreated with the inhibitors for 1 h.

2.3. ELISA

U937 macrophages were incubated in 6-well plates, and stimulated with the different concentrations of NE for the indicated time. After the treatment, the cells were washed with PBS, lysed and scraped with 200 μl of ice-cold RIPA lysis buffer. Then, the cell lysates were centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant containing the protein fraction was extracted. Protein concentration was quantified with the BCA protein assay kit [1]. Finally, equal amount of protein extract was assayed for IL-6 by ELISA kit specific for IL-6 according to the manufacturer's instruction.

2.4. RT-PCR

Total RNA was isolated from U937 macrophages after the treatment using Trizol (Invitrogen, NY, USA) according to manufacturer's instruction. One microgram of isolated RNA from each sample was transcribed into complementary (cDNA) using oligo $(dT)_{18}$ Primer and RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). The cDNA was amplified using 1.5 μl of each of the IL-6 forward primer (5′-ATGAACTCCTTCTCCACAAGCGC-3′) and reverse primer (5′-GAAGAGCCCTCAGGCTGGACTG-3′). This resulted in a 629 bp PCR product (40 amplification cycles of 94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min). The housekeeping gene (GAPDH) was amplified as an internal control for normalization using the forward primer (5′-GGATTTGGTCGTATTGGG-3′) and the reverse primer (5′-GGAAGA TGGTGATGGGATT-3′). This resulted in a 205 bp product (40 amplification cycles of 94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min). PCR products were separated on 1.5% agarose gels. Results were expressed as relative to control.

2.5. Measurement of ROS

Intracellular ROS production was detected using the 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). To evaluate inhibition of ROS generation, U937 macrophages were pretreated with Phentolamine (3×10^{-9} M), Propranolol (3×10^{-9} M), DPI (10^{-5} M), or TIFA (10^{-5} M) for 1 h before exposed to NE (10^{-7} M) for 12 h. Then, the cells were loaded with H_2DCF-DA (10 μM) for 30 min at 37 °C, and washed twice with PBS. Finally, the cells were imaged with fluorescence microscope (Olympus BX51, Japan). Fluorescence images were acquired at the excitation and emission wavelengths of 488 and 525 nm, respectively.

2.6. Statistical analysis

All values were shown as mean ± S.E.M. Statistical significance between groups was assessed by one-way ANOVA, followed by Tukey's multiple comparison tests. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. NE induces IL-6 expression in macrophages

Fig. 1A showed that NE at 10^{-8} M to10^{-5} M evidently upregulated IL-6 mRNA expression in U937 macrophages in a concentration-dependent manner compared to control. The maximal response of 4.1 folds over control was reached at a concentration of 10^{-5} M. The results from ELISA indicated that the unstimulated U937 macrophages exhibited a low basal IL-6 release of 47.92–54.53 ng/ml in the protein extracts. NE at 10^{-8} M up to10^{-5} M caused an apparent time- and concentration-dependent increase of IL-6 production in U937 macrophages as compared to control. IL-6 level reached a maximum after stimulating the cells with NE at 10^{-5} M for 48 h, which was 5.22 times over baseline (Fig. 1B).

3.2. The β-adrenoreceptor signaling pathway modulates IL-6 secretion in macrophages

To test the role of adrenoreceptor in NE-induced IL-6 expression in U937 macrophages, the cells were pretreated with α-ADR inhibitor phentolamine or β-ADR inhibitor propranolol for 1 h before NE stimulation. The results showed that β-ADR but not α-ADR mediated NE-induced IL-6 expression in U937 macrophages, since propranolol almost abolished NE-induced mRNA and protein expression of IL-6, while phentolamine did not produce a significant effect on IL-6 expression (Fig. 2A and B).

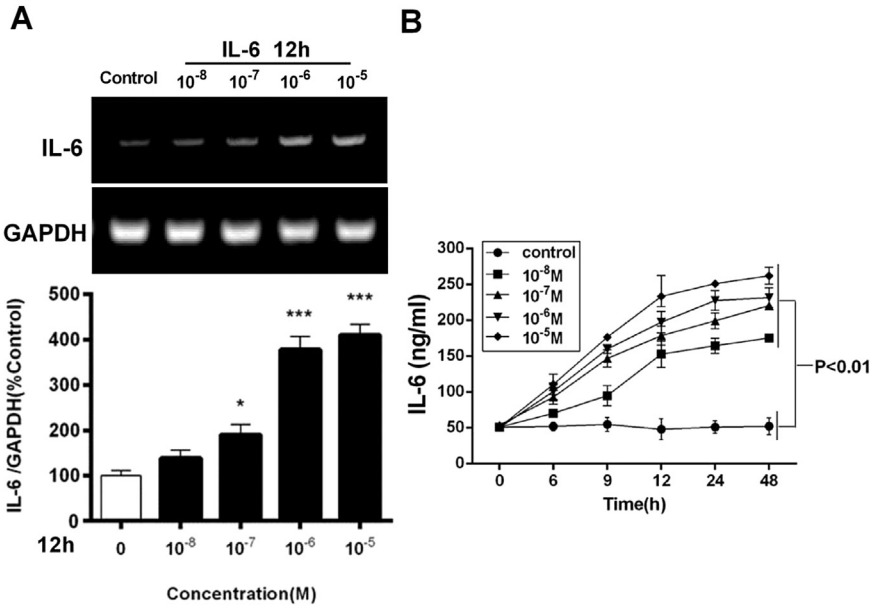

Fig. 1. NE stimulates IL-6 generation in U937 macrophages. (A) Concentration-dependent expression of IL-6 mRNA, (B) Concentration- and time-dependent increase of IL-6 protein in U937 macrophages. The cells were incubated with the different concentrations of NE for 12 h or with NE (10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M) for the different time (0 h, 6 h, 9 h, 12 h, 24 h, 48 h). Then, IL-6 mRNA level was identified by RT-PCR, and IL-6 concentration was measured by ELISA. Data from three independent experiments are expressed as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

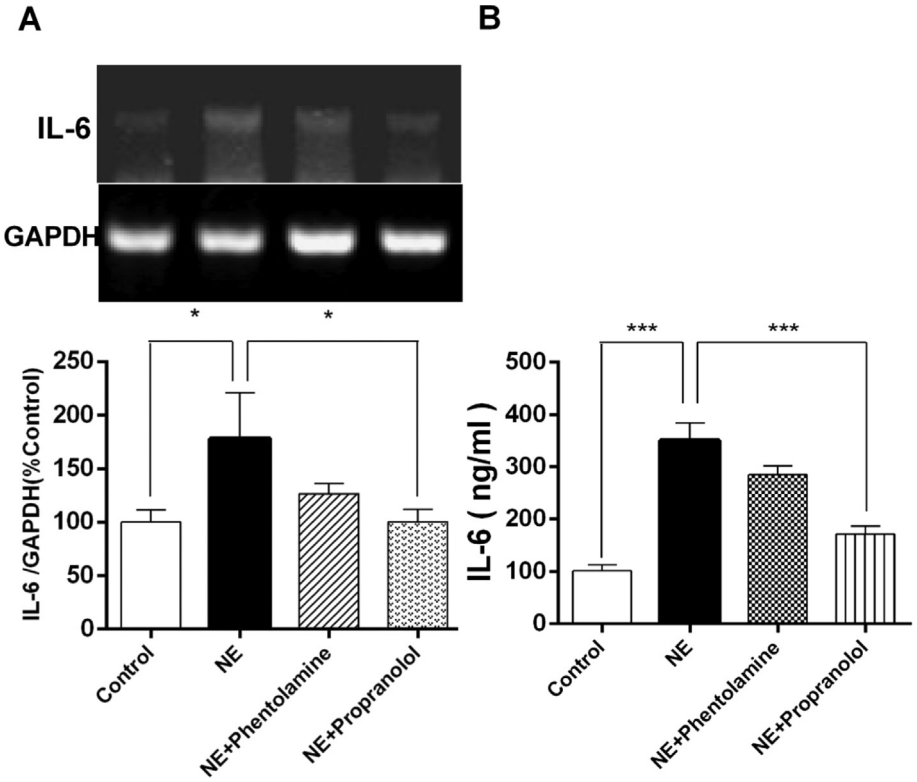

Fig. 2. NE induces IL-6 expression in U937 macrophages via β-adrenoreceptor. The cells were pretreated with α-ADR inhibitor phentolamine (3×10^{-9} M) or β-ADR inhibitor propranolol (3×10^{-9} M) or for 1 h prior to stimulation with NE (10^{-7} M) for 12 h for mRNA expression and protein generation. Then, mRNA expression (A) and protein level (B) of IL-6 were analyzed by RT-PCR and ELISA, respectively. Data from three independent experiments are expressed as means ± S.E.M. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. NE alone.

3.3. Involvement of ROS activation in NE-induced IL-6 expression in macrophages

To determine whether intracellular ROS participate in NE-induced production of IL-6, intracellular ROS generation was observed. Exposure of U937 macrophages to NE resulted in a noticeable raise of DCF fluorescence as compared to control, whereas phentolamine, propranolol and DPI [NAD(P)H oxidase inhibitor] obviously attenuated the effect. Although TIFA (complex II inhibitor) tended to depress NE-stimulated ROS production, there was no statistical significance (Fig. 3A). Further investigation revealed that antioxidant NAC and DPI significantly inhibited NE-induced expression of IL-6 in macrophages (Fig. 3B).

3.4. NE induces IL-6 expression in macrophages through ADR-ROS-NF-κB signal pathway

To investigate if the NF-κB pathway is involved in NE-induced expression of IL-6 in U937 macrophages, the cells were stimulated with 10^{-7} M NE for 12 h after pretreatment with PDTC (NF-κB inhibitor) for 1 h. As shown in Fig. 4A and B, PDTC substantially reduced NE-induced mRNA and protein expression of IL-6 in macrophages. The above-mentioned results indicate that ADR and ROS participate in NE-induced expression of IL-6 in U937 macrophages. To elucidate whether ADR mediated NE-induced activation of ROS in U937 macrophages, the ROS was determined. As shown in Fig. 4C there was a marked increase of the ROS was detected following stimulation of the cells with NE. However, pretreatment of the cells with Pht or Pro for 1 h prior to exposure of the cells to NE for 1 h, Pro but not Pht markedly inhibited NE-induced expression of ROS.

4. Discussion

Recent study suggests that proinflammatory cytokines are capable of modulating cardiovascular function by a variety of mechanisms, including contractile dysfunction [11,12], left ventricular remodeling [13,14], and uncoupling of myocardial β-ADR receptors [15,16]. IL-6 as a mediator of inflammation and a major alarm hormone signaling tissue damage and infection to the body's host defense system, in particular to the heart and liver, where it induces the synthesis of acute phase plasma proteins [1,2]. It was reported that intracellular IL-6 is involved in the platelet derived growth factor-induced proliferation of human fibroblasts, mesangial cells, vascular smooth muscle cells and in the proliferation of synovial fibroblastic cells [4,5]. Moreover, IL-6 in the vessel wall may play a direct and essential role in the whole inflammatory process of atherosclerosis and the development of cardiovascular complications.

NE is a well known major vasoconstricting factor, but growing evidence suggests that norepinephrine also plays an important role via the adrenergic α and/or β-adrenoceptors which are expressed in macrophages [10]. NE contributes not only to pathogenesis of hypertension but also to inflammatory process of many diseases, such as atherosclerosis, coronary heart disease, heart failure, brain ischaemia. Therefore, α and/or β-ADR have been successfully used in treatment of a pathologic inflammatory process of the diseases.

As the main inflammatory cells, macrophages play a critical role in successful tissue repair as key mediators of the inflammatory phase of atherosclerosis [17]. In addition, monocytes are recruited to the vascular wall during the process of atherogenesis where they differentiate into macrophages and acquire the capacity to synthesize an array of growth factors, chemokine and other

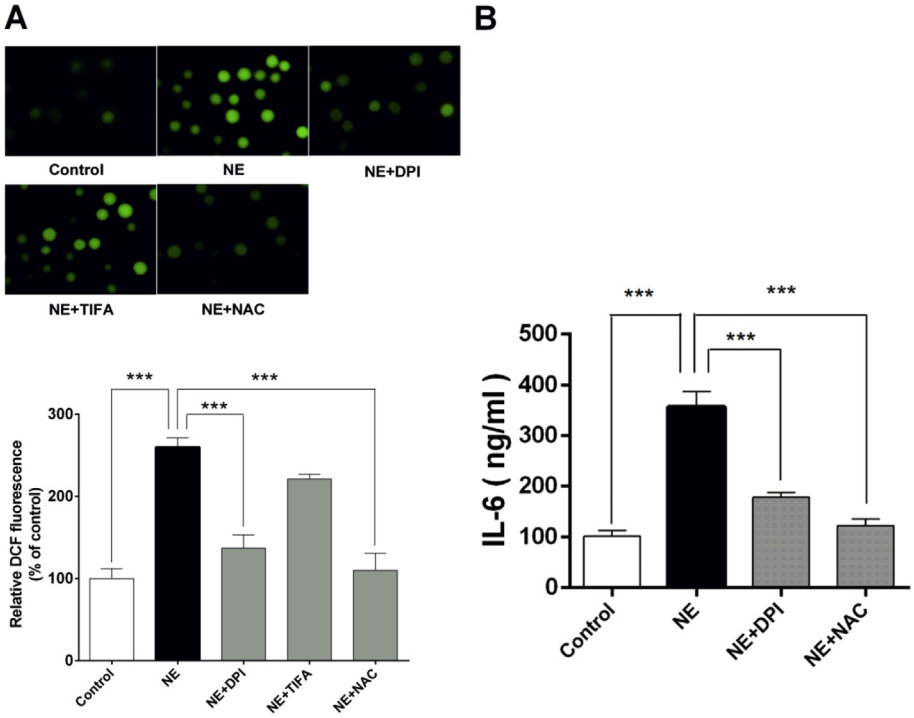

Fig. 3. Involvement of ROS in NE-induced IL-6 expression in U937 macrophages. (A) Effect of the different inhibitors on NE-stimulated generation of ROS. The cells were stimulated with NE (10^{-7} M) for 12 h after pretreatment with NAC (antioxidant, 10 Mm), DPI [NAD(P)H oxidase inhibitor, 10^{-5} M] or TIFA (complex II inhibitor, 10^{-5} M) for 1 h, and then, incubated for 30 min with H_2DCF-DA (10 μM). Finally, the cells were observed under fluorescence microscope. (B) Effect of NAC and DPI on NE-induced protein expression of IL-6. The cells were stimulated with NE (10^{-7} M) for 12 h after pretreatment with NAC (antioxidant, 10 mM) or DPI (10^{-5} M) for 1 h. Then, IL-6 expression was assayed by ELISA. Results from three independent experiments are expressed as means ± S.E.M. $^{***}P < 0.001$ vs. NE alone.

inflammatory factors which contribute to the development of atherosclerosis [18]. In our study, we found that NE induced mRNA and protein expression of IL-6 in U937 macrophages in time- and concentration-dependent manners, which is consistent with previous results in cardiac fibroblasts and Epithelial Cells [19,20]. This result further enriches the proinflammatory action of NE.

Numerous studies have demonstrated functional α and β-ADR on macrophages [10]. To our knowledge, NE up-regulates IL-6 expression in cardiac fibroblasts through β-ADR [19] and NE stimulates IL-6 secretion via theα2 and β-ADR pathway in Spleen [9]. Therefore, adrenoceptors possibly mediate the proinflammatory effect of NE in macrophages. In the experiment, pretreatment of

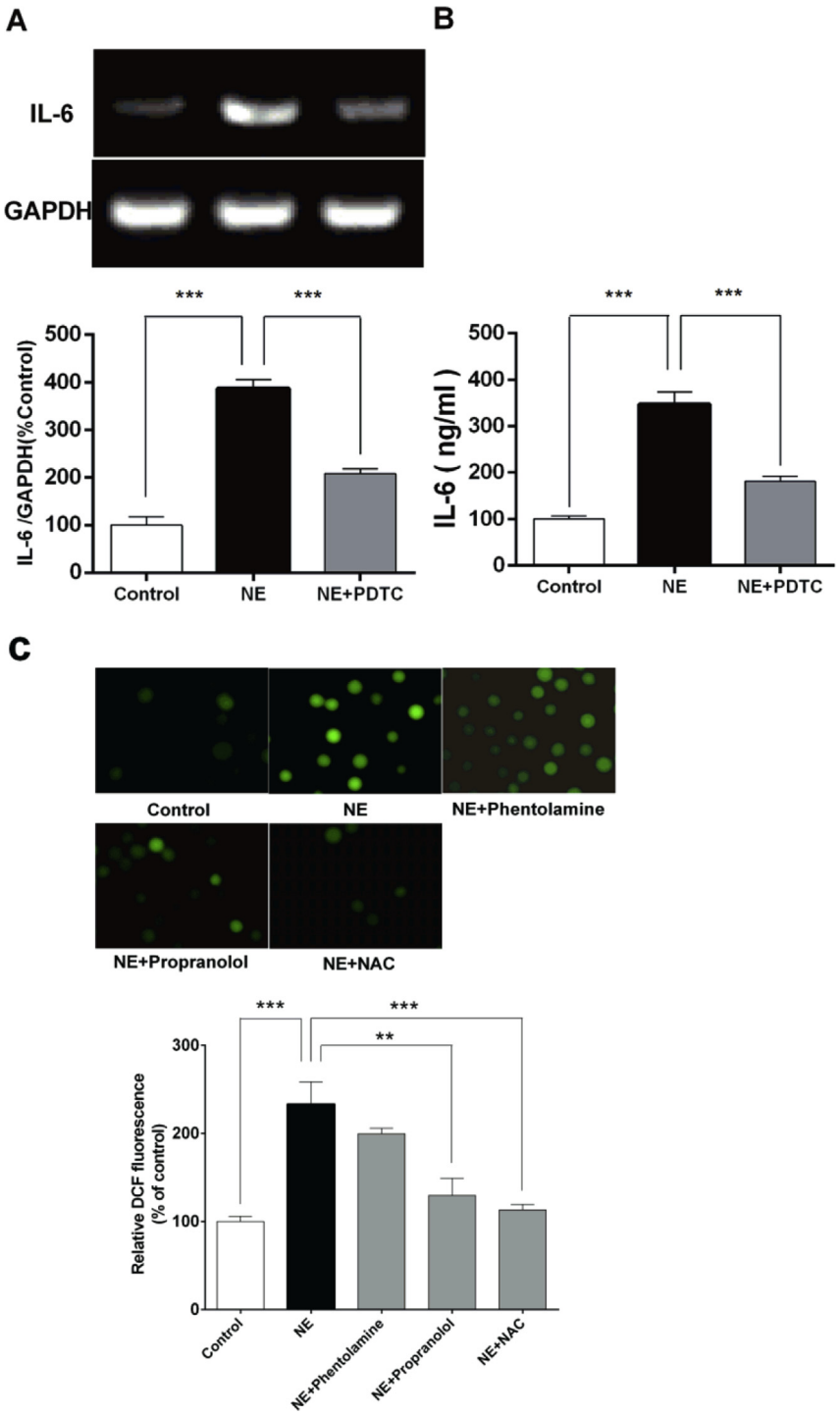

Fig. 4. NE induces IL-6 expression in U937 macrophages through β-ADR-ROS-NF-κB signal pathway. The cells were pretreated with PDTC (NF-κB inhibitor, 100 μM) for 1 h prior to stimulation with NE (10^{-7} M) for 12 h. Then, mRNA expression (A) and protein level (B) of IL-6 were detected by RT-PCR and ELISA, respectively. The cells were stimulated with NE (10^{-7} M) for 1 h after pretreated with phentolamine (3×10^{-9} M), propranolol (3×10^{-9} M) or NAC (antioxidant, 10 mM) for 1 h. Then, the cells were observed under fluorescence microscope (C). Data from three independent experiments are expressed as the mean ± S.E.M. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. NE alone.

U937 macrophages with the specific β-ADR inhibitor propranolol almost completely abolished NE-induced increase of IL-6 in mRNA and protein levels. But, the specific inhibitor of α-ADR, phentolamine, did not alter the effect of NE on IL-6. These demonstrate that NE induces IL-6 expression in macrophages via β-ADR.

It is reported that ROS mediate production of the inflammatory cytokines through the multiple signal pathways including MAPK and NF-κB signaling, and NE stimulates peroxide production in adult rat ventricular myocytes predominantly through activation of α_1-ADR [21]. The results from the present experiment indicated that NE-induced ROS generation in U937 macrophages involved an β-ADR/NAD(P)H oxidase system, but not mitochondria-derived pathway, since β-ADR inhibitor propranolol and NAD(P)H oxidase inhibitor DPI remarkably antagonized the stimulatory effect of NE on ROS production, whereas α-ADR inhibitor phentolamine and complex II inhibitor TIFA did not show the significant inhibitory effect. These data are also consistent with the study of Remondino et al. in endothelial cells, who report that β-ADR -mediated ROS generation is involved in NE-stimulated apoptosis [22]. But the finding is not entirely the same as previous results in adult rat cardiac myocytes, which indicate that α-ADR stimulated ROS production as measured by DCF fluorescence is inhibited by DPI [21]. This different result suggests that adrenoceptor mediated ROS generation is cell-specific and pathway-specific. In addition, we also found that antioxidant NAC completely inhibited NE-induced mRNA expression of IL-6. These imply that NE stimulates IL-6 generation in macrophages via β-ADR mediated ROS signaling.

A great number of investigations document that the NF-κB pathway is considered to be an important “bridge” between NE and its proinflammatory actions in atherosclerosis, and ROS is an upstream event of NF-κB activation [23]. Moreover, NE can enhance NF-κB activity in cardiac myocyte [23]. Our further study displayed that NF-κB participated in NE-induced IL-6 expression in U937 macrophages, since the selective NF-κB inhibitor PDTC remarkably antagonized NE-stimulated IL-6 production.

In conclusion, our data demonstrate the ability of NE to initiate an inflammatory response by stimulating IL-6 production in macrophages, which is mediated through β-ADR-NAD(P)H oxidase system -NF-κB signal pathway. The finding may contribute to better understanding of the proinflammatory and proatherosclerotic actions of NE.

Conflict of interest

None.

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