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Activation of AMPA receptor promotes TNF-α release via the ROS-cSrc-NFκB signaling cascade in RAW264.7 macrophages

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ABSTRACT

The relationship between glutamate signaling and inflammation has not been well defined. This study aimed to investigate the role of AMPA receptor (AMPAR) in the expression and release of tumor necrosis factor-alpha (TNF-α) from macrophages and the underlying mechanisms. A series of approaches, including confocal microscopy, immunofluorescency, flow cytometry, ELISA and Western blotting, were used to estimate the expression of AMPAR and downstream signaling molecules, TNF-α release and reactive oxygen species (ROS) generation in the macrophage-like RAW264.7 cells. The results demonstrated that AMPAR was expressed in RAW264.7 cells. AMPA significantly enhanced TNF-α release from RAW264.7 cells, and this effect was abolished by CNQX (AMPAR antagonist). AMPA also induced elevation of ROS production, phosphorylation of c-Src and activation of nuclear factor (NF)-κB in RAW264.7 cells. Blocking c-Src by PP2, scavenging ROS by glutathione (GSH) or inhibiting NF-κB activation by pyrrolidine dithiocarbamate (PDTC) decreased TNF-α production from RAW264.7 cells. We concluded that AMPA promotes TNF-α release in RAW264.7 macrophages likely through the following signaling cascade: AMPAR activation → ROS generation → c-Src phosphorylation → NF-κB activation → TNF-α elevation. The study suggests that AMPAR may participate in macrophage activation and inflammation.

1. Introduction

Glutamate (Glu) is a well-defined excitatory neurotransmitter in the central nervous system and exerts a variety of functions depending on their specific glutamate receptors. Glu is also a nutritional amino acid existed in the blood stream. There are two groups of glutamate receptors, they are the ionotropic receptors (iGluRs) and the metabotropic receptors (mGluRs). The iGluRs are subdivided into three subtypes according to their selectivity to agonists: the N-methyl-D-aspartate receptor (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), and kainate receptor (KAR) [1]. Glu receptors have been found expressed in most peripheral tissues, including the myocardium [2–4]. However, the roles of Glu receptors in the function of immune cells, are not well defined.

AMPAR, which belongs to the non-NMDA-type iGluRs, mediates most of the fast excitatory synaptic transmission in the central nervous system (CNS) [5]. Apart from this, AMPAR was found important in the excitable cells of peripheral tissues. For example, AMPA stimulates insulin release from β cells [6] in the pancreas; AMPAR activation induces oxidative stress and apoptosis in cardiomyocytes [7]. AMPAR is also found expressed and having functions in the non-excitable cells including immune cells, for example, platelet AMPAR mediates platelet activation [8]; AMPA enhances the proliferation of autoreactive T cells in patients with multiple sclerosis in response to myelin proteins [9]. However, the expression and function of AMPAR in macrophages are seldom investigated.

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine and mainly produced by macrophages. Accumulated evidences proved that cytokines, especially TNF-α, take important

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roles in the progress of inflammation and atherosclerosis [10–12]. Besides, AMPAR signaling and TNF-α have interactions in some conditions. Bliss et al. [13] reported that TNF-α augments the AMPA-induced toxicity in Purkinje neurons by increasing calcium influx through calcium permeable AMPA receptor and caused loss of Purkinje neurons. He et al. [14] reported that type 1 TNF receptor (TNFR1) plays a critical role in modulating AMPAR clustering, implying a novel approach in targeting TNFR1 gene to preventing neuronal AMPAR-mediated excitotoxicity. These studies suggest that AMPAR and TNF-α correlate with each other and take part in pathogenesis. The present study aimed to test the hypothesis that AMPAR activation may promote TNF-α release from macrophages and thus may contribute to the initiation of inflammation. We used the macrophage-like RAW264.7 cell line as a cell model in this study, because this cell line presents many characters of human macrophages and has a homogeneous and reproducible property.

2. Materials and methods

2.1. Reagents

Reagents were purchased from the following suppliers: AMPA (Santa Cruz); 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2), glutathione (GSH), pyrrolidine dithiocarbamate (PDTC) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma); FITC-conjugated rabbit anti-AMPAR antibody (Bioss Biotechnology, Ltd., Beijing); mouse TNF-α ELISA kit (R&D Systems); p-c-Src and IκB antibodies (Cell Signaling Technology); antibodies against NF-κB and p65, goat anti-rabbit secondary antibody and rabbit anti-mouse secondary antibody (Beijing Zhongshan Golden Bridge Biotechnology).

2.2. Cell culture

RAW264.7 cell line was obtained from the Cell Center of Peking Union Medical College. Cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were then passaged every two or three days in a 1:3–1:6 ratio and discarded after culture for one month. All the experiments were performed using the logarithmic growth phase of cells.

2.3. Confocal microscopy

To evaluate the expression of AMPAR in RAW264.7 macrophages, cells grown on coverslips were fixed by 4% paraformaldehyde, permeabilized with 0.1% Triton-X 100 for 15 min, and blocked by 5% BSA. Cells were then incubated with FITC-conjugated rabbit anti-AMPAR antibody overnight at 4 °C, followed by washing with PBS. Coverslips were mounted on slides with antifade mounting medium (Dako, Denmark) to slow down the fading of fluorescence. The presence of AMPAR in RAW264.7 cells was examined under an Olympus confocal microscope with excitation and emission waves at 488 nm and 507 nm respectively for FITC fluorescence. Twenty cells were randomly chosen from each group (control and AMPA treatment) and the FITC fluorescence intensities were quantitatively analyzed by ImageJ software to reflect the AMPAR expression level.

2.4. Enzyme linked immunosorbent assay (ELISA)

ELISA was conducted with a mouse TNF-α ELISA Kit to detect the release of TNF-α into the culture medium. Briefly, after treatment with respective stimuli, RAW264.7 cell culture medium was collected and centrifuged to acquire the supernatant. ELISA was performed according to the manufacturers' instructions. Optical absorbing values were read at 450 nm and corrected by 570 nm. Data were analyzed using SoftMax Pro4.8 software.

2.5. Flow cytometry

To determine the intracellular ROS level of RAW264.7 macrophages, DCFH-DA was introduced to cells and incubated at 37 °C for 45 min, followed by triple washes. After different stimuli were applied, cells were collected and flow cytometry was performed with excitation wavelength at 488 nm and emission wavelength at 525 nm. The fluorescence intensity represented the quantity of ROS, stronger fluorescence intensity represented larger ROS production. A rightward shift of the trace meant an increasing of fluorescence intensity.

2.6. Western blotting

The levels of phosphorylated c-Src (p-cSrc, at Tyr-418), IκB, p65 and phosphorylated p65 (p-p65) in RAW264.7 macrophages were determined by Western blotting using specific antibodies. Briefly, after washing with ice-cold PBS, cells were scraped off and collected by centrifugation at 1000 rpm for 5 min at 4 °C and resuspended in RIPA lysis buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and a series of protease inhibitors. After lysed thoroughly on ice, centrifugation was performed at 12,000 rpm for 10 min at 4 °C to acquire the supernatant which contained the total proteins. Protein quantity was determined by the BCA method and protein samples were aliquoted and stored at −80 °C until for detection. For Western blotting, fifty microgram (μg) of total proteins were separated by a 10% SDS-PAGE and transferred to PVDF membrane, followed by blocking with 5% skim milk at room temperature for 1 h. Respective primary antibodies against proteins mentioned above were diluted using blocking buffer to suitable concentrations and incubated at 4 °C overnight. After washing with TBST, a horseradish peroxidase-conjugated secondary antibody was applied and incubated at room temperature for 1 h, then blot underwent washes for three times each for five minutes, finally the reaction system was developed with the ECL system (Engreen Biosystem Co., Ltd., China). Images were captured using the EC3 Imaging System (UVP, Inc., upland, CA, USA) and quantified by Quantity One software.

2.7. Statistical analysis

Data were expressed as mean ± S.E.M. Student's *t* test and ANOVA were used for statistical analyses where suitable. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. AMPAR is expressed in RAW264.7 macrophages and upregulated by AMPA

Confocal immunofluorescence microscopy was used to estimate the expression of AMPAR in RAW264.7 cells. Control RAW264.7 cells (without AMPA stimulation) showed weak green fluorescence (Fig. 1A). However, treatment with AMPA (10^{-4} mol/L) for 4 h significantly increased the intensity of AMPAR fluorescence in the cell membrane compared with the control (Fig. 1). These results indicate that the AMPAR expression level of RAW264.7 cells was

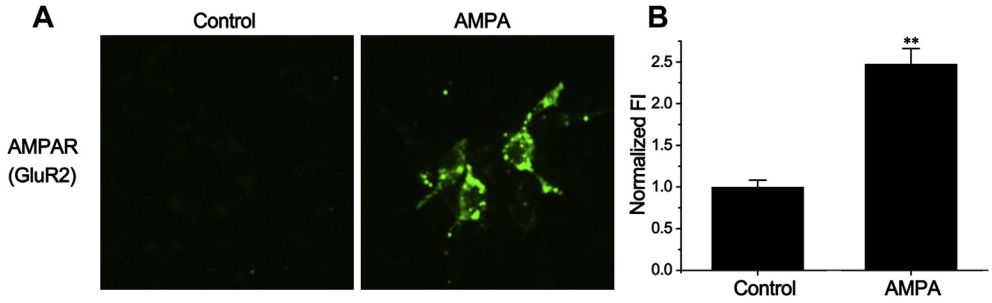

Fig. 1. Confocal immunofluorescency showing the AMPAR expression in RAW264.7 macrophages at baseline and upon AMPA stimulation. AMPAR was detected by the FITC-conjugated anti-AMPAR antibody. *A*, confocal images showing lower level AMPAR expression in quiescent RAW264.7 cells and the significant upregulation of AMPAR expression in response to AMPA (10^{-4} mol/L) stimulation for 4 h. The AMPAR was likely located mainly in the cell membrane. Control cells were treated with the solvent PBS. *B*, statistical bar graph showing the relative AMPAR expression levels. FI, fluorescence intensity. $^{**}P < 0.01$ *vs.* control. N = 20.

relatively low at baseline, while was significantly upregulated in response to agonist stimulation.

3.2. AMPA induces ROS generation in RAW264.7 cells

Using the DCFH-DA fluorescence probe in flow cytometry, we observed the effect of AMPA on ROS generation in RAW264.7 macrophages. Fig. 2A showed representative recordings of ROS production in response to different stimuli, a rightward shift of the tracing indicated an increase of ROS level. AMPA at 10^{-4} mol/L significantly increased the ROS level compared with the control (Fig. 2A). As a positive control, H_2O_2 significantly increased the ROS production (Fig. 2A). Selective blockade of AMPAR with CNQX totally abolished the AMPA-induced ROS production (Fig. 2A). Fig. 2B showed the quantified results (DCF intensity) after normalization, which was compatible with Fig. 2A. These results indicated that AMPA-induced ROS elevation was mediated by AMPAR.

3.3. AMPA induces TNF-α release in RAW264.7 macrophages, and this effect is abolished by blocking AMPAR, ROS, c-Src and NF-κB

The ELISA assay showed that AMPA significantly increased the TNF-α level in the supernatant (Fig. 3A), indicating an increased release of TNF-α from RAW264.7 cells after AMPAR activation. In the preliminary experiments, we observed that the AMPA concentration that induced most significant TNF-α release was 10^{-4} mol/L and at 24 h after AMPA treatment. Thus, unless otherwise illustrated, AMPA was applied at 10^{-4} mol/L for 24 h in the following experiments. Blocking AMPAR with CNQX (10^{-4} mol/L), eliminating ROS by GSH (10^{-2} mol/L), inhibiting c-Src by PP2 (10^{-4} mol/L), or suppressing NF-κB by PDTC (10^{-4} mol/L), all abolished the stimulating effect of AMPA on TNF-α release (Fig. 3A). These results indicate that the AMPA-induced TNF-α release is mediated by AMPAR in RAW264.7 macrophages, and ROS, c-Src and NF-κB are involved in this effect.

3.4. AMPA induces c-Src phosphorylation and NF-κB activation in RAW264.7 macrophages

Because AMPA induced ROS elevation as shown above, and c-Src is sensitive to ROS [15], we therefore examined whether AMPA would induce c-Src phosporylation in RAW264.7 macrophages using Western blotting. Fig. 3B shows that AMPA exposure increased the phosporylated level of c-Src (p-c-Src) (at Tyr418) in RAW264.7 cells (Fig. 3B), and this effect was completely blocked by AMPAR antagonist CNQX (Fig. 3B), indicating that AMPA could induce c-Src phosphorylation (activation).

It is also known that NF-κB activation process includes dissociation of IκB from NF-κB, degradation of IκB by the proteosome which is required for the translocation of activated NF-κB from the cytoplasm to the nucleus, and phosphorylation of p65 (a major subunit of NF-κB) [16]. Based on these findings, we determined the levels of IκB and phosphorylated p65 (p-p65) in RAW264.7 macrophages after AMPA exposure. AMPA reduced the IκB level compared with the control. Pretreatment with PP2, an inhibitor of c-Src, rescued the IκB level (Fig. 3C), suggesting that AMPAR activation potentially induces NF-κB dissociation and/or IκB degradation, and thus intensifies the action of NF-κB on TNF-α expression and releasing. In addition, AMPA increased the level of p-p65, and

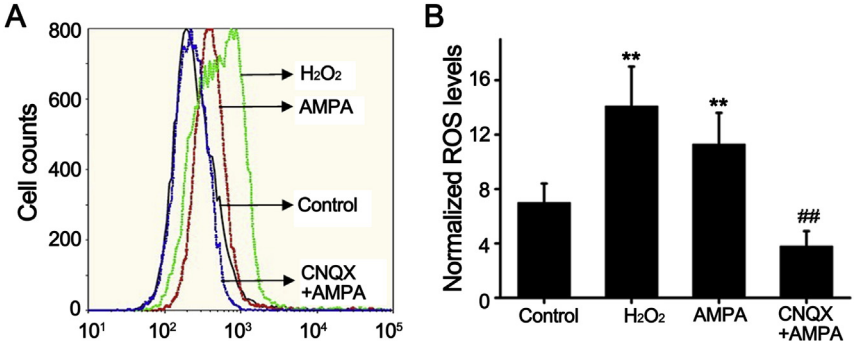

Fig. 2. Flow cytometry results showing the ROS production in the RAW264.7 macrophages upon different stimuli. *A*, original recordings of DCF fluorescence intensity reflecting the ROS level. *Black*, control. *Green*, H_2O_2 as positive control. *Red*, AMPA (10^{-4} mol/L). *Blue*, CNQX (10^{-4} mol/L) + AMPA (10^{-4} mol/L). *B*, statistical data (normalized units) of DCF fluorescence intensities based on individual experiments (N = 5 for each treatment). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

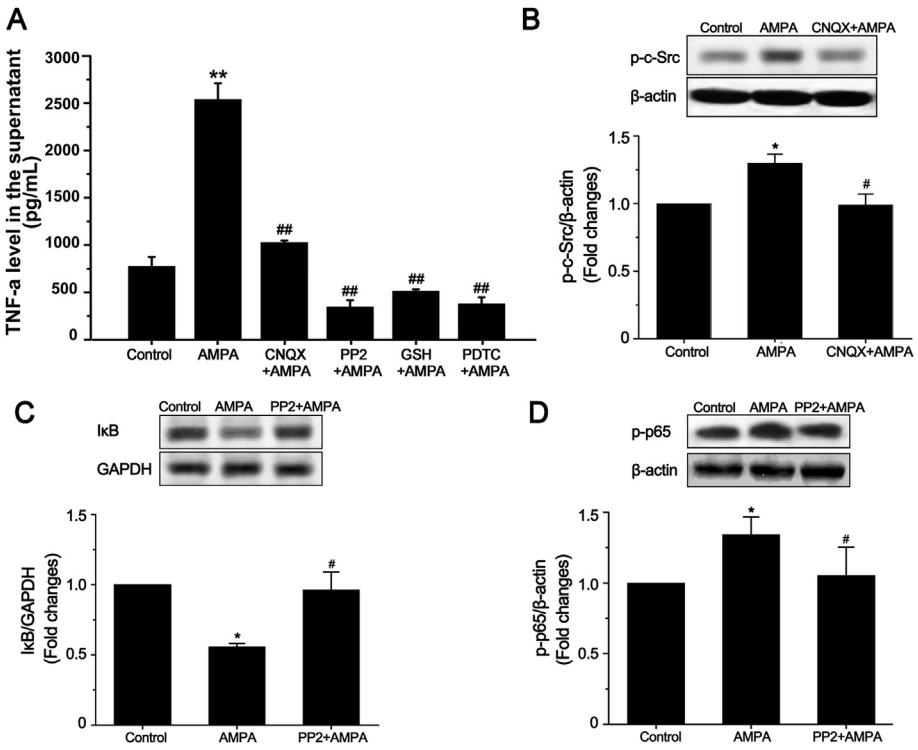

Fig. 3. ELISA results of TNF-α release and Western blotting of the expression levels of p-c-Src, IκB and p-p65 in RAW264.7 macrophages in response to different drug treatments. *A*, TNF-α levels in the culture medium representing TNF-α release by RAW264.7 macrophages in response to different stimuli. AMPA was applied at 10^{-4} mol/L for 24 h; CNQX (10^{-4} mol/L), PP2 (10^{-4} mol/L), GSH (10^{-2} mol/L) or PDTC (10^{-4} mol/L) was applied 2 h before AMPA treatment. $^{**}P < 0.01$ *vs.* control, $^{\#\#}P < 0.01$ *vs.* AMPA, N = 5. *B, C and D*, the upper panels are the representative electrophoresis bands of p-c-Src, IκB and p-p65, respectively, with β-actin or GAPDH as loading controls; the lower panels show the respective quantitative fold changes of p-c-Src, IκB and p-p65 relatively to control. $^{**}P < 0.01$ *vs.* control, $^{\#\#}P < 0.01$ *vs.* AMPA. N = 3 for each treatment.

this effect was also blocked by the Src family kinase inhibitor PP2 (Fig. 3D). These results revealed that AMPA activated the c-Src/NF-κB signaling and thus promote TNF-α expression and releasing in RAW264.7 macrophages as we showed in Fig. 3A.

3.5. Scavenging ROS suppresses the phosphorylations of c-Src and p65 induced by AMPA in RAW264.7 macrophages

We further tested if ROS generation is the initial step of the signal chain by which AMPA induces TNF-α elevation. Pretreatment with GSH (a ROS scavenger) (10^{-2} mol/L) decreased the AMPA-induced elevation of p-c-Src in RAW264.7 macrophages (Fig. 4A), suggesting that ROS is an upstream molecule relatively to c-Src in AMPA-induced TNF-α elevation. GSH also blocked the AMPA-induced increase of phosphorylated p65 (p-p65) (Fig. 4B). These results support our hypothesis that ROS production acts as the initial step in the process of AMPA-induced TNF-α elevation, and the signaling cascade may be AMPAR activation → ROS generation → c-Src phosphorylation → NF-κB activation → TNF-α elevation.

4. Discussion

Glutamate receptors are expressed not only in the CNS but also in the periphery non-neural tissues/cells [17], such as heart [7], pancreas [18], lung [19], keratinocyte [20], osteoblast [21], platelets

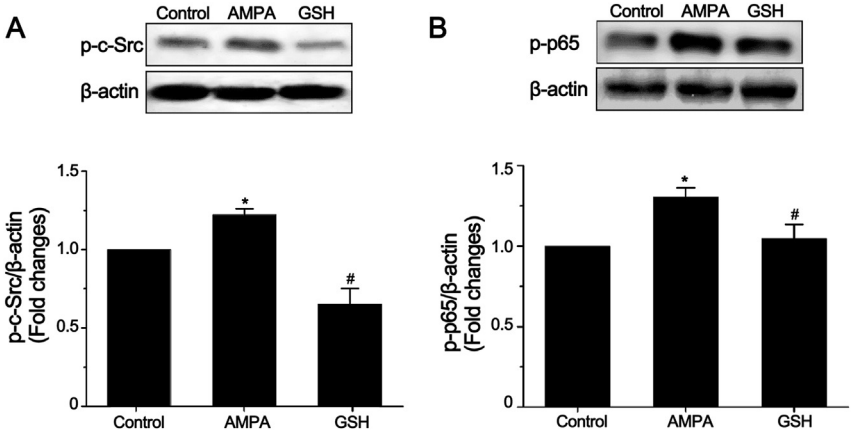

Fig. 4. Western blotting showing the effects of AMPA and GSH on the phosphorylations of c-Src and p65 in RAW264.7 macrophages. *A*, p-c-Src. *B*, p-p65. Drug concentrations: AMPA 10^{-4} mol/L, GSH 10^{-2} mol/L. $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* AMPA. N = 3 in each group.

[8] and immune cells such as T cells [9]. As an important member of the immune system, macrophages take important roles in the immune activities and the generation and development of inflammation. However, the potential expression and function of iGluRs in macrophages are not yet well elucidated. The present study investigated the expression and function of AMPAR in a macrophage-like RAW264.7 cell line, with a special focus on whether and how AMPAR activation induces TNF-α release from this cell line. We identified for the first time that AMPAR was expressed in the RAW264.7 macrophages, and the AMPAR-ROS-cSrc-NFκB signaling chain may be used in the AMPA-induced TNF-α release from this macrophage model. The study suggests that AMPAR may serve as a target for the modulation of the inflammatory behaviors of macrophages.

ROS production is a destructive aspect of oxidative stress that may cause cell death by triggering apoptosis and necrosis [22]. ROS production has been documented to be involved in the glutamate induced NMDAR activation in cultured forebrain neurons [23]. Besides, we have reported that ROS generation participates in the NMDA and AMPA mediated apoptosis in the neonatal cardiomyocytes [7,24]. However, whether ROS is involved in the AMPAR mediated TNF-α release in macrophages remains unknown. In the present study, we demonstrated that ROS was elevated in response to AMPA exposure, and pretreatment with GSH blocked the elevation of TNF-α induced by AMPA, suggesting that ROS is involved and acts as the upstream signaling molecule in the AMPA induced TNF-α release in RAW264.7 macrophages.

NF-κB, a transcription factor, can promote the transcription of TNF-α. In mammalian cells, there are five NF-κB family members constituting homo- or heterodimers. Among these members, p50/p65 showed the highest expression level nearly in all types of the mammalian cells [25]. Under resting (un-activated) condition, NF-κB complexes are retained in the cytoplasm by the inhibitor named IκB. Upon stimulation, IκB was phosphorylated by kinase and followed by degradation, allowing the translocation of NF-κB from the cytoplasm to the nucleus to function as a transcription factor [26]. The present study showed that upon treatment with AMPA, IκB was down-regulated and p65 was phosphorylated, suggesting the involvement of NF-κB in AMPA-induced TNF-α release from RAW264.7 macrophages. We also verified that pretreatment with PDTC (NF-κB inhibitor) decreased the TNF-α release. As mentioned above, p65 phosphorylation reflects NF-κB activation. We showed here that blocking ROS by GSH decreased p65 phosphorylation, suggesting that ROS is involved in NF-κB signaling and functions as an upstream signaling molecule of this process. Thus, our data suggest that ROS and NF-κB participate in the AMPA-induced TNF-α release in the RAW264.7 macrophages.

c-Src is a non-receptor tyrosine kinase and is ROS sensitive, and has been reported to participate in hypoxia signaling [27]. Sato et al. reported that hypoxia induced the ROS-mediated and c-Src-dependent signaling cascades and they were closely associated with angiogenesis and thrombosis in atherosclerotic vasculature [15]. The present study demonstrated that AMPA treatment enhanced the c-Src activity, and this effect was blocked by the selective AMPAR antagonist CNQX in the macrophages. Besides, pretreatment with ROS scavenger GSH inhibited the AMPA-stimulated increase of c-Src activity. Thus, the ROS-promoted TNF-α release was likely mediated by the c-Src activity. Furthermore, blocking c-Src signaling by PP2 rescued the IκB degradation induced by AMPA, and decreased the level of p-p65, revealing that c-Src may take its role by regulating the NF-κB activity and promoting its translocation from the cytoplasm to nucleus. Thus, these results imply that upon AMPA stimulation, ROS activates c-Src, c-Src in turn promotes NF-κB activity and cytoplasm-nucleus translocation, resulting in the increase of TNF-α release from the RAW264.7 macrophages.

Taken together, the present study demonstrated for the first time that AMPAR is expressed in the RAW264.7 macrophages, and activation of AMPAR stimulates the TNF-α release, and this effect is likely mediated by the AMPAR → ROS → c-Src → NF-κB signaling cascade. The study suggests an important role of AMPAR in the inflammatory activities of macrophages and AMPAR activation may participate in inflammation and inflammation-related diseases such as atherosclerosis.

Conflict of interest

None.

Acknowledgments

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