and Biochemistry

Accepted: May 29, 2015

1421-9778/15/0365-2025$39.50/0

Original Paper

Acetylcholine Inhibits LPS-Induced MMP-9 Production and Cell Migration via the α7 nAChR-JAK2/STAT3 Pathway in RAW264.7 Cells

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Key Words
Acetylcholine • Macrophage • MMP-9 • Migration • α7 nAChR

Abstract
Background: Excessive activation of matrix metalloproteinase 9 (MMP-9) has been found in several inflammatory diseases. Previous studies have shown that acetylcholine (ACh) reduced the levels of pro-inflammatory cytokines and decreased tissue damage. Therefore, this study was designed to explore the potential effects and mechanisms of ACh on MMP-9 production and cell migration in response to lipopolysaccharide (LPS) stimulation in RAW264.7 cells. ***Methods:*** MMP-9 expression and activity were induced by LPS in RAW264.7 cells, and examined by real-time PCR, western blotting and gelatin zymography, respectively. ELISA was used to determine the changes in MMP-9 secretion among the groups. Macrophage migration was evaluated using transwell migration assay. Knockdown of α7 nicotinic acetylcholine receptor (α7 nAChR) expression was performed using siRNA transfection. ***Results:*** Pre-treatment with ACh inhibited LPS-induced MMP-9 production and macrophage migration in RAW264.7 cells. These effects were abolished by the α7 nAChR antagonist methyllycaconitine (MLA) and α7 nAChR siRNA. The α7 nAChR agonist PNU282987 was found to have an effect similar to that of ACh. Moreover, ACh enhanced the expression of JAK2 and STAT3, and the JAK2 inhibitor AG490 and the STAT3 inhibitor static restored the effect of ACh. Meanwhile, ACh decreased the phosphorylation and nuclear translocation of NF-κB, and this effect was abrogated in the presence of MLA. In addition, the JAK2 and STAT3 inhibitor abolished the inhibitory effects of ACh on phosphorylation of NF-κB. ***Conclusions:*** Activation of α7 nAChR by ACh inhibited LPS-induced MMP-9 production and macrophage migration through the JAK2/STAT3 signaling pathway. These results provide novel insights into the anti-inflammatory effects and mechanisms of ACh.

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Introduction

Macrophages serve as major effector cells in inflammation, including the recognition and processing of foreign materials, activation of the adaptive immune response, and the regulation of tissue repair [1, 2]. These activities require the migration of macrophages to lesion sites. Migration, an important characteristic of macrophages, is mediated by matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM) [3-5]. It has been acknowledged that MMPs, a family of highly conserved zinc-dependent endopeptidases, can regulate aspects of inflammation and contribute to the development of many inflammatory diseases [6]. MMP-9, also known as gelatinase B, has received the most attention among all MMPs because of its immunomodulatory role [7], especially in monocytes and macrophages [5, 8, 9].

MMP-9 is mainly secreted in a latent zymogenic form, and is cleaved by other MMPs or proteases (e.g., plasmin) to yield the activated form. Once activated, MMP-9 is not only able to readily digest denatured collagens, gelatins, and other elements of the ECM but it can also cleave several non-ECM components, such as cytokines and growth factors. However, excessive activation of MMP-9 is involved in the pathogenesis and development of several inflammatory diseases, including arthritis and atherosclerosis. Therefore, the inhibition of MMP-9 expression and activity may be an attractive therapeutic target in these diseases [6, 7]. Unfortunately, limited efficacy of MMP inhibitors has been demonstrated in clinical trials due to their side effects and low specificity. Thus, the development of future therapies would require an exploration of the underlying mechanisms that control MMP-9 expression and activity [7]. MMP-9 is tightly regulated at several levels, including transcription, translation, proenzyme activation, and the tissue inhibitor of metalloproteinases (TIMPs); the transcriptional regulation is particularly important [6, 7, 10]. Several transcription factors have been shown to bind to the promoter region of the MMP-9 gene, including nuclear factor-κ B (NF-κB) and activating protein 1 (AP-1), which leads to MMP-9 transcription [11, 12]. In contrast, signal transducer and activator of transcription 3 (STAT3), a potential anti-inflammatory mediator, can inhibit MMP-9 expression [13]. Other studies have demonstrated that activation of p38 mitogen-activated protein kinase (MAPK) and extracellular regulated protein kinases 1/2 (ERK1/2) up-regulated MMP-9 expression [12, 14, 15], while JNK (c-Jun N-terminal kinase) phosphorylation reduced MMP-9 expression [15, 16]. Thus, upstream regulators of MMP-9, such as STAT3 and NF-κB, may be utilized as therapeutic targets and can provide new insights into the treatment of many inflammatory diseases.

Vagal nerve stimulation (VNS) has been shown to exert anti-inflammatory effects, as well as other protective effects, in several diseases [17]. Acetylcholine (ACh), an important vagal neurotransmitter, inhibited the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and high-mobility group box 1 (HMGB1), via the activation of the α7 nicotinic acetylcholine receptor (α7 nAChR) in macrophages, leading to beneficial effects [17-19]. However, it is unclear whether the Janus tyrosine kinase (JAK2)/STAT3 pathway is involved in the regulation of MMP-9 production and cell migration. Additionally, the role of ACh in the modulation of MMP-9 is also unknown. In this study, we explored the effect of ACh on lipopolysaccharide (LPS)-stimulated MMP-9 expression and potential activity in macrophages. Furthermore, we investigated the possible signaling pathways involved in this process.

Materials and Methods

Reagents

LPS (*Escherichia coli* 0111:B4 L4391), ACh, PNU282987 (P6499), methyllycaconitine citrate salt hydrate (MLA M168), AG490 (T3434), and stattic (S7947) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-JAK2 (BS2432) and anti-STAT3 (Tyr^{705}, P699) antibodies were obtained from Bioworld Technology Inc (Louis Park, MN). Anti-phospho-JAK2 (Tyr$^{1007/1008}$ 3771S), anti-phospho-STAT3 (Tyr^{705}, 9131S), anti-NF-

κB (C22B4), and anti-phospho-NF-κB p65 (Ser^{536} 93H1) antibodies were from Cell Signaling Technology (Danvers, MA). Anti-MMP-9 antibody was obtained from Abcam (Cambridge, MA, Ab38898). Anti-p38 MAPK (sc-728), anti-phospho-p38 MAPK (Tyr^{7182}, sc-101759) and anti-α7 nAChR antibodies (sc-5544) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse total MMP-9 Quantikine ELISA Kit was purchased from R&D Systems (Minneapolis, MN).

Cell culture

RAW264.7 (a murine macrophage cell line, ATCC, TIB-71) cells were cultured in high-glucose DMEM (Hyclone Louis, MO, USA) containing 10% fetal bovine serum (FBS; Hyclone), 100 U/mL penicillin (Sigma, USA) and 100 μg/mL streptomycin (Sigma) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C until 70%-80% confluence (2-3 days) and split 1 to 4.

For experiments, macrophages were cultured in six-well plates at 5×10^5 cells/well for 24 h and starved with FBS-free DMEM for 8 h. To determine the role of LPS, cells were treated with 0, 25, 50, 100 or 200 ng/mL LPS for 0, 1, 2, 4, 8, 12, 24 or 36 h. To confirm its effects, ACh (10^{-8}-10^{-5} mol/L) was added to the medium 30 min prior to LPS stimulation. PNU282987 (a specific α7 nAChR agonist, 10^{-6} mol/L) or pyrrolidinedithiocarbamic acid (PDTC, an NF-κB inhibitor, 10^{-4} mol/L) was used as a positive control for ACh and added to the medium 30 min before LPS treatment. In some experiments RAW264.7 cells were incubated with methyllycaconitine (MLA, a specific α7 nAChR antagonist, 10^{-6} mol/L), Tyrphostin AG490 (a JAK2-specific inhibitor, 10^{-5} mol/L), or stattic (a STAT3 inhibitor, 10^{-5} mol/L) 10 min prior to the addition of ACh.

Total RNA extraction and quantitative real-time PCR

Total RNA was prepared from cultured RAW264.7 cells using RNAiso Plus (TaKaRa). Reverse transcription of RNA was performed with the PrimeScript RT reagent kit (TaKaRa) according to the manufacturer's instruction. The resulting cDNA was subjected to real-time PCR using the following specific primers (Kingsy Biological Technology, Nanjing China): MMP-9 (forward: TGT GTG TTC CCG TTC ATC TT; reverse: GGT CAT AGT TGG CTG TGG TG; 103 bp) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, as an endogenous control) (forward: ACA ACT TTG GCA TTG TGG AA; reverse: GAT GCA GGG ATG ATG TTC TG; 133 bp). Real-time PCR was performed using the iQ5 (Bio-Rad, Hercules, CA) with SYBR Premix Ex Taq™ (TaKaRa).

Western Blotting

Cells were lysed with RIPA (Beyotime, Jiangsu, China) containing 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) after treatment. Culture medium was centrifuged at 12,000 g for 10 min at 4 °C. The protein content in the supernatants was measured using the BCA protein assay kit (Beyotime), and the supernatants were then boiled in 5x loading buffer for 10 min. Equal sample amounts (20 μg) were subjected to 8% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA). The membranes were blocked in 5-8% skim milk or 3% BSA in TBST (25 mmol/L Tris-HCl, 150 mmol/L NaCl and 0.2% Tween-20) for 1-3 h at room temperature. Subsequently, the membranes were incubated with specific primary antibodies at room temperature for 1-2 h and then at 4 °C overnight. After washing five times in TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) for 30-40 min at room temperature. After five washes with TBST, the blots were developed using an enhanced chemiluminescence (ECL) reagent (Millipore) in accordance with the manufacturer's instructions, and the band density was analyzed using the Gel-Pro Analyzer 4.0 software (Media Cybernetics, Bethesda, MD).

ELISA

The concentration of MMP-9 in supernatants was determined using the mouse total MMP-9 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. All samples and standards were prepared in triplicate. MMP-9 levels were expressed as pg/ml. The detection threshold for this assay was 3 pg/mL.

Gelatin Zymography

The protein content of supernatants from each group was determined using the BCA protein assay kit (Beyotime) following centrifugation at 12,000 g at 4 °C. Equal amounts of protein for each sample were

mixed with 5× loading buffer (50 mmol/L Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue), and loaded onto a 10% SDS-PAGE containing 0.1% gelatin. After electrophoresis, gels were rinsed a total of two times in 0.25% Triton X-100 for 30 min at room temperature, and then incubated at 37°C in developing buffer (50 mmol/L Tris–HCl pH 7.4, 5 mM CaCl2, 0.02% Brij-35, 200 mmol/L NaCl) for 20 h. Then, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 in 25% isopropanol and 10% glacial acetic acid for 30 min, and subsequently destained in 50% methanol and 10% glacial acetic acid for 5-10 min. The images were obtained using the Gel Image Formation System and analyzed using the Gel Protein Analyser 4.0 software (Media Cybernetics, Bethesda, MD). Both pro-MMP-9 and the activated gelatinase develop gelatinolytic activity using gelatin zymography [20]. Thus, the term "activity" was used to describe the total gelatinolytic capacity detected in the cell supernatant, which, in consistent with previous studies, is attributed entirely to pro-form of MMP-9 in our experimental conditions [8, 16].

Transwell migration assay

Migration assays were performed using 24-transwell inserts with 8-μm microporous membranes (Corning, Beijing, China). Cell suspensions (0.1 ml, 2×10^5 cells/mL) were added to the upper chamber and incubated for 12 h at 37 °C. Following starvation in FBS-free DMEM for 8 h, the cells were treated with the indicated treatments and allowed to migrate for 24 h. Non-migrating cells were removed from the upper surface of the insert using a cotton swab. Cells were fixed with 4% formalin and stained with DAPI. Migrated cells were visualized using an inverted fluorescence microscope (Nikon, TE-2000U) and then enumerated manually per high-power field (HPF).

Small interfering RNA

RAW264.7 cells were seeded into six-well plates. Upon reaching 70-80% confluence, the cells were transiently transfected with α7 nAChR siRNA (100 nmol/L) or negative control (NC) siRNA (GenePharm, Shanghai, China) using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Gene silencing was monitored by performing western blots on cell extracts isolated 24 h post transfection. Following transfection, the cells were treated with LPS and ACh and MMP-9 mRNA and protein levels, secretion and activity were determined.

Immunofluorescence Assay

To assess the intracellular location of NF-κB p65subunit, RAW264.7 cells were cultured on sterile cover slips. After indicated treatments, the cells were fixed with 4% formaldehyde diluted in warm PBS for 15 min at room temperature and washed three times in PBS. Then the cells were blocked in 5% normal serum/0.3% Triton X-100 in PBS for 1 h at room temperature. Subsequently, the cells were incubated with anti-NF-kB p65 (1:300) overnight at 4°C. Rinsed three times in PBS for 5 min each, the specimen were incubated in a 1:50 dilution of Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:100, abcam, ab150077) for 1 h at room temperature in the dark and washed three times in PBS again. PI counterstaining was performed to stain the nuclei. Finally, the cover slips were mounted and images were viewed by a Leica TCS-SP2 confocal scanning microscope (Leica, Heidelberg, Germany). Fluorescence intensity of NF-κB in nucleus was measured by Image-Pro Plus 6.0 software in 6 fields per each experimental group.

Statistical analysis

All experiments were performed at least three times. All results are expressed as means ± SEM. GraphPad Prism version 5.01 (GraphPad Software Inc., La Jolla, CA, USA) was utilized to analyze the results using a one-way ANOVA followed by a Tukey's post hoc test. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

LPS induces MMP-9 production and cell migration in RAW264.7 cells

We first examined MMP-9 mRNA expression and activity in RAW264.7 cells over time. MMP-9 mRNA and activity were endogenously expressed in RAW264.7 cells, and both were increased by LPS in a time-dependent manner. After treatment with LPS, MMP-9 mRNA was

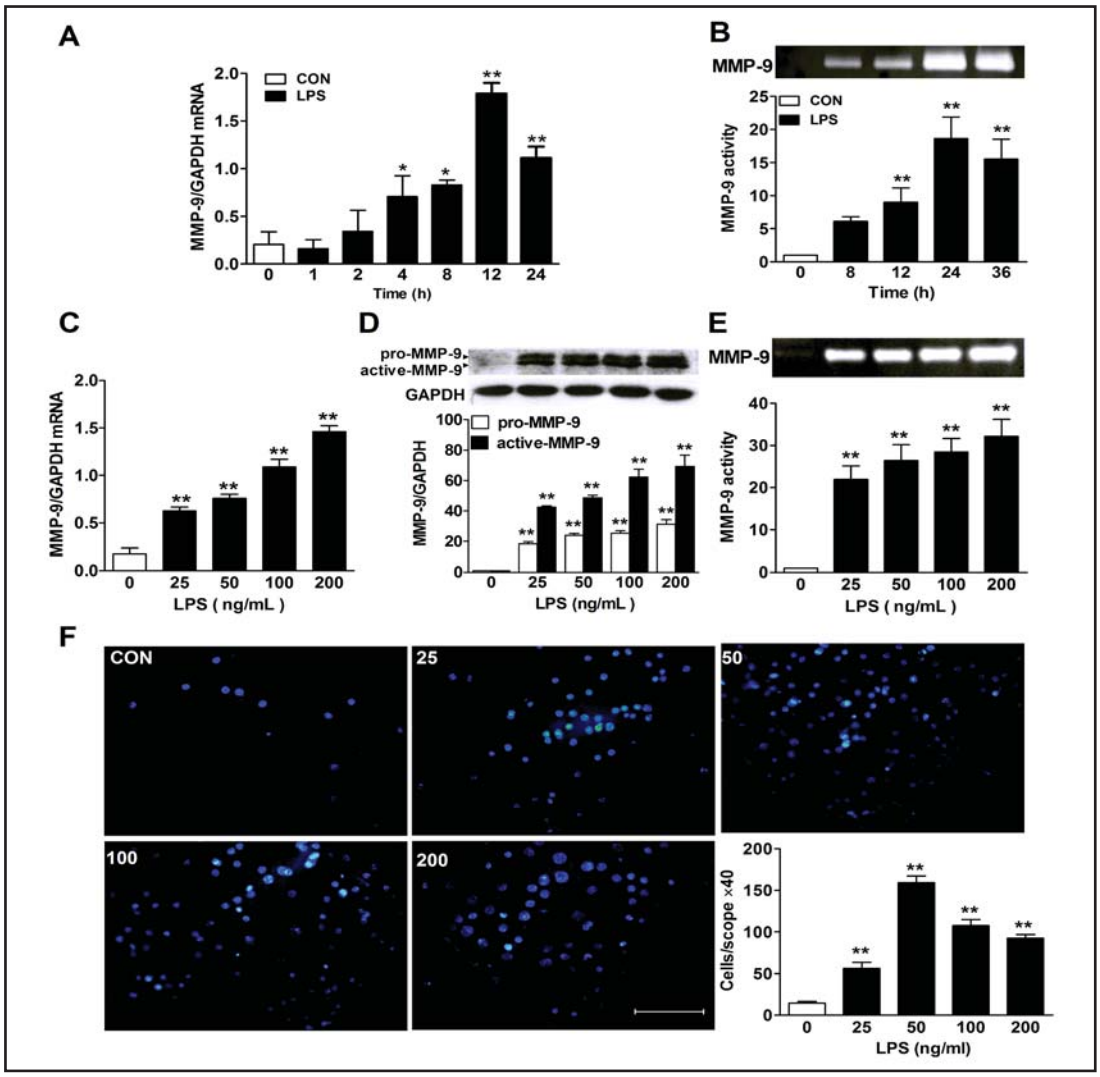

Fig. 1. LPS treatment induces MMP-9 production and migration by RAW264.7 cells. LPS (100 ng/mL) induced (A) MMP-9 mRNA expression and (B) activity over time. LPS increased (C) MMP-9 mRNA, (D) protein, (E) activity, and (F) cell migration in a concentration-dependent manner. Macrophage nuclei stained with DAPI represent migrated cells. Scale bar = 100 μm. Data are expressed as means ± SEM, $n = 4$, $^{*}P < 0.05$, versus control group; $^{**}P < 0.01$, versus control group.

significantly increased at 8 h, compared to the control, maximally expressed at 12 h, and decreased at 24 h (Fig. 1A). Moreover, MMP-9 activity was significantly increased after LPS stimulation at 12 h, peaked at 24 h, and decreased at 36 h (Fig. 1B). Next, we measured MMP-9 production and activity in response to various concentrations of LPS (Fig. 1C-E). The data demonstrated that MMP-9 mRNA, active-MMP-9 and pro-MMP-9 protein expression, as well as MMP-9 activity were induced by LPS in a concentration-dependent manner; significant effects were evident at concentrations of 25-200 ng/mL. Based on these results, in subsequent experiments, 25 ng/mL LPS was used to stimulate the cells, mRNA was assayed at 12 h, and MMP-9 activity at 24 h.

Since MMPs, especially MMP-9, are required for cell migration [3, 4], we assessed the migration of RAW264.7 cells. LPS at 25-200 ng/mL induced a significant increase in cell migration following 24-h incubation (Fig. 1F). Thus, 25 ng/mL LPS was subsequently used for cell migration studies.

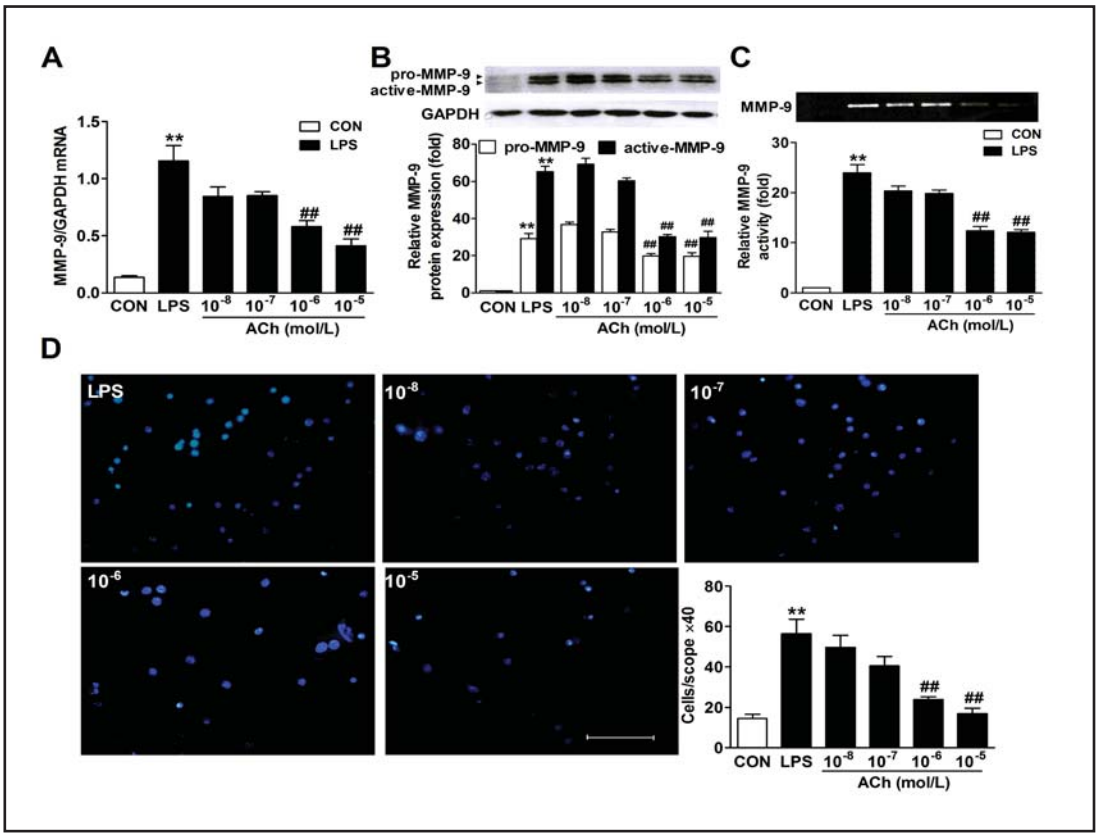

Fig. 2. ACh inhibits MMP-9 production and RAW264.7 cell migration induced by LPS. ACh (10^{-8}-10^{-5} mol/L) decreased LPS-induced (25 ng/mL) MMP-9 (A) mRNA, (B) protein expression, (C) activity, and (D) cell migration in a concentration-dependent manner. Macrophage nuclei stained with DAPI represent migrated cells. Scale bar = 100 μm. Data are expressed as means ± SEM, $n = 6$, $^{**}P < 0.01$, versus control group; $^{\#\#}P < 0.01$, versus LPS group.

ACh reduces LPS-induced MMP-9 production and cell migration

To investigate the effects of ACh on MMP-9 production, RAW264.7 cells were pretreated with 10^{-8}-10^{-5} mol/L ACh 30 min before LPS stimulation. The results showed that ACh (10^{-6} and 10^{-5} mol/L) dramatically decreased the LPS-induced MMP-9 mRNA expression, active-MMP-9 and pro-MMP-9 protein expression, as well as the MMP-9 activity (Fig. 2A-C). Similarly, ACh (10^{-6} and 10^{-5} mol/L) significantly inhibited RAW264.7 cell migration (Fig. 2D). Based on these results, 10^{-6} mol/L ACh was used in the subsequent experiments.

α7 nAChR mediates the inhibitory effect of ACh on LPS-stimulated MMP-9 production and cell migration

To determine the role of α7 nAChR in MMP-9 production, the specific α7 nAChR antagonist MLA was used. As shown in Fig. 3, MLA pretreatment abolished the inhibitory effects by ACh on LPS-induced MMP-9 production, activity, and cell migration in RAW264.7 cells. In addition to the pharmacologic inhibitor, α7 nAChRs siRNA was utilized to confirm this finding. Western blot analysis indicated that α7 nAChRs siRNA treatment considerably decreased the expression of α7 nAChR (Fig. 4A). Following the analysis of transfection efficiency, RAW264.7 cells transfected with α7 nAChR siRNA were stimulated with LPS and then treated with ACh as before. The effects of ACh on MMP-9 were attenuated by α7 nAChR siRNA, including mRNA, protein expression and activity (Fig. 4B-E). Similarly, ACh failed to inhibit migration of RAW64.7 cells transfected with α7 nAChR siRNA (Fig. 4F).

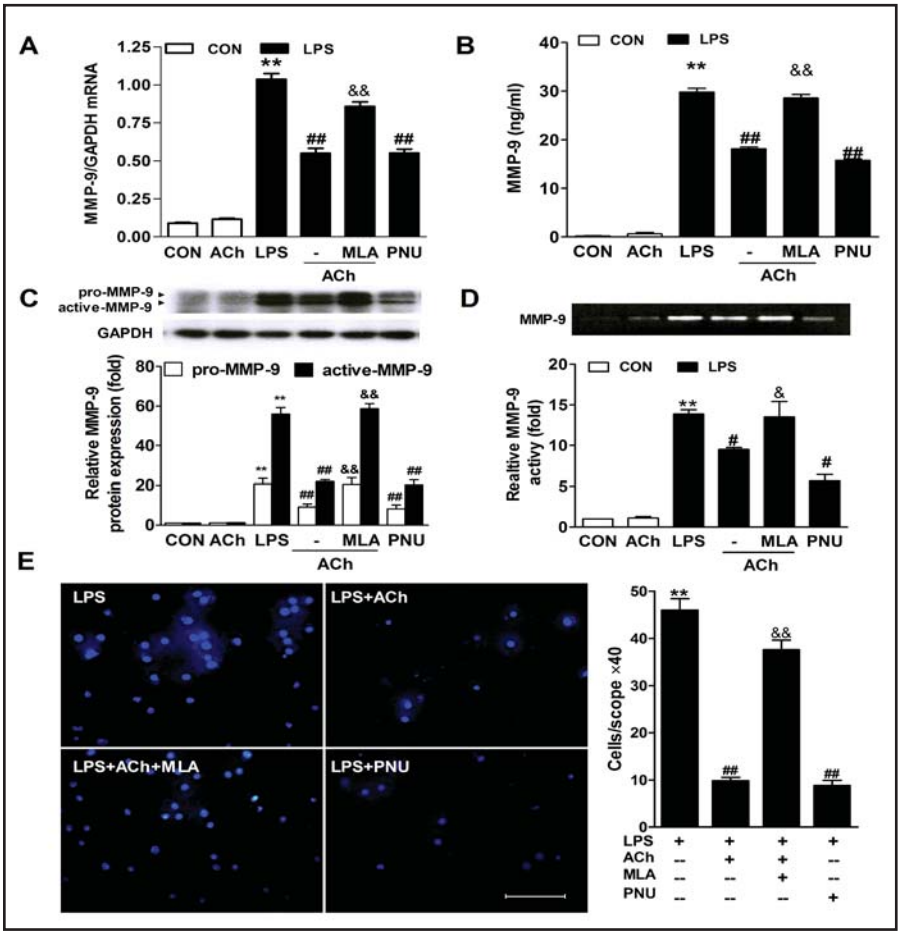

Fig. 3. ACh suppressed LPS-induced MMP-9 production and activity via α7 nAChR. ACh (10^{-6} mol/L) decreased LPS-induced MMP-9 (A) mRNA, (B) protein expression, (C) secretion, (D) activity, and (E) cell migration via α7 nAChR. PNU: PNU282987. Data are expressed as means ± SEM, n = 4, $^{**}P < 0.01$, versus control group; $^{\#}P < 0.05$, versus LPS group; $^{\#\#}P < 0.01$, versus LPS group; $^{\&}P < 0.05$ versus, ACh+LPS group; $^{\&\&}P < 0.01$, versus ACh+LPS group.

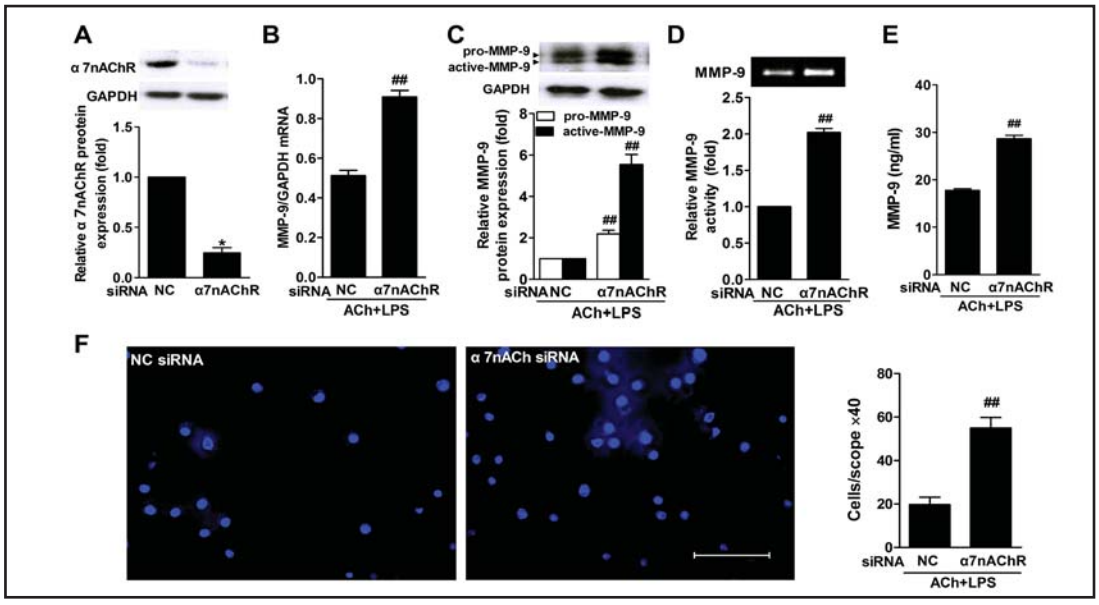

Fig. 4. α7 nAChR siRNA abolished the inhibitory effects of ACh. (A) The silencing efficiency of α7AChR siRNA was detected by Western blotting. α7 nAChR siRNA abolished the inhibitory effects of ACh on MMP-9 (B) mRNA, (C) protein expression, (D) secretion, (E) activity, and (F) cell migration. Macrophage nuclei stained with DAPI represent migrated cells. Scale bar = 100 μm. NC: negative control. Data are expressed as means ± SEM, n = 4, $^{*}P < 0.05$, versus NC group; $^{\#\#}P < 0.01$, versus NC group.

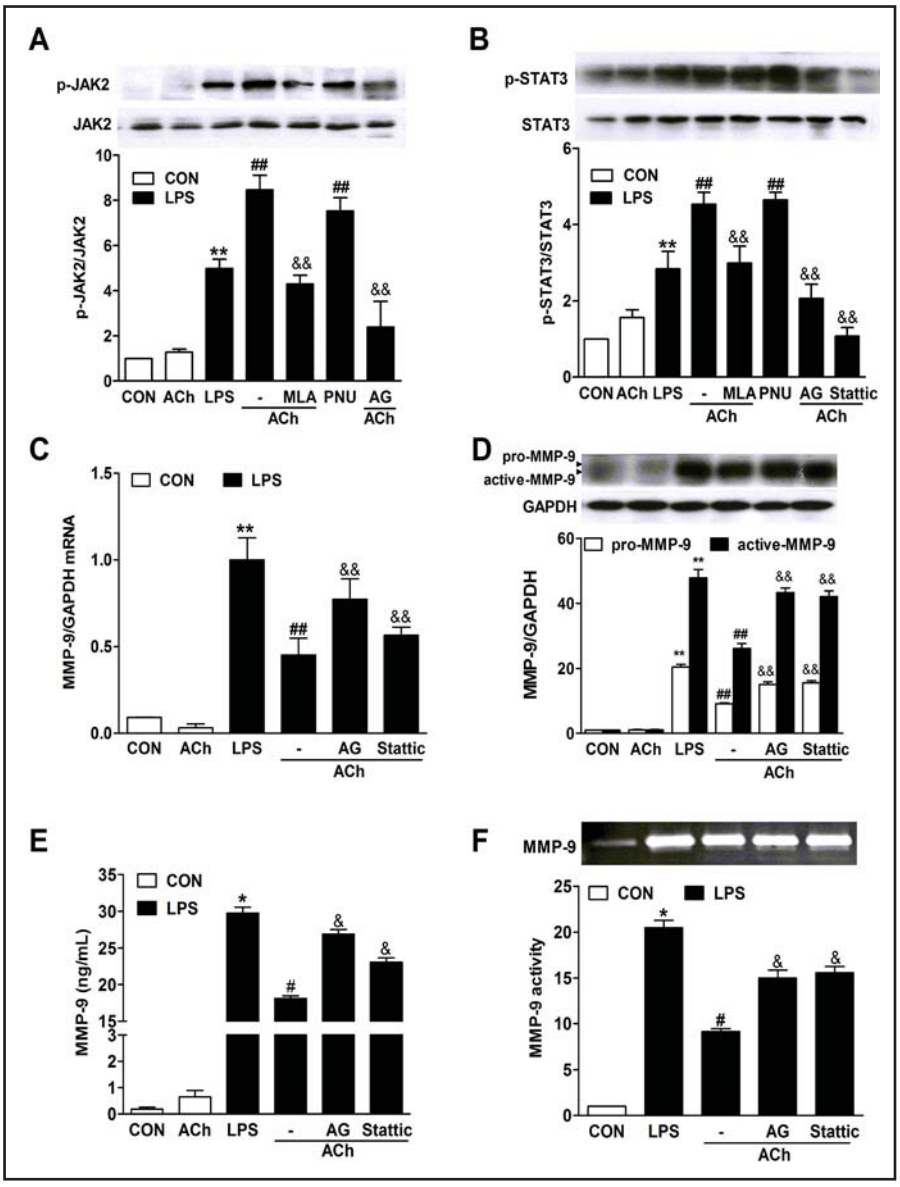

Fig. 5. The JAK2-STAT3 pathway mediated the inhibitory effects of ACh on LPS-induced MMP-9 production and cell migration. ACh enhanced the phosphorylation of (A) JAK2 and (B) STAT3. AG490 and stattic reversed the effects of ACh on MMP-9 (C) mRNA, (D) protein production, (E) secretion, and (F) activity. Data are expressed as means ± SEM, n = 4, $^{*}P < 0.05$, versus control group; $^{**}P < 0.01$, versus control group; $^{\#}P < 0.05$, versus control group; $^{\#\#}P < 0.01$, versus LPS group; $^{\&}P < 0.05$, versus ACh+LPS group; $^{\&\&}P < 0.01$, versus ACh+LPS group.

Additionally, we determined that the specific α7 nAChR agonist PNU282987 exerted effects on macrophages as those of ACh. Therefore, these data suggest that α7 nAChR plays an essential role in LPS-induced MMP-9 production and cell migration.

ACh decreases MMP-9 production and cell migration via JAK2/STAT3 pathway

To further explore the potential mechanisms by which ACh exerted on LPS-stimulated MMP-9 production and macrophage migration, the JAK2/STAT3 pathway was investigated. The results demonstrated that following LPS stimulation the phosphorylation levels of JAK2/STAT3 were significantly higher than in the control group. However, treatment with ACh further increased the levels of their phosphorylation while the α7 nAChR antagonist MLA and the JAK2 inhibitor AG490 dramatically decreased STAT3 phosphorylation (Fig. 5A, B). In addition, the JAK2 inhibitor AG490 and the STAT3 inhibitor, stattic, resulted in decreases in the production, activity of MMP-9 as well as cell migration induced by ACh (Fig. 5C-F and Fig. 6). These data demonstrated that the JAK2/STAT3 pathway likely plays an essential role in the inhibitory effect of ACh on LPS-induced MMP-9 production and cell migration.

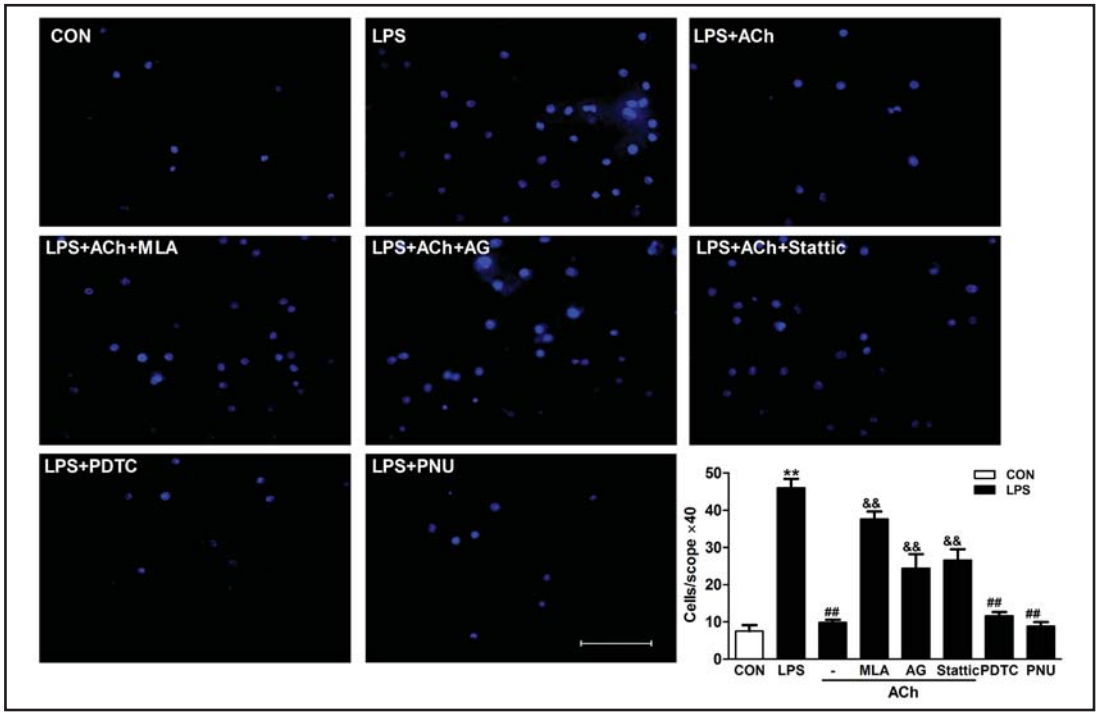

Fig. 6. ACh suppressed LPS-induced RAW264.7 cell migration via the JAK2-STAT3 pathway and inhibition of NF-κB. Macrophage nuclei stained with DAPI represent migrated cells. Scale bar=100μm. Data are expressed as means ± SEM, $n = 6$, $^{**}P < 0.01$, versus control group; $^{\#\#}P < 0.01$, versus LPS group; $^{\&\&}P < 0.01$ versus ACh+LPS group.

NF-κB is involved in the inhibitory effect of ACh on MMP-9 production and cell migration

It has previously been shown that NF-κB and p38 MAPK play an important role in the regulation of MMP-9 and macrophage migration [11, 14]. In the present study, we determined that NF-κB and p38 MAPK activation were markedly increased in RAW264.7 cells in response to LPS stimulation. Treatment with both ACh and the NF-κB inhibitor PDTC prevented the increases in p38 MAPK and NF-κB phosphorylation (Fig. 7A, B). Moreover, immunofluorescent staining indicated that ACh decreased nuclear translocation of NF-κB in response to LPS treatment (Fig. 8). While the α7 nAChR antagonist, MLA, recovered the preventive effect of ACh (Fig. 7A, B). Additionally, similar to ACh, PDTC treatment decreased LPS-induced MMP-9 production and cell migration (Fig. 7E-H and Fig. 8). These results suggest the effect of ACh may be associated with reduced NF-κB activity. In addition, as shown in Fig. 7C and 7D, treatment with AG490 and stattic diminished the ACh-induced suppression of NF-κB phosphorylation, suggesting that NF-κB is likely negative regulated by JAK2/STAT3 pathway.

Discussion

The present study has shown that ACh ameliorated LPS-induced MMP-9 overexpression and cell migration via α7 nAChR-mediated JAK2/STAT3 signalling as well as inhibition of NF-κB in RAW264.7 cells. The major findings were summarized as the following: 1) ACh inhibited MMP-9 expression and activity, and cell migration in a concentration-dependent manner after LPS administration; 2) α7 nAChR antagonist MLA or α7 nAChR siRNA abolished the benefits elicited by ACh; 3) ACh enhanced JAK2/STAT3 phosphorylation and attenuated NF-κB activation, resulting in the inhibitory of MMP-9 production and cell migration. Our data provides evidence for a possible mechanism that might contribute to the protective effect afforded by ACh on MMP-9 regulation (Fig. 9).

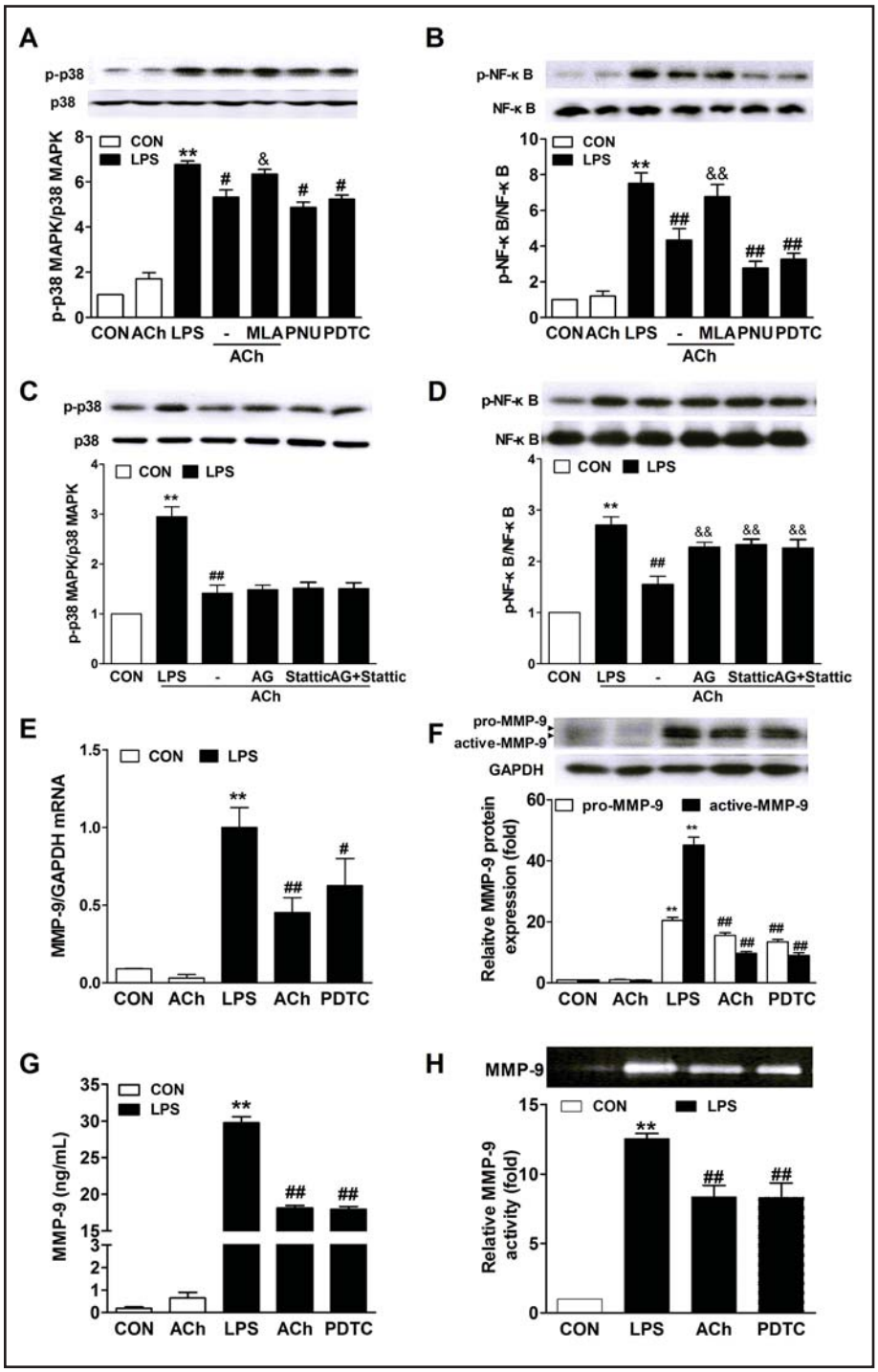

Fig. 7. NF-κB is involved in the inhibitory effect of ACh on LPS-induced MMP-9 production and activity. ACh and PDTC decreased (A) p38 MAPK phosphorylation and (B) NF-κB phosphorylation. JAK2 and STAT3 inhibitors regulate (C) p38 MAPK phosphorylation and (D) NF-κB phosphorylation. ACh and PDTC inhibited MMP-9 (E) mRNA, (F) protein expression, (G) secretion and (H) activity. Data are expressed as means ± SEM, n = 4, $^{**}P < 0.01$, versus control group; $^{\#}P < 0.05$, versus LPS group; $^{\#\#}P < 0.01$, versus LPS group; $^{\&}P < 0.05$ versus ACh+LPS group; $^{\&\&}P < 0.01$ versus ACh+LPS group.

MMP-9 has been use as a marker of various inflammatory diseases, such as atherosclerosis, arthritis and systemic lupus erythematosus [21]. MMP-9 production and activity could be induced by LPS, which is a common potent activator of inflammatory responses [22-25]. In addition, MMP-9 was closely associated with cell migration [4, 5]. In the current study, we verified that LPS not only induced the production of MMP-9 mRNA and protein expression, but also induced the potential activity of MMP-9, as well as cell migration. MMP-9 gene expression is primarily regulated at the transcriptional level via transcription

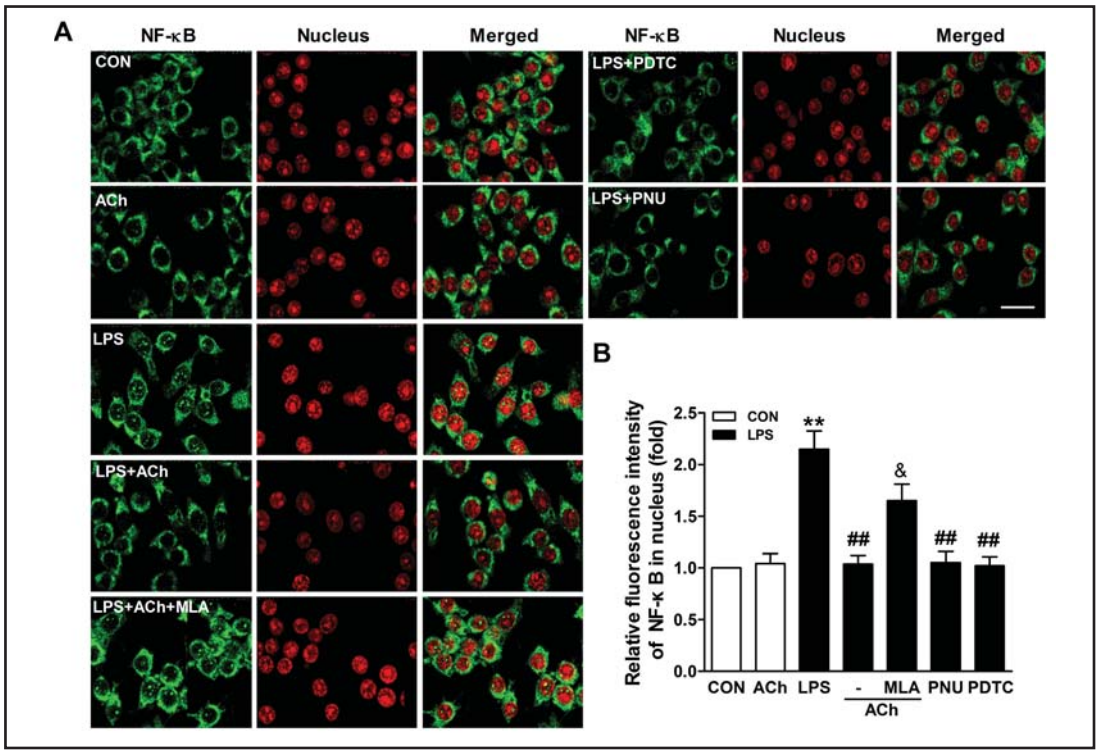

Fig. 8. ACh attenuates LPS-induced NF-κB nuclear translocation. After indicated treatments, RAW264.7 cells were fixed for immunocytochemical staining. NF-κB and nuclei were visualized using Alexa Fluor 488 (green) and PI (red), respectively. (A) Representative micrographs were shown: NF-kB immunofluorescence (left), nuclei stained with Hoechst (middle), and the merged images (right). Scale bar = 20 μm. (B) The relative immunofluorescence intensity of nuclear NF-κB. Data are expressed as means ± SEM, n = 4, $^{**}P < 0.01$, versus control group; $^{\#\#}P < 0.01$, versus LPS group; $^{\&}P < 0.05$, versus ACh+LPS group.

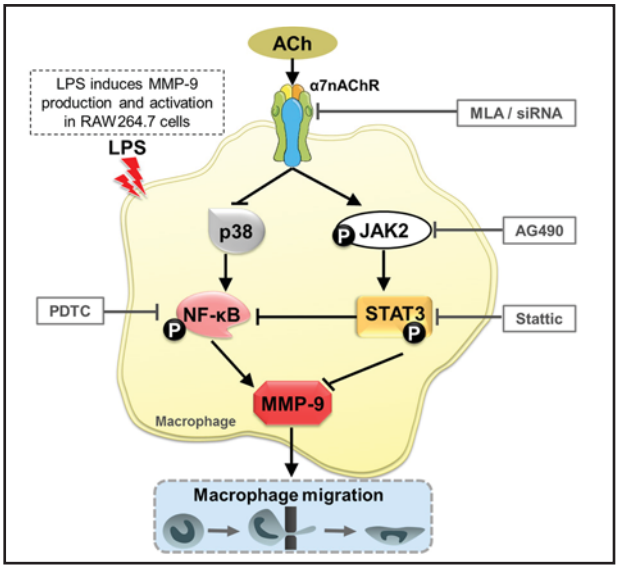

Fig. 9. Proposed schematic of the possible mechanisms by which ACh exerts inhibitory effects on LPS-induced MMP-9 production and macrophage migration. LPS induces MMP-9 production and macrophage migration, which is inhibited by ACh via α7 nAChR-mediated activation of the JAK2/STAT3 pathway and inhibition of NF-κB signaling. MLA:α7 nAChR inhibitor, AG490: JKA2 inhibitor, stattic: STAT3 inhibitor, PDTC: NF-κB inhibitor.

factors such as NF-κB and STAT3 [7, 10]. Mutation of NF-κB binding sites decreased the activity of MMP-9 promoter in endothelial cells and A549 cells [26, 27]. In our study, LPS enhanced the phosphorylation of NF-κB. Treatment with NF-κB inhibitor PDTC dimished the MMP-9 overexpression triggered by LPS, suggesting that NF-κB is required for this response. In contrast to NF-κB, transcriptional factor STAT3 was able to decline the expression of

MMP-9 [10, 13,]. Morever, The inhibitor of JAK, the upper kinase of STAT3, enhanced MMP-9 expression [28]. Thus, NF-κB and STAT3 could be dual targets of MMP-9 regulation.

Accumulating evidence indicated that the activation of efferent vagal nerve fibers and cholinergic agonists could modulate local and systemic inflammatory responses via α7 nAChR [17, 18]. ACh, the vagal neurotransmitter, attenuated both the early and late phase of LPS-induced TNF-α release in human macrophages [29]. Activation of α7 nAChR attenuates LPS-induced acute lung injuries and improves the survival of mice with sepsis [30, 31]. In contrast, vagus nerve stimulation in α7 nAChR-deficient mice failed to reduce serum TNF levels [32]. Consistent with these results, we previously demonstrated that VNS protected against remote vascular dysfunction induced by myocardial infarction through α7 nAChR [33]. The present study found that α7 nAChR knockdown using siRNA or MLA abolished the inhibitory effects of ACh on LPS-induced MMP-9 production and cell migration. These data suggested that α7 nAChR played an essential role in ACh-mediated inhibition of MMP-9. Therefore, stimulation of α7 nAChR with cholinergic agonists has become an accepted novel therapeutic target in inflammation-based diseases [34]. Futhermore, the tissue inhibitor of metalloproteinase (TIMPs) family, a specific endogenous inhibitor of MMPs, can suppress macrophage migration by inhibiting the active form of MMP-9 [6, 7, 12]. A recent study demonstrated that ACh increased the expression of TIMP-1 in the myocardium [35]. In the present study, ACh inhibited MMP-9 expression and activity induced by LPS. Therefore, TIMPs may contribute to the effects of ACh on MMP-9 and cell migration, although further studies are required to elucidate the details of its role.

The anti-inflammatory activity of ACh involves JAK2/STAT3 signaling [36, 37]. Previous studies have demonstrated that nicotine acted on macrophages via the recruitment of JAK2 to the α7 nAChR and activation of JAK2, thereby initiating the anti-inflammatory STAT3 signaling cascade [36]. Similarly, our current data showed that ACh upregulated JAK2 and STAT3 phosphorylation in the presence of LPS. The beneficial effect of ACh was blocked by AG490 and stattic. Furthermore, down-regulation of NF-κB was also shown to contribute to the action of cholingergic agonists [19, 29]. The present study found that ACh, similar with PDTC, inhibited the activation of NF-κB and then led to anti-inflammatory effects. Interestingly, AG490 and stattic diminished the ACh-induced suppression of NF-κB phosphorylation. Recent studies suggested that JAK2/STAT3 acted as negative regulator of NF-kB activation [38, 39]. The potential mechanisms are required to be clarified in the future and dual targets of them has become a significant therapeutic strategy [39].

In conclusion, the present study demonstrated that ACh inhibited LPS-induced MMP-9 production and cell migration by activating JAK2/STAT3 signaling pathway and decreasing NF-κB activation, which depends on α7 nAChR. Inhibition of MMP-9 overexpression is a major point of focus for modulating relevant diseases. Our findings provided the novel insights into the anti-inflammatory effects and mechanisms of ACh, allowing vagal regulation to serve as a novel target for preventing and treating inflammatory diseases.

Acknowledgements

This work is supported by National Natural Science Foundation of China (Major International Joint Research Project, No. 81120108002; General Project, No. 81473203; Young Project No. 81300114), Specialized Research Fund for the Doctoral Program of Higher Education (No. 20130201130008).

Disclosure Statement

None.

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