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ORIGINAL RESEARCH

Benidipine Hydrochloride Inhibits NLRP3 Inflammasome Activation by Inhibiting LPS-Induced NF-κB Signaling in THP-1 Macrophages

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Introduction: NLRP3, ASC, and procaspase-1 form the multiprotein complex known as the NLRP3 inflammasome. Following the priming of NLRP3 by TLR4 ligand, the activation of the NLRP3 inflammasome causes caspase-1 maturation, which results in the release of IL-1β. Calcium channel antagonists are commonly employed as antihypertensive medications and have anti-inflammatory properties through the inhibition of cytokine release, specifically IL-1β. The impact of calcium channel antagonists on NLRP3 inflammasomes, however, has not been well studied. This study aimed to investigate the effect of the calcium channel blocker benidipine hydrochloride on LPS-induced NLRP3 inflammasome activation in THP-1 macrophages and its possible mechanism.

Methods: Firstly, the cytotoxicity of benidipine hydrochloride was determined by MTT. The effect of benidipine hydrochloride on LPS-induced IL-1β release was determined by ELISA. Then, the effect of benidipine hydrochloride on the expression of IL-1β, NLRP3, ASC, and Caspase-1 induced by LPS was determined by QPCR, and the expression of IL-1β, GSDMD, Caspase-1, and their active forms was determined by Western blot, and the activation of NF-κB was determined by Western blot and immunofluorescence. Finally, the production of ROS was determined by flow cytometry and fluorescence microscopy.

Results: Benidipine hydrochloride was found to drastically lower the expression of NLRP3, ASC, and caspase 1, which in turn decreased the amount of IL-1β secreted by THP-1 macrophages. Benidipine hydrochloride dramatically reduced the phosphorylation level of NF-κB p65 and its nuclear translocation in THP-1 macrophages. Furthermore, benidipine hydrochloride significantly decreased the generation of ROS.

Discussion: Based on these results, we deduced that benidipine hydrochloride prevents ROS formation in THP-1 macrophages and LPS-induced NF-κB signaling, which in turn prevents the activation of NLRP3 inflammasomes and the release of IL-1β.

Keywords: benidipine hydrochloride, NLRP3, NF-κB, ROS, THP-1

Introduction

A two-edged sword, inflammation is essential to host defense reactions, but uncontrolled inflammation produces a large number of inflammatory cytokines that can lead to tissue damage and severe diseases.[1] One important inflammatory cytokine implicated in both local and systemic reactions to injury and infection is IL-1β. An excess of IL-1β can also result in a variety of autoinflammatory disorders. An intracellular polyprotein complex known as an inflammasome, of which the NLRP3 inflammasome is the most extensively researched, is necessary for the development of IL-1β.

NLRP3 inflammasome is a family member of NLR, composed of the LRR domain, NACHT, and pyrin domain, which can recruit ASC and pro-caspase-1 to complete the assembly of inflammasomes and activate the NLRP3 function.[2] There are two phases involved in activating the NLRP3 inflammasome. Firstly, LPS activates the TLR and the NF-κB signaling pathway, which increases the transcription level of inflammasome-related components such as NLRP3 and IL-1β.[3] The second stage involves the formation and activation of the NLRP3 inflammasome in response to external stimuli. NLRP3, ASC, and pro-caspase-1 combine to form the inflammasome complex that facilitates the production and

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maturation of IL-1β.[4,5] Pro-inflammatory factor production is regulated by the NLRP3 inflammasome, and if the NLRP3 inflammasome is excessively activated, it will lead to a homeostasis imbalance in the body, leading to inflammatory diseases. Therefore, inhibiting the initiation and assembly of NLRP3 inflammasome has become the target for the treatment of inflammatory diseases.

In recent years, some NLRP3 inflammasome blockers have been studied, such as MCC950,[6] INF200,[7] alantolactone,[8] etc., but most NLRP3 inflammasome blockers have not been used in clinical practice due to strong toxicity and side effects or poor bioavailability. Benidipine hydrochloride is a dihydropyridine calcium channel blocker that blocks calcium influx and has been frequently used to treat angina pectoris and hypertension.[9] Notably, a large body of research has demonstrated the anti-inflammatory properties of calcium channel antagonists,[10] but less research has examined the impact of these agents on NLRP3 inflammasomes. The objective of this investigation was to assess the impact of benidipine hydrochloride on the activation of the NLRP3 inflammasome and investigate the underlying mechanisms.

Material and Methods

Chemicals and Reagents

Benidipine hydrochloride (BH) (Cat# T6227) and MCC950 (Cat# T3701) were purchased from Topscience. Phorbol 12-myristate 13-acetate (PMA) (Cat# HY18739) was purchased from MedChemExpress. Lipopolysaccharides (LPS) (Cat# L2880) and adenosine 5'-triphosphate (ATP) (Cat# A2383) were purchased from Sigma. MTT (Cat# M8180) was obtained from Solarbio. IL-1β ELISA kit (Cat# 88–7261-22) was purchased from ThermoFisher. Antibodies against β-actin (Cat# AC006), IL-1β (Cat# A16288), GSDMD (Cat# A20197), NF-κB p65 (Cat# A2547) and phospho-NF-κB p65 (Cat# AP0475) were obtained from ABclonal. Antibody against Caspase-1 (Cat# WL03450) was obtained from Wanleibio. DAPI (Cat# C1005) and DCFH-DA fluorescent probe (Cat# S0033S) were purchased from the Beyotime.

Cell Culture

THP-1 cell lines were obtained from ATCC and cultured in 1640 medium containing 5% penicillin-streptomycin solution and 10% fetal bovine serum and placed in a cell incubator containing 5% CO_2 at 37°C.

Cell Viability Assay

MTT assay was employed to assess the proliferation of cells. THP-1 cells were seeded in 96-well plates, and 100 ng/mL PAM was added to induce cell adherence. The fresh culture medium was replaced after 48 h, and multiple concentrations of benidipine hydrochloride were added for 24 h. After treating the cells with MTT (5 mg/mL) and allowing them to incubate for an additional 4 h, DMSO was added to dissolve the formazan precipitate. Finally, a TECAN Spark & Infinite 200 pro plate reader was used to detect the absorbance at 490 nm.

ELISA Assay

THP-1 cells were seeded onto 6-well plates and differentiated for 48 h with 100 ng/mL PMA. The fresh culture medium was replaced, and benidipine hydrochloride was added 1 h in advance. Subsequently, the cells were stimulated for 4 h with 1 μg/mL LPS, and for an additional 2 h, 5 mM ATP was added. After collecting the cell supernatant, the levels of IL-1β were determined using an ELISA kit in accordance with the manufacturer's instructions. The TECAN Spark & Infinite 200 pro plate reader was used to measure the absorbance at 450 nm. By comparing the results with the standard curve, the amount of IL-1β in the supernatant was ascertained.

Real-Time PCR (RT-PCR)

THP-1 cells were treated in the same way as described in 2.4. After the cells were gathered, Trizol (Vazyme) was used to extract the cells' total RNA. The cDNA was then obtained by reverse transcription using the TaKaRa enzyme. AceQ qPCR SYBR Green Master Mix (Vazyme) was used for RT-PCR and the relative expression of the target gene was determined by the $2^{-\Delta\Delta CT}$ method. The primers specifically used in this investigation were listed in Table 1.

Table 1 Primers Used in This Study

Gene	Primer sequence (5'-3')
GAPDH	F-TGCACCACCAACTGCTTAGC
	R-GGCATGGACTGTGGTCATGAG
IL-1β	F-TCGCCAGTGAAATGATGGCT
	R-TGGAAGGAGCACTTCATCTGTT
NLRP3	F-CTACACACGACTGCGTCTCATCAA
	R-GGGTCAAACAGCAACTCCATCTTA
ASC	F-GTCACAAACGTTGAGTGGCT
	R-AAGTCCTTGCAGGTCCAGTT
Caspase-1	F-GCACACGTCTTGCTCTCATT
	R-GCCTCCAGCTCTGTAGTCAT
TLR4	F-AGACCTGTCCCTGAACCCTAT
	R-CGATGGACTTCTAAACCAGCCA

Western Blot Analysis

THP-1 cells were treated in the same way as described in 2.4. The cells were collected and lysed with RIPA. The resulting protein samples were quantified, and the protein was separated using SDS-PAGE. Subsequently, the protein that had been isolated was placed onto membranes made of polyvinylidene difluoride (PVDF) from Millipore, USA. The membranes were sealed using 5% skim milk for 1 h, incubated with the designated antibodies for a whole night at 4 °C, and then with the secondary antibody for 1 h at room temperature. Finally, the Bio-Rad Chemiluminescence image system was used to observe the immunological complexes.

Immunofluorescence Analysis

THP-1 cells were treated in the same way as described in 2.4. The cells were fixed for 30 min with 4% paraformaldehyde, and permeabilized for 15 min with 0.1% Triton X-100. Subsequently, the cells were blocked for 1 h with 5% BSA, incubated overnight at 4°C with the anti-p65 antibody (1:1000), and then incubated for 1 h with a FITC goat anti-rabbit lgG secondary antibody. Ultimately, the cells underwent a 5-minute room temperature DAPI staining process before being captured on fluorescence microscopy images.

ROS Assay

THP-1 cells were treated in the same way as described in 2.4. Subsequently, the cells were treated with 10 μM DCFH-DA solution (Beyotime, China) for 30 min in complete darkness. Ultimately, the fluorescence intensity was measured using the BD AccuriTM C6 Plus flow cytometer.

Statistical Analysis

One-way ANOVA was used for statistical analysis, and Dunnett's post hoc test was performed after. The expression of all the data is means ± SD. *** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Results

Effect of Benidipine Hydrochloride on Cell Survival

We employed the MTT test to gauge the cytotoxicity of benidipine hydrochloride on THP-1 macrophages. Benidipine hydrochloride, when compared to the control group, did not significantly affect cell survival at doses of 40 μM and below, according to the data (Figure 1). Subsequently, we used 10, 20, and 40 μM of benidipine hydrochloride in the following experiments.

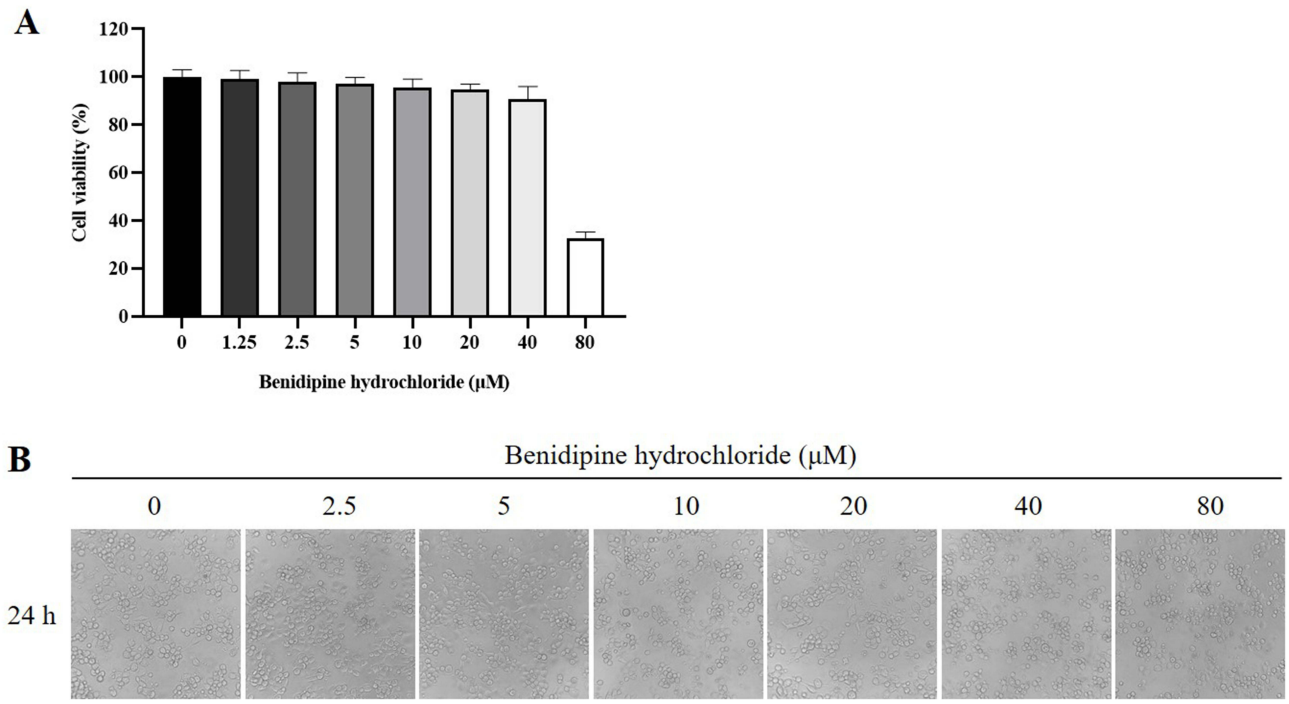

Figure 1 Effect of benidipine hydrochloride on the survival of THP-1 macrophages. (**A**) MTT was used to detect the cell viability of THP-1 macrophages after benidipine hydrochloride (0–80 μM) was applied for 24 h. (**B**) Cell morphology after benidipine hydrochloride treatment.

Benidipine Hydrochloride Inhibited the Expression and Release of IL-1β

After LPS and ATP treatment, samples are collected to determine the level of IL-1β. ELISA was used to measure the amount of IL-1β in the supernatant, as indicated in Figure 2A. In comparison to the control group, the LPS+ATP-stimulated group exhibited a considerably increased quantity of IL-1β. The concentration of LPS+ATP-induced IL-1β is markedly reduced by benidipine hydrochloride, suggesting that the compound inhibited the release of IL-1β. The transcriptional expression of IL-1β was detected by real-time PCR. The findings indicated that the IL-1β mRNA level was also significantly lower in the LPS+ATP +benidipine hydrochloride group compared to the LPS+ATP group (Figure 2B). Pro-IL-1β, pro-caspase-1 in cell lysates, and IL-1β, caspase-1 protein in cell supernatants were measured by Western blotting, with quantitative data displayed (Figure 2C and D). It was shown that the LPS+ATP group had a significantly higher level of IL-1β protein than the control group, while the LPS+ATP+benidipine hydrochloride group had a significantly lower level of IL-1β protein than the LPS+ATP group.

Benidipine Hydrochloride Attenuated the Activation of NLRP3 Inflammasomes and Pyroptosis

We also looked into the impact of benidipine hydrochloride on NLRP3 inflammasome activation in THP-1 macrophages to confirm if the elevation in IL-1β production triggered by LPS resulted from the activation of NLRP3 inflammasome. The findings demonstrated that LPS stimulation markedly enhanced NLRP3 mRNA expression, and that benidipine hydrochloride administration could reverse this increase in NLRP3 mRNA expression (Figure 3A). Additionally, benidipine hydrochloride also dramatically decreased the expression of ASC and caspase 1 (Figure 3B and C).

Cell lysis and swelling are hallmarks of pyroptosis, a type of controlled cell death. NLRP3 inflammasome assembly triggers pyroptosis, leading to caspase-1 activation. The cleaved caspase-1 then promotes N-terminal Gasdermin D maturation, forming pores in the plasma membrane and contributing to pyroptosis. Our results showed that benidipine hydrochloride inhibited the cleavage of GSDMD (Figure 3D and E) and the processing and secretion of IL-1β (Figure 2) by downregulating NLRP3.

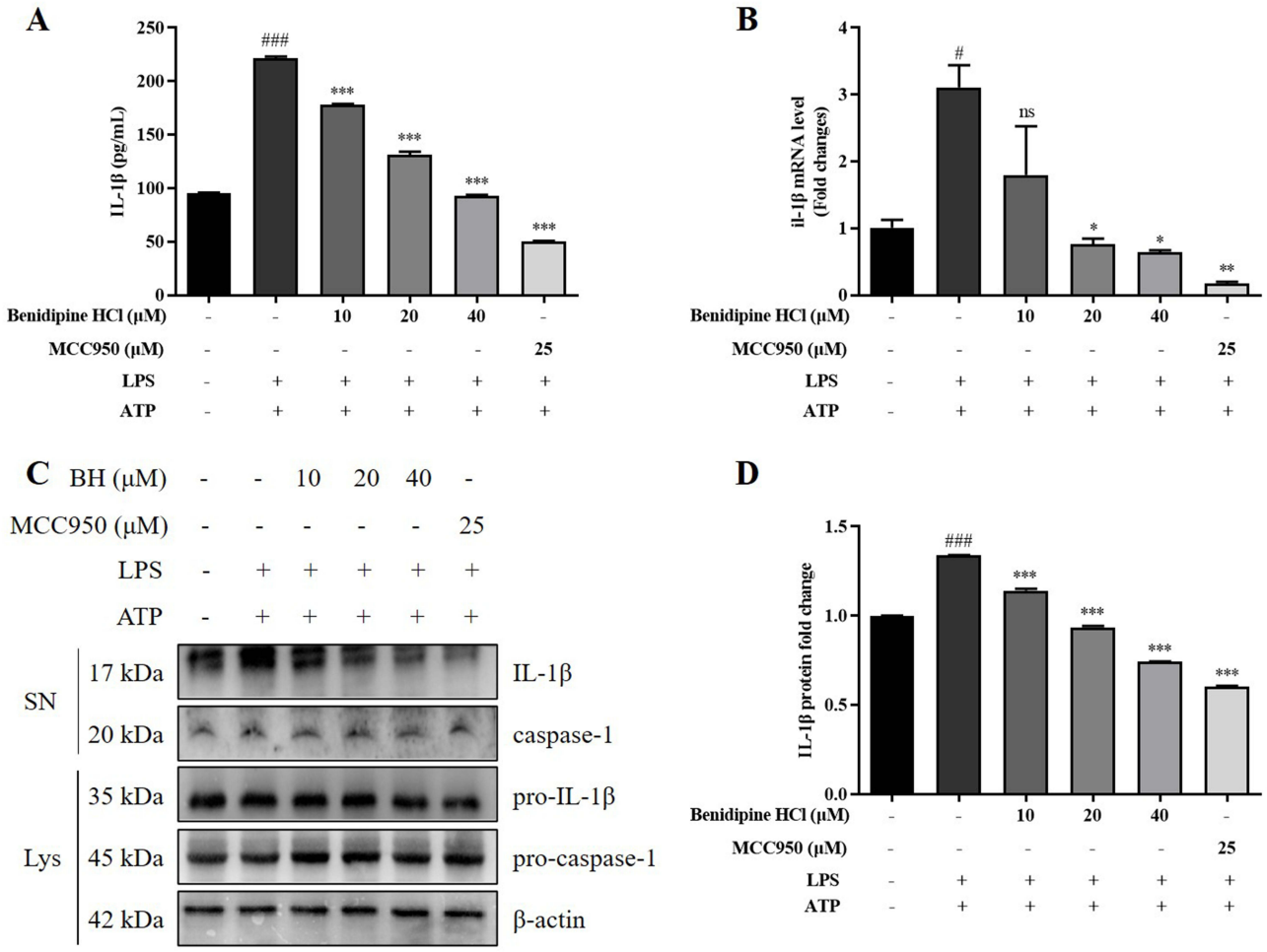

Figure 2 Benidipine hydrochloride decreases IL-1β production in LPS and ATP-stimulated THP-1 macrophages. (**A**) The IL-1β levels of THP-1 macrophages were detected after pretreatment with benidipine hydrochloride or MCC950 for 1 h, incubation with LPS (1 μg/mL) for 4 h, and ATP (5 mM) incubation for 2 h. (**B**) Analysis of IL-1β mRNA levels by qRT-PCR. (**C**) Western blot of IL-1β and caspase-1 levels in cell lysates (Lys) and supernatants (SN). (**D**) Quantitative analysis results of (**C**). The results represent the mean ± SD for three experiments. # p<0.05, ### p< 0.001 vs the control group. * p<0.05, ** p<0.01 and *** p< 0.001 vs the LPS+ATP group.

Benidipine Hydrochloride Reduced the Phosphorylation and Nuclear Translocation of NF-κB p65

We looked into whether the suppression of benidipine hydrochloride on LPS-induced NLRP3 priming was linked to a weakening of the NF-κB pathway or a decrease in TLR4 expression. Using Western blotting, the expression of phosphorylated NF-κB p65 was ascertained. Phosphorylated NF-κB p65 was expressed at a considerably higher level in the LPS group compared to the control group, while phosphorylated NF-κB p65 was expressed at a lower level in the LPS+benidipine hydrochloride group than in the LPS group (Figure 4A and B). Using immunofluorescence microscopy, the nuclear translocation of NF-κB p65 was identified (Figure 4C), and Figure 4D displays the quantitative findings of this translocation. The immunofluorescence signal was higher in the LPS group and NF-κB p65 was mostly found in the nuclear region than in the control group. In the control group, immunostained NF-κB p65 was rarely seen in the nuclear region. The degree of nuclear translocation of NF-κB p65 in the LPS+benidipine hydrochloride group was much lower than that of the LPS group, which is consistent with the Western blot data.

Furthermore, using real-time PCR to measure TLR4 transcriptional expression, we discovered that the LPS group had considerably higher TLR4 mRNA levels than the control and LPS+benidipine hydrochloride groups did (Figure 4E).

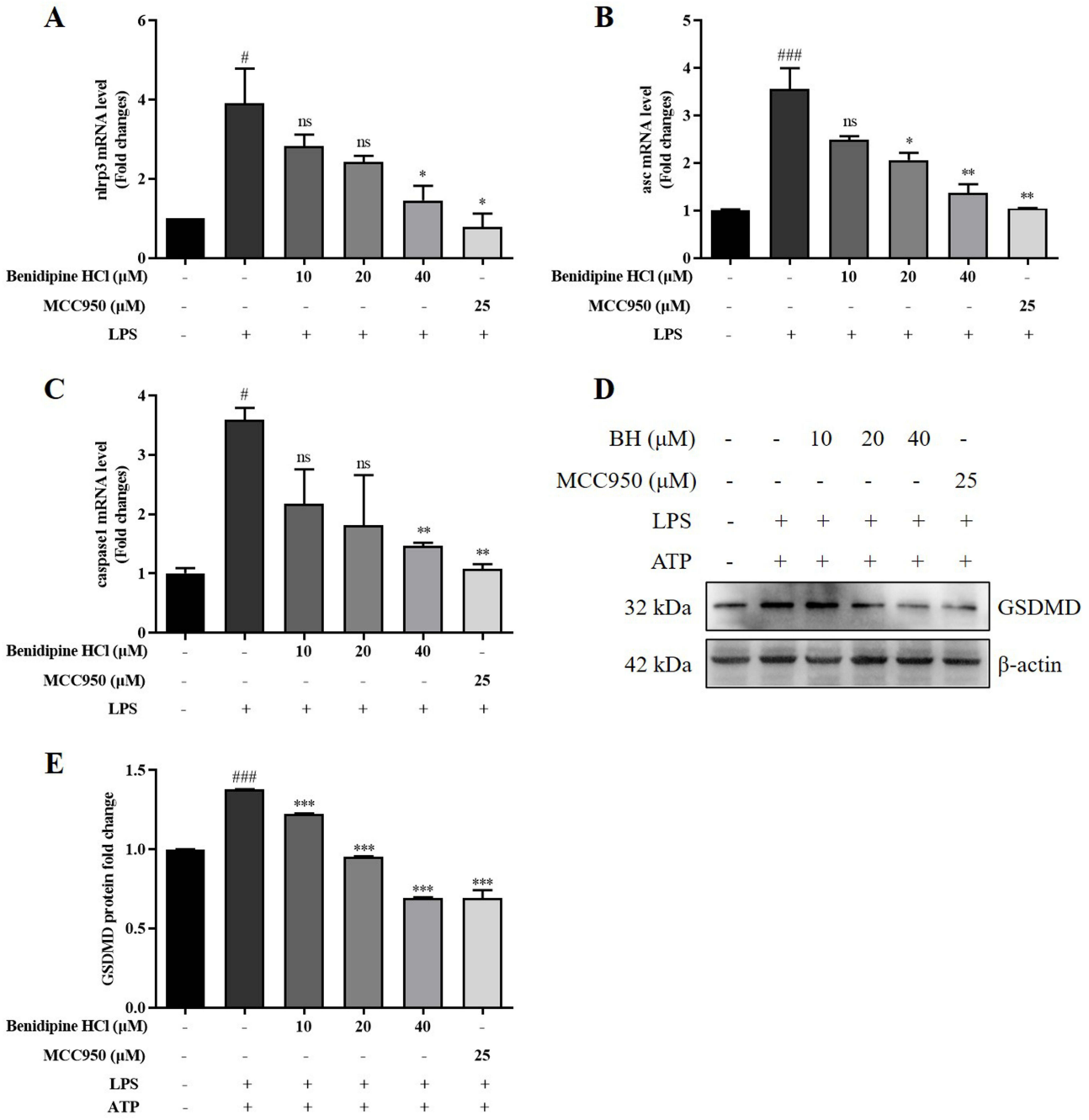

Figure 3 Benidipine hydrochloride inhibited LPS+ATP-stimulated NLRP3 inflammasome activation in THP-1 macrophages. (**A**) THP-1 macrophages were pretreated with benidipine hydrochloride or MCC950 for 1 h, then incubated with LPS (1 μg/mL) for 4 h, followed by ATP (5 mM) for 2 h, and qRT-PCR was used to analyze the mRNA expression levels of (**A**) nlrp3, (**B**) asc, (**C**) caspase 1. (**D**) The expression level of GSDMD was detected by Western blotting. (**E**) Quantitative analysis results of (**D**). The results represent the mean ± SD for three experiments. # p<0.05, ### p< 0.001 vs the control group. * p<0.05, ** p<0.01 and *** p< 0.001 vs the LPS+ATP group.

Benidipine Hydrochloride Inhibited the Production of ROS

Given that ROS are necessary for NLRP3 inflammasome activation,[11] we further determined the effect of benidipine hydrochloride treatment on ROS production. The results showed that benidipine hydrochloride also inhibited ROS production (Figure 5), suggesting that benidipine hydrochloride inhibited the activation of NLRP3 inflammasome by inhibiting ROS production.

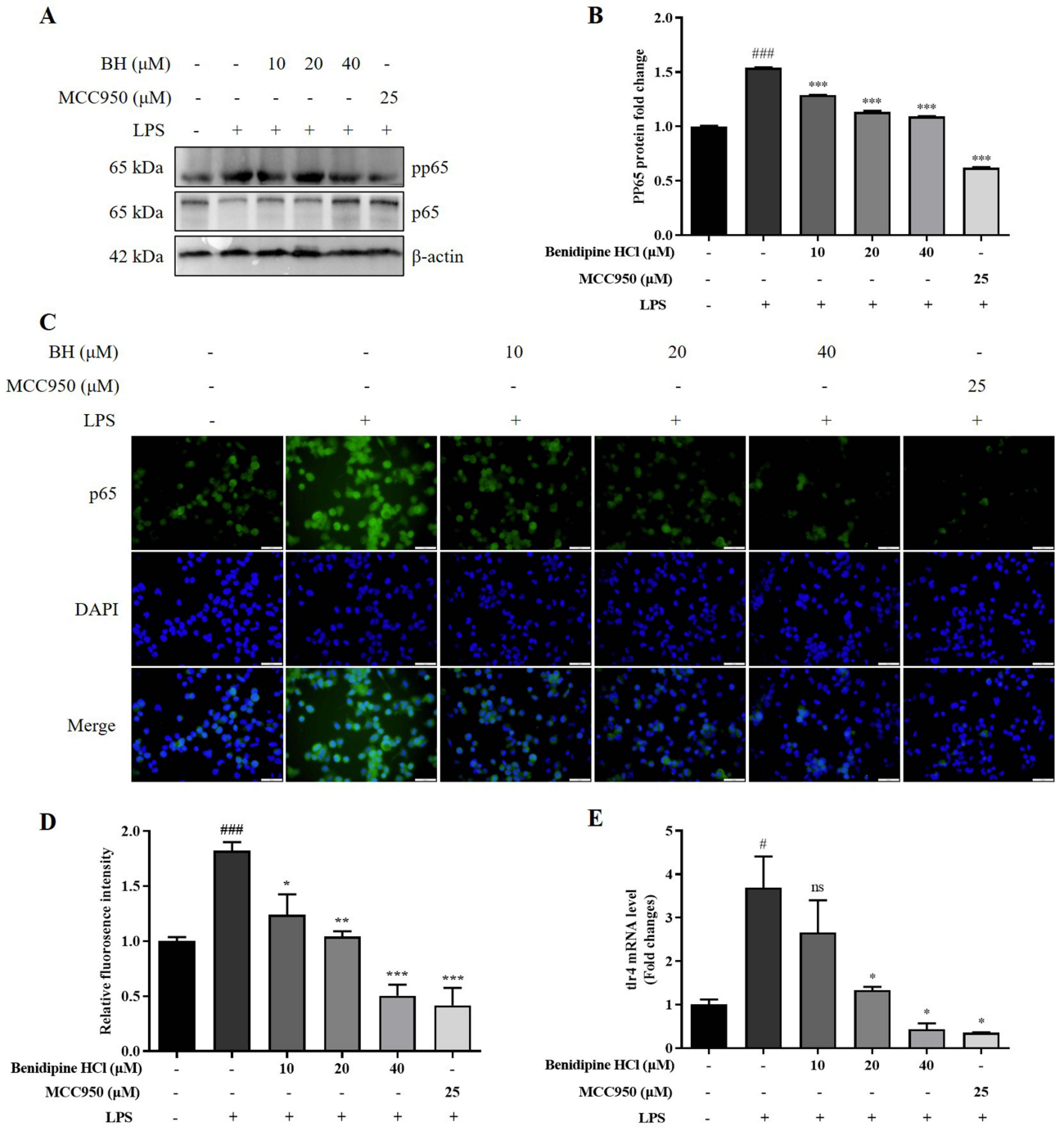

Figure 4 Benidipine hydrochloride inhibited LPS-stimulated NF-κB activation in THP-1 macrophages. (**A**) THP-1 macrophages were pretreated with benidipine hydrochloride for 1 h and incubated with LPS (1 μg/mL) for 4 h, and the pp65 protein level was analyzed by Western blotting. (**B**) Quantitative analysis results of (**A**). (**C**) The nuclear localization of p65 in THP-1 macrophages was observed by immunofluorescence with anti-p65 antibody, and with DAPI as a nuclear marker. Scale bar: 50 μm. (**D**) Quantitative analysis results of (**C**). (**E**) The mRNA expression levels of tlr4. The results represent the mean ± SD for three experiments. # p<0.05, ### p< 0.001 vs the control group. * p<0.05, ** p<0.01 and *** p< 0.001 vs the LPS group.

Discussion

Previous studies have found that in addition to exerting antihypertensive effects, benidipine also prevents cardiac hypertrophy, fibrosis, and inflammation.[12] Hassan et al demonstrated that benidipine could reduce levels of oxidative stress and myocardial apoptosis in isoprenaline-induced infarcted rats.[13] Servi et al found that benidipine hydrochloride

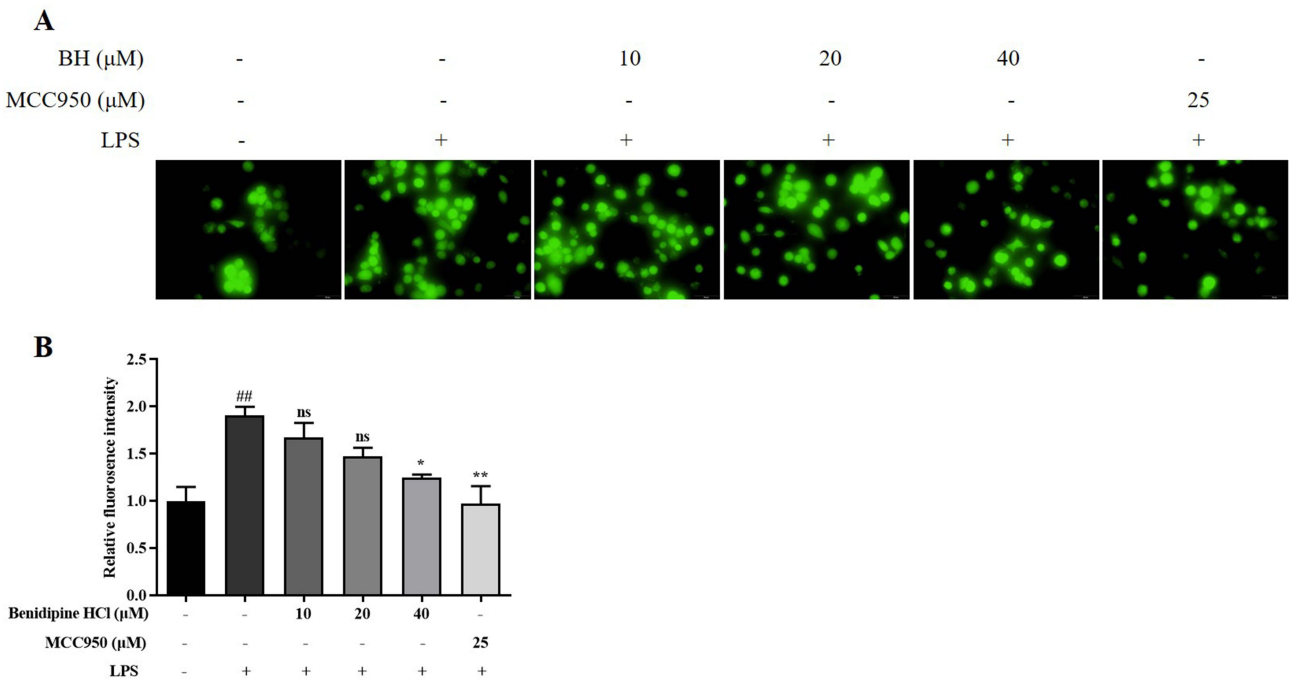

Figure 5 Benidipine hydrochloride reduced the production of ROS required for NLRP3 activation in THP-1 macrophages. (**A**) THP-1 macrophages pretreated with benidipine hydrochloride for 1 h and incubated with LPS (1 μg/mL) for 4 h, and ROS levels were recorded by fluorescence microscopy. (**B**) Quantitative analysis results of (**A**). The results represent the mean ± SD for three experiments. $^{\#\#}p<0.01$ vs the control group. *p<0.05, **p<0.01 vs the LPS group.

could reduce the production of pro-inflammatory cytokines IL-6, IL-12, TNF-α, and GM-CSF in J774.2 mouse macrophages induced by LPS.[14] Although the anti-inflammatory properties of benidipine hydrochloride have been well studied, whether benidipine hydrochloride can inhibit the NLRP3 inflammasome remains unknown. In this study, we investigated the effect of benidipine hydrochloride on the NLRP3 inflammasome and its mechanism of action. Our results showed that benidipine hydrochloride inhibited the activation of NLRP3, decreased the expression of NLRP3, ASC, and caspase-1, and the release of IL-1β.

According to previous reports, during the priming phase, LPS-induced transcriptional upregulation of NLRP3 is facilitated by TLR4 signaling-induced NF-κB activation.[15,16] Moreover, elevated TLR4 expression levels are linked to the upregulation of the NF-κB pathway.[17,18] Numerous inducible transcription factors that regulate the immunological and inflammatory responses to different cellular stressors make up the NF-κB family. Inflammatory cytokine expression is greatly increased with nuclear translocation and activation of NF-κB. NF-κB activation inhibitors can prevent the activation of NLRP3,[19,20] suggesting that NF-κB is involved in the regulation of NLRP3 expression and activation. Meanwhile, benidipine hydrochloride has been reported to have the ability to inhibit the NF-κB pathway.[21] In this study, we demonstrated that benidipine hydrochloride attenuates LPS-induced TLR4 expression, and reduces NF-κB p65 phosphorylation, and nuclear translocation of p65 to inhibit NLRP3 transcriptional initiation (Figure 6). Still, more studies are needed to confirm the mechanism by which benidipine hydrochloride inhibits NLRP3 inflammasome activation.

Taken together, our data suggested that benidipine hydrochloride inhibited NLRP3 inflammatory-mediated release of the pro-inflammatory cytokine IL-1β. Mechanistic studies showed that benidipine hydrochloride blocked NLRP3 inflammasome activation by inhibiting the phosphorylation of p65 and its entry into the nucleus, scavenging intracellular ROS, and inhibiting the expression of NLRP3, ASC, caspase-1, and IL-1β.

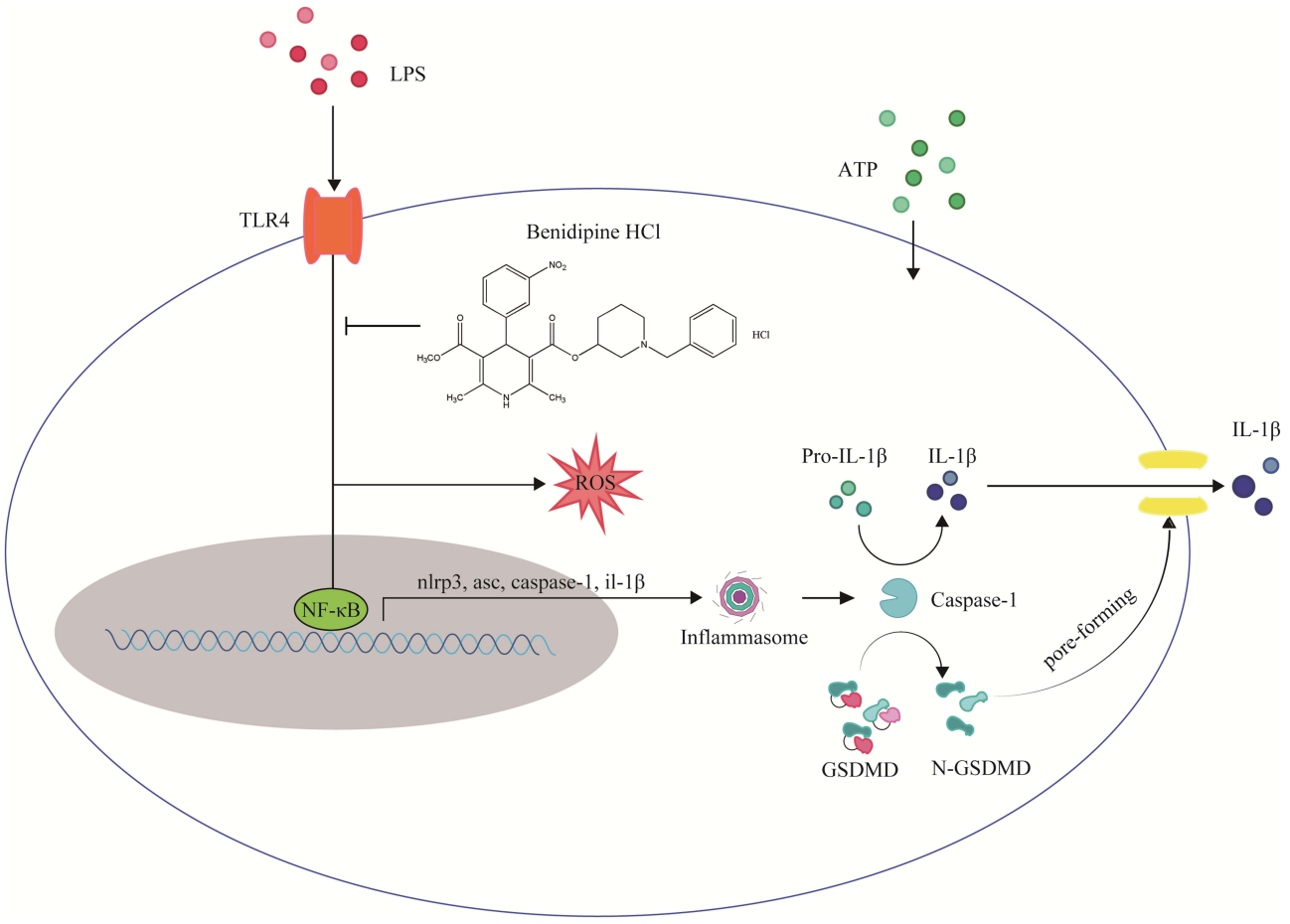

Figure 6 Schematic diagram of the effect of benidipine hydrochloride on NLRP3 inflammasome activation in LPS-stimulated THP-1 macrophages. Benidipine hydrochloride reduced LPS-induced TLR4 expression, which in turn inhibited NF-κB activation, thereby preventing NLRP3 transcription initiation and activation.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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