International Immunopharmacology 68 (2019) 137–144

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Notoginsenoside R1 up-regulates microRNA-132 to protect human lung fibroblast MRC-5 cells from lipopolysaccharide-caused injury

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ARTICLE INFO

Keywords:
Lung fibroblast cells
Notoginsenoside R1
Lipopolysaccharide
MicroRNA-132
NF-κB pathway
JNK pathway

ABSTRACT

Background: Pneumonia is a common lung disease in children with high fatality rate. Notoginsenoside R1 (NGR1) is the main active component extracted from the roots of *Panax notoginseng* (Burk.) F.H. Chen (Araliaceae). Here, we carefully explored the potential anti-inflammatory and protective effects of NGR1 on lipopolysaccharide (LPS)-induced lung fibroblast MRC-5 cell injury.

Methods: Viability and apoptosis of MRC-5 cells after different treatment or transfection were respectively assessed using CCK-8 assay and Annexin V-FITC/PI staining. The expression levels of microRNA-132 (miR-132), IL-1β, IL-6 and TNF-α in MRC-5 cells were measured using qRT-PCR. MicroRNA transfection was conducted to reduce the expression level of miR-132. Western blotting was used to analyze the protein expression levels of key factors involving in cell proliferation, apoptosis, NF-κB pathway and JNK pathway.

Results: LPS treatment caused MRC-5 cell proliferation inhibition, apoptosis and over-production of inflammatory cytokines. NGR1 treatment had no significant effects on MRC-5 cell proliferation, apoptosis and production of inflammatory cytokines, but protected MRC-5 cells from LPS-caused cell proliferation inhibition, apoptosis and over-production of inflammatory cytokines. In addition, NGR1 increased the expression level of miR-132 in MRC-5 cells. Knockdown of miR-132 reversed the protective effects of NGR1 on LPS-treated MRC-5 cells. Furthermore, NGR1 attenuated LPS-activated NF-κB and JNK pathways in MRC-5 cells via up-regulation of miR-132.

Conclusion: This research confirmed the protective roles of NGR1 in lung fibroblast cell inflammatory injury. NGR1 protected MRC-5 cells from LPS-caused inflammatory injury through up-regulating miR-132 and then inactivating NF-κB and JNK pathways.

1. Introduction

Pneumonia is a common lung disease in children and the main reason for the dying of infants and children all over the world [1,2]. Its pathogens are complex and different in diverse countries, regions and ages [2,3]. The main clinical features of pneumonia are fever, cough dyspnea, somnolence and loss of appetite [4]. For pneumonia treatment, the most common medicine is antibiotic treatment, such as cephalosporins, penicillins, erythromycin and tetracycline [5,6]. However, due to the immune system of infants and children is very fragile, the use of antibiotic in the treatment of pneumonia has a very negative effect on patient's health [6,7]. Considering that anti-inflammatory response plays key roles in the treatment of pneumonia, it is worthy believing that searching for other anti-inflammatory medicine for alleviating lung cell inflammatory injury may provide more effective and safe novel medicine for pneumonia treatment.

A number of compounds isolated from plants have been reported to exert anti-inflammatory activity [8,9]. Notoginsenoside R1 (NGR1, CAS number: 80418-24-2) is the main active component extracted from the roots of *Panax notoginseng* (Burk.) F.H. Chen (Araliaceae) with outstanding anti-inflammatory activity in vitro and in vivo [10–12]. Su et al. reported that NGR1 suppressed inflammatory cytokines production in oxidized low-density lipoprotein (oxLDL)-treated human endothelial EA.hy926 cells [11]. Xiao et al. proved that NGR1 protected $ApoE^{-/-}$ mice from cardiac hypertrophy by inhibiting pro-inflammatory monocytes [12]. However, whether NGR1 has a good anti-inflammatory role in lung cells remains unclear yet. More experimental researches are still needed.

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https://doi.org/10.1016/j.intimp.2018.12.065
Received 10 October 2018; Received in revised form 14 December 2018; Accepted 30 December 2018
Available online 08 January 2019

MicroRNAs are small RNA transcripts in eukaryotic cells without protein-coding potential [13]. The discovery of microRNAs provides new objects for the research of molecular biology [14]. Numerous studies have demonstrated that microRNAs participate in the regulation of many cellular biological processes, including cellular inflammatory response [15,16]. Many plant-derived medicines, including NGR1, can exert anti-inflammatory effects via regulating microRNAs expression [17,18]. MicroRNA-132 (miR-132) has been found to be involved in the regulation of cellular inflammatory response and exert anti-inflammatory activity [19,20].

In this research, human lung fibroblast MRC-5 cells were treated by lipopolysaccharide (LPS) to establish lung fibroblast cell injury model. Then, the possible protective effect of NGR1 on LPS-induced MRC-5 cell inflammatory injury was investigated. Moreover, the potential internal molecular mechanism related to anti-inflammatory activity of miR-132, as well as signaling pathways were also analyzed. This study will provide evidence for understanding the anti-inflammatory effect of NGR1 on lung fibroblast cell inflammatory injury.

2. Materials and methods

2.1. Cell culture and treatment

Human lung fibroblast MRC-5 cells were kindly provided by Stem Cell Bank, Chinese Academy of Science (Shanghai, China) and cultured in Minimum Essential Medium (MEM, Gibco, Life Technologies, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (FBS, Gibco, Life Technologies), 1% (v/v) Gluta-max (Gibco, Life Technologies), 1% (v/v) Non-Essential Amino Acids (NEAA) solution (Gibco, Life Technologies) and 1% (v/v) Sodium Pyruvate solution (100 mM, Gibco, Life Technologies) in 75 cm^2 cell culture flask. Flasks were maintained at 37 °C in humidity incubator (Sanyo, Jencons, United Kingdom) with 5% CO_2.

NGR1 and LPS powders were both purchased from Sigma-Aldrich (catalog number: N3915 and L3012, St Louis, MO, USA). NGR1 was dissolved into ultrapure water to a storage concentration of 10 mM in line with the manufacturer's instruction. The chemical structure of NGR1 was shown in Fig. 1. LPS was dissolved into ultrapure water to a storage concentration of 5 mg/ml according to the manufacturer's protocol. MRC-5 cells were exposed to 10 μg/ml LPS for 6 h to stimulate inflammatory injury in this research. NGR1 (10, 20, 30, 40 or 50 μM) was added into culture medium for 24 h before LPS stimulation.

2.2. MicroRNA transfection

MicroRNA transfection was conducted to reduce the expression level of miR-132 in MRC-5 cells. miR-132 inhibitor and its negative control (NC) were both synthesized by GenePharma Corporation (Shanghai, China). The sequence of miR-132 inhibitor was 5′-CGACC AUGGCUGUAGACUGUUA-3′. Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was used to microRNA transfection. Quantitative reverse transcription PCR (qRT-PCR) was performed to verify transfection efficiency.

2.3. qRT-PCR

The expression levels of miR-132, interleukin 1β (IL-1β), IL-6 and tumor necrosis factor α (TNF-α) in MRC-5 cells were measured using qRT-PCR. Briefly, after relevant treatment and/or transfection, total RNAs in MRC-5 cells were isolated using RiboPure™ RNA Purification kit (Invitrogen). Then, the expression level of miR-132 was measured using *mir*Vana™ qRT-PCR miRNA Detection kit (Invitrogen). The expression levels of IL-1β, IL-6 and TNF-α were measured using SuperScript™ III Platinum™ One-Step qRT-PCR kit (Invitrogen). The expression levels of U6 snRNA and β-actin respectively acted as endogenous controls. Data were analyzed using classic $2^{-\triangle\triangle Ct}$ method [21].

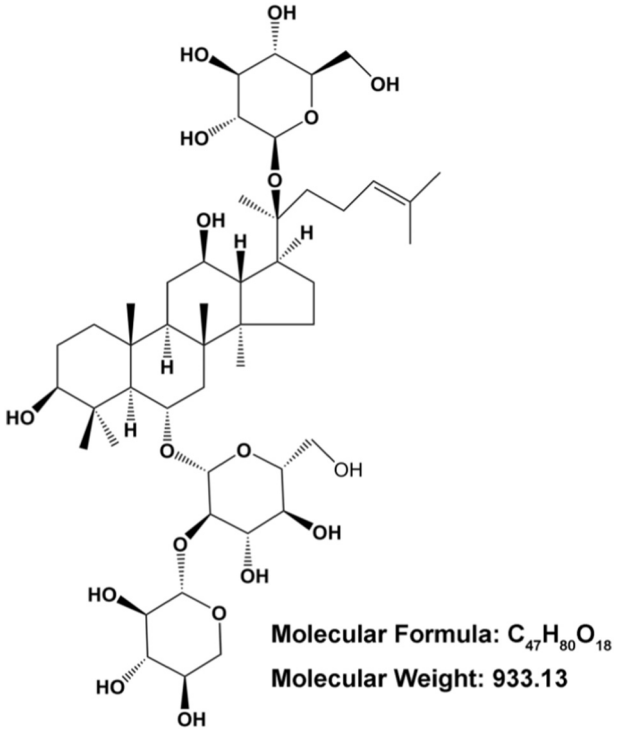

Fig. 1. The chemical skeleton structure of NGR1. NGR1: Notoginsenoside R1. CAS number: 80418-24-2.

2.4. Cell viability assay

Viability of MRC-5 cells was detected using cell counting kit-8 (CCK-8) assay (Beyotime Biotechnology, Shanghai, China) after LPS and/or NGR1 treatment or miR-132 inhibitor transfection. Briefly, MRC-5 cells were seeded into 96-well plate (Corning Incorporated, New York, NY, USA) with 5×10^3 cells per well and exposed to different treatment or transfection. Then, 10 μl CCK-8 kit solution was added into the culture medium of each well. The plate was incubated at 37 °C in humidity incubator for 1 h. Subsequently, the absorbance of each well at 450 nm was recorded using Micro-plate Reader (Bio-Tek Instruments, Winooski, VT, USA). Cell viability (%) was calculated by average absorbance of treatment (transfection) group / average absorbance of control group × 100%.

2.5. Cell apoptosis assay

Apoptosis of MRC-5 cells was measured using Annexin V-FITC/PI apoptosis detection kit (BD Bioscience, Franklin Lakes, NJ, USA) after LPS and/or NGR1 treatment or miR-132 inhibitor transfection. Briefly, MRC-5 cells were seeded into 6-well plate (Corning Incorporated) with 5×10^4 cells per well and exposed to different treatment or transfection. Then, cells in each group was collected, washed with phosphate buffered saline (PBS) for twice and stained using 5 μl Annexin V-FITC and 5 μl PI for 25 min at 37 °C in the dark. Subsequently, the rate of apoptotic cells in each group was recorded using flow cytometer (Beckman Coulter, Fullerton, CA, USA). Data were analyzed using FCS Express software (De Novo software, Los Angeles, CA, USA).

2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to determine the concentrations of IL-1β, IL-6 and TNF-α in culture supernatant of MRC-5 cells. Briefly, MRC-5 cells were seeded into 6-well plate (Corning Incorporated) with 5×10^4 cells

per well and exposed to different treatment or transfection. Then, culture supernatant of each group was respectively collected. The concentrations of IL-1β, IL-6 and TNF-α in culture supernatant were analyzed using Human IL-1β ELISA kit (Abcam Biotechnology, Cambridge, MA, USA, catalog number: ab100562), Human IL-6 ELISA kit (Abcam Biotechnology, catalog number: ab46027) and Human TNF-α ELISA kit (Abcam Biotechnology, catalog number: ab181421), respectively.

2.7. Western blotting

After LPS and/or NGR1 treatment or miR-132 inhibitor transfection, total proteins in MRC-5 cells were isolated using M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitors (Roche, Basel, Switzerland). The concentration of total proteins was calculated by BCA Protein Assay kit (Beyotime Biotechnology). Then, proteins in equal concentration were electrophoresed on polyacrylamide gels using Bio-Rad Bis-Tris Gel system (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). All primary antibodies were purchased from Abcam Biotechnology and prepared in 1% bovine serum albumin (BSA) solution (Beyotime Biotechnology) with a dilution of 1:1000. The information of catalog was as follows: Cyclin D1 (ab226977), Anti-procaspase 3 (ab32150), Cleaved-caspase 3 (ab2302), Pro-caspase 9 (ab135544), Cleaved-caspase 9 (ab2324), t-p65 (ab16502), p-p65 (ab86299), t-IκBα (ab7217), p-IκBα (ab24783) t-JNK (ab179461), p-JNK (ab201864) and β-actin (ab8226). After blocking with 5% BSA solution for 1 h at room temperature, the PVDF membranes were incubated with primary antibodies for 4 °C overnight. Subsequently, the PVDF membranes were washed with Tris-Buffer-Solution-Tween (TBST, Beyotime Biotechnology) for twice and incubated with Goat anti-Rabbit (or anti-Mouse) IgG H&L (HRP) (ab205718, ab205719, Abcam Biotechnology) for 1 h at room temperature. Followed by adding 200 μl Immubilon Western Chemiluminescent HRP Substrate (Millipore) to the surface of PVDF membranes, the signals of proteins were captured using Bio-Rad ChemiDoc™ XRS system (Bio-Rad Laboratories). The intensities of bands were quantified using Image Lab™ software (Bio-Rad Laboratories).

2.8. Statistical analysis

All experiments in this research were repeated three times in triplicate. The results of multiple experiments were presented as mean ± standard deviation (SD). Statistical analyses were conducted using Graphpad 6.0 statistical software (Graphpad, San Diego, CA, USA). The *P*-values between two groups were calculated using Student's *t*-test. The *P*-values between more than three groups were calculated using one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be significant different.

3. Results

3.1. The effects of NGR1 on MRC-5 cell viability

Firstly, we detected the viability of MRC-5 cells after 10, 20, 30, 40 or 50 μM NGR1 treatment by using CCK-8 assay. The results in Fig. 2 showed that 10, 20, 30 or 40 μM NGR1 treatment had no significant effects on MRC-5 cell viability, while 50 μM NGR1 treatment reduced the viability of MRC-5 cells ($P < 0.05$). These findings suggested that NGR1 in low concentration had no effects on MRC-5 cell viability, but inhibited the viability of MRC-5 cells in high concentration.

The viability of MRC-5 cells after 10, 20, 30, 40 or 50 μM NGR1 treatment was detected using CCK-8 assay. NGR1: Notoginsenoside R1. N = 3. Results were expressed as mean ± standard deviation (SD). $^{*}P < 0.05$ vs. 0 μM NGR1 treatment group.

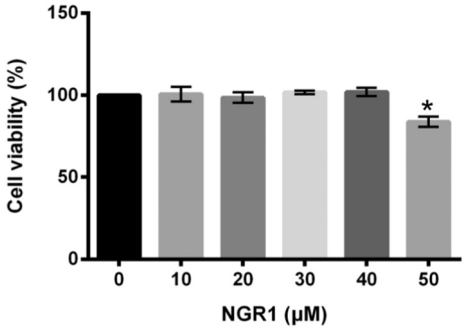

Fig. 2. The effects of NGR1 on MRC-5 cell viability.

3.2. LPS stimulated MRC-5 cell inflammatory damage

Then, we measured the viability, proliferation, apoptosis and inflammatory cytokines expression of MRC-5 cells after 10 μg/ml LPS treatment for 6 h in this study. Fig. 3A presented that 10 μg/ml LPS treatment remarkably inhibited the viability of MRC-5 cells ($P < 0.01$). Fig. 3B showed that 10 μg/ml LPS treatment significantly reduced the protein expression level of Cyclin D1 in MRC-5 cells ($P < 0.01$). The results of Fig. 3C displayed that 10 μg/ml LPS treatment notably induced MRC-5 cell apoptosis ($P < 0.01$). The protein expression levels of Cleaved-caspase 3 and Cleaved-caspase 9 in MRC-5 cells were both increased after 10 μg/ml LPS treatment (Fig. 3D). Moreover, Fig. 3E showed that 10 μg/ml LPS treatment dramatically up-regulated the mRNA expression levels of IL-1β, IL-6 and TNF-α in MRC-5 cells ($P < 0.01$ or $P < 0.001$). The concentrations of IL-1β, IL-6 and TNF-α in culture supernatant of MRC-5 cells were also dramatically increased after 10 μg/ml LPS treatment (Fig. 3F, $P < 0.01$ or $P < 0.001$). Taken together, these above results suggested that LPS could cause MRC-5 cell inflammatory damage.

After 10 μg/ml LPS treatment for 6 h, the viability of MRC-5 cells (A), the protein level of Cyclin D1 in MRC-5 cells (B), the apoptosis of MRC-5 cells (C), the protein levels of Pro-caspase 3, Cleaved-caspase 3, Pro-caspase 9 and Cleaved-caspase 9 in MRC-5 cells (D), the mRNA levels of IL-1β, IL-6 and TNF-α in MRC-5 cells (E) and the concentrations of IL-1β, IL-6 and TNF-α in culture supernatant of MRC-5 cells (F) were assessed using CCK-8 assay, western blotting, Annexin V-FITC/PI staining, qRT-PCR and ELISA, respectively. LPS: Lipopolysaccharide; IL-1β: Interleukin 1β; TNF-α: Tumor necrosis factor α. N = 3. Results were expressed as mean ± standard deviation (SD). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group.

3.3. NGR1 protected MRC-5 cells from LPS-caused inflammatory damage

To explore the potential protective effects of NGR1 on LPS-caused MRC-5 cell inflammatory damage, we assessed the viability, proliferation, apoptosis, and inflammatory cytokines expression of MRC-5 cells after LPS and/or NGR1 treatment. Fig. 4A showed that 30 or 40 μM NGR1 treatment remarkably alleviated the 10 μg/ml LPS-induced MRC-5 cell viability loss ($P < 0.05$). Fig. 4B displayed that 30 μM NGR1 treatment had no significant effect on Cyclin D1 expression in MRC-5 cells, while notably attenuated the 10 μg/ml LPS-induced down-regulation of Cyclin D1 in MRC-5 cells ($P < 0.01$). Similar result was found in Fig. 4C, which presented that the apoptosis of MRC-5 cells was not changed after 30 μM NGR1 treatment, while 30 μM NGR1 treatment noticeably alleviated the 10 μg/ml LPS-induced MRC-5 cell apoptosis ($P < 0.05$). Compared to Control group, the protein expression levels of Cleaved-caspase 3 and Cleaved-caspase 9 in MRC-5 cells were not changed after 30 μM NGR1 treatment group. Relative to LPS group, the protein expression levels of Cleaved-caspase 3 and Cleaved-caspase 9 were both decreased in LPS + NGR1 group (Fig. 4D). In addition, Fig. 4E and F showed that 30 μM NGR1 treatment had no significant

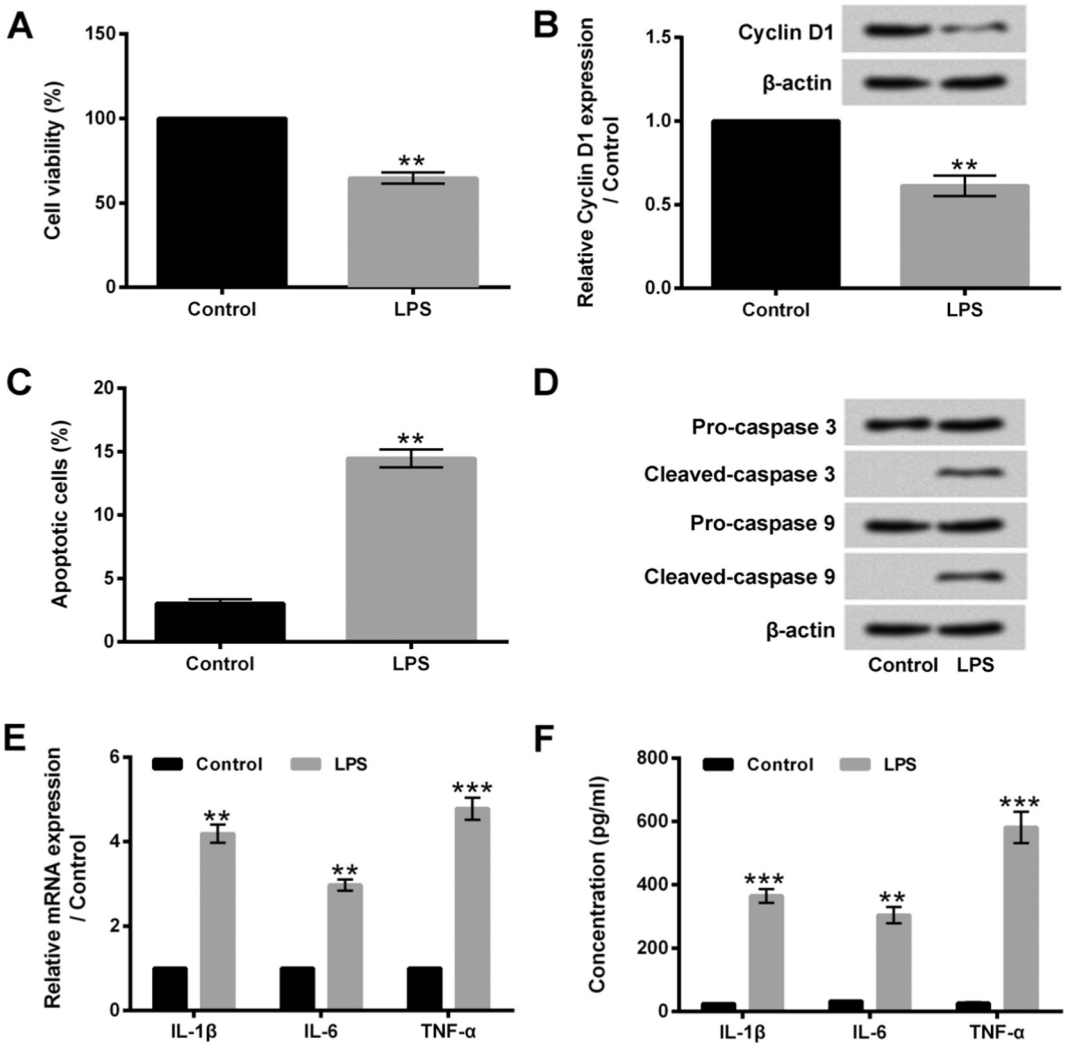

Fig. 3. LPS stimulated MRC-5 cell inflammatory damage.

effects on the mRNA expression levels of IL-1β, IL-6 and TNF-α in MRC-5 cells, as well as the concentrations of IL-1β, IL-6 and TNF-α in culture supernatant of MRC-5 cells. Compared to LPS group, the mRNA expression levels of IL-1β, IL-6 and TNF-α in MRC-5 cells, as well as the concentrations of IL-1β, IL-6 and TNF-α in culture supernatant of MRC-5 cells were all reduced inLPS + NGR1 treatment ($P < 0.05$ or $P < 0.01$). Taken together, these above findings indicated that NGR1 had no significant effects on MRC-5 cell proliferation, apoptosis and inflammatory cytokines production, but could protect MRC-5 cells from LPS-caused proliferation inhibition, apoptosis and inflammatory response.

(A) After 10 μg/ml LPS and/or 20, 30 or 40 μM NGR1 treatment, the viability of MRC-5 cells was measured using CCK-8 assay. After 10 μg/ml LPS and/or 30 μM NGR1 treatment, the protein level of Cyclin D1 in MRC-5 cells (B), the apoptosis of MRC-5 cells (C), the protein levels of Pro-caspase 3, Cleaved-caspase 3, Pro-caspase 9 and Cleaved-caspase 9 in MRC-5 cells (D), the mRNA levels of IL-1β, IL-6 and TNF-α in MRC-5 cells (E) and the concentrations of IL-1β, IL-6 and TNF-α in culture supernatant of MRC-5 cells (F) were evaluated using western blotting, Annexin V-FITC/PI staining, qRT-PCR and ELISA, respectively. NGR1: Notoginsenoside R1; LPS: Lipopolysaccharide; IL-1β: Interleukin 1β; TNF-α: Tumor necrosis factor α. N = 3. Results were expressed as mean ± standard deviation (SD). ns: no significance. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. 10 μg/ml LPS treatment group.

3.4. NGR1 up-regulated the expression level of miR-132 in MRC-5 cells

The expression level of miR-132 in MRC-5 cells after NGR1 treatment was measured using qRT-PCR. As shown in Fig. 5, 20, 30 or 40 μM NGR1 treatment significantly enhanced the expression level of miR-132 in MRC-5 cells ($P < 0.05$ or $P < 0.01$), which implied that miR-132 might be related to the protective effects of NGR1 on LPS-caused MRC-5 cell inflammatory damage.

The expression level of miR-132 in MRC-5 cells after 20, 30 or 40 μM NGR1 treatment was detected using qRT-PCR. NGR1: Notoginsenoside R1; miR-132: MicroRNA-132. N = 3. Results were expressed as mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 μM NGR1 treatment group.

3.5. miR-132 was related to the protective effects of NGR1 on LPS-caused MRC-5 cell inflammatory damage

To investigate the roles of miR-132 in the protective effects of NGR1, miR-132 inhibitor was transfected into MRC-5 cells. Fig. 6A displayed that miR-132 inhibitor transfection notably reduced the expression level of miR-132 in MRC-5 cells ($P < 0.01$). Fig. 6B presented that miR-132 inhibitor transfection significantly reversed the protective effects of NGR1 on LPS-caused MRC-5 cell viability loss ($P < 0.05$). Fig. 6C showed that compared to LPS + NGR1 + NC group, the protein expression level of Cyclin D1 was remarkably decreased in

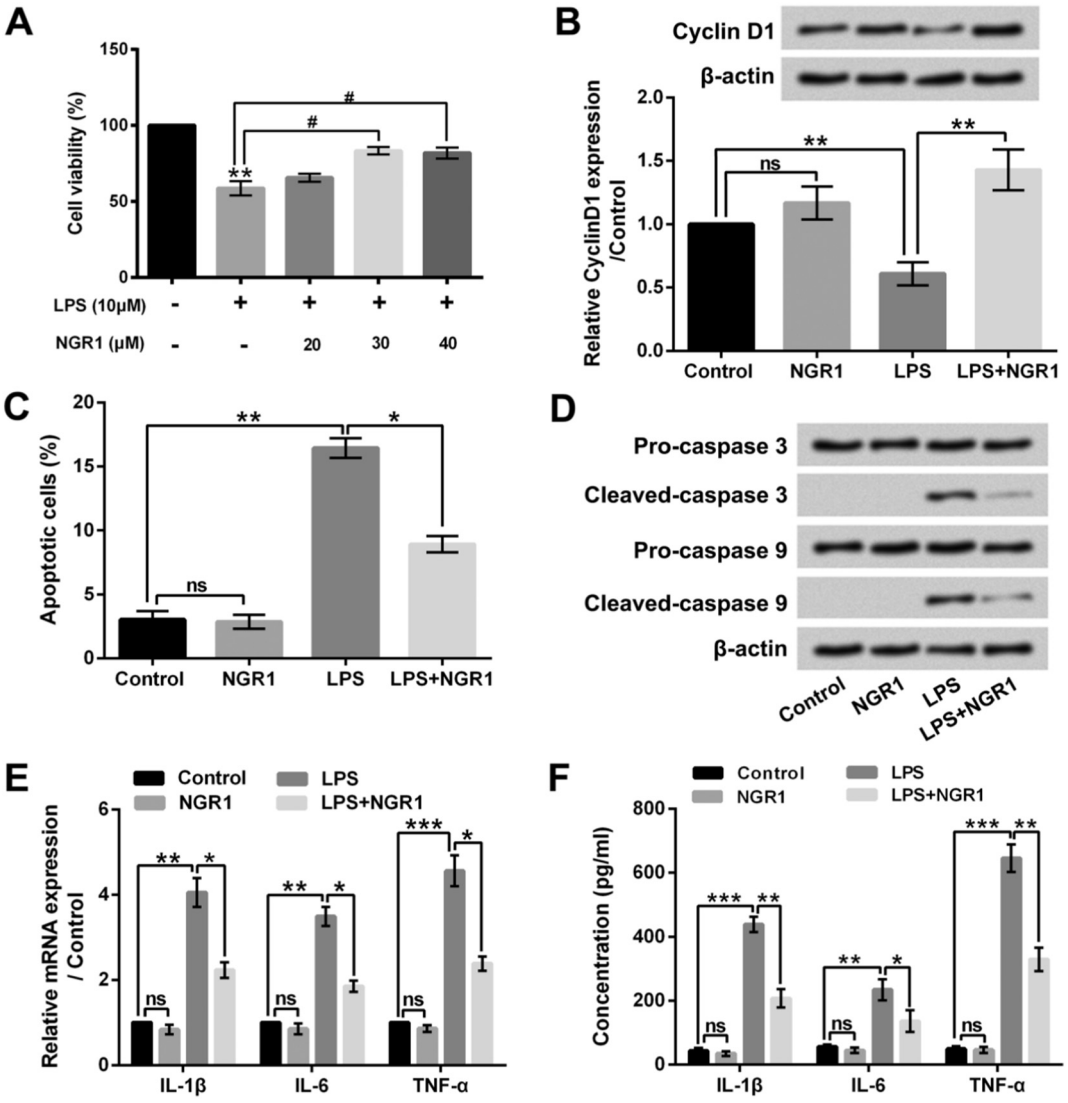

Fig. 4. NGR1 protected MRC-5 cells from LPS-caused inflammatory damage.

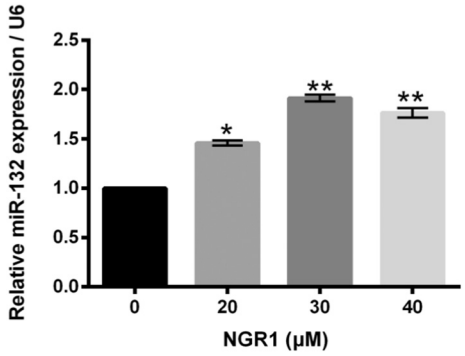

Fig. 5. NGR1 up-regulated the expression level of miR-132 in MRC-5 cells.

LPS + NGR1 + miR-132 inhibitor group ($P < 0.05$). The results of Fig. 6D presented that miR-132 inhibitor transfection significantly reversed the protective effects of NGR1 on LPS-caused MRC-5 cell apoptosis ($P < 0.05$). Compared to LPS + NGR1 + NC group, the protein expression levels of Cleaved-caspase 3 and Cleaved-caspase 9 were both increased in LPS + NGR1 + miR-132 inhibitor group (Fig. 6E). Moreover, Fig. 6F and G showed that miR-132 inhibitor transfection noticeably reversed the effects of NGR1 on LPS-caused over-production of IL-1β, IL-6 and TNF-α in MRC-5 cells and culture supernatant of MRC-5 cells ($P < 0.05$ or $P < 0.01$). Taken together, these above findings suggested that miR-132 was related to the protective effects of NGR1 on LPS-caused MRC-5 cell inflammatory damage.

(A) After miR-132 inhibitor transfection, the expression level of miR-132 in MRC-5 cells was measured using qRT-PCR. After 10 μg/ml LPS and/or 30 μM NGR1 treatment or miR-132 inhibitor transfection, the viability of MRC-5 cells (B), the protein level of Cyclin D1 in MRC-5 cells (C), the apoptosis of MRC-5 cells (D), the protein levels of Pro-caspase 3, Cleaved-caspase 3, Pro-caspase 9 and Cleaved-caspase 9 in MRC-5 cells (E), the mRNA levels of IL-1β, IL-6 and TNF-α in MRC-5 cells (F) and the concentrations of IL-1β, IL-6 and TNF-α in culture supernatant of MRC-5 cells (G) were assessed using CCK-8 assay, western blotting, Annexin V-FITC/PI staining, qRT-PCR and ELISA, respectively. LPS: Lipopolysaccharide; NGR1: Notoginsenoside R1; miR-132: MicroRNA-132; IL-1β: Interleukin 1β; TNF-α: Tumor necrosis factor α. N = 3. Results were expressed as mean ± standard deviation (SD). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. 10 μg/ml LPS treatment or 10 μg/ml LPS + 30 μM NGR1 treatment group.

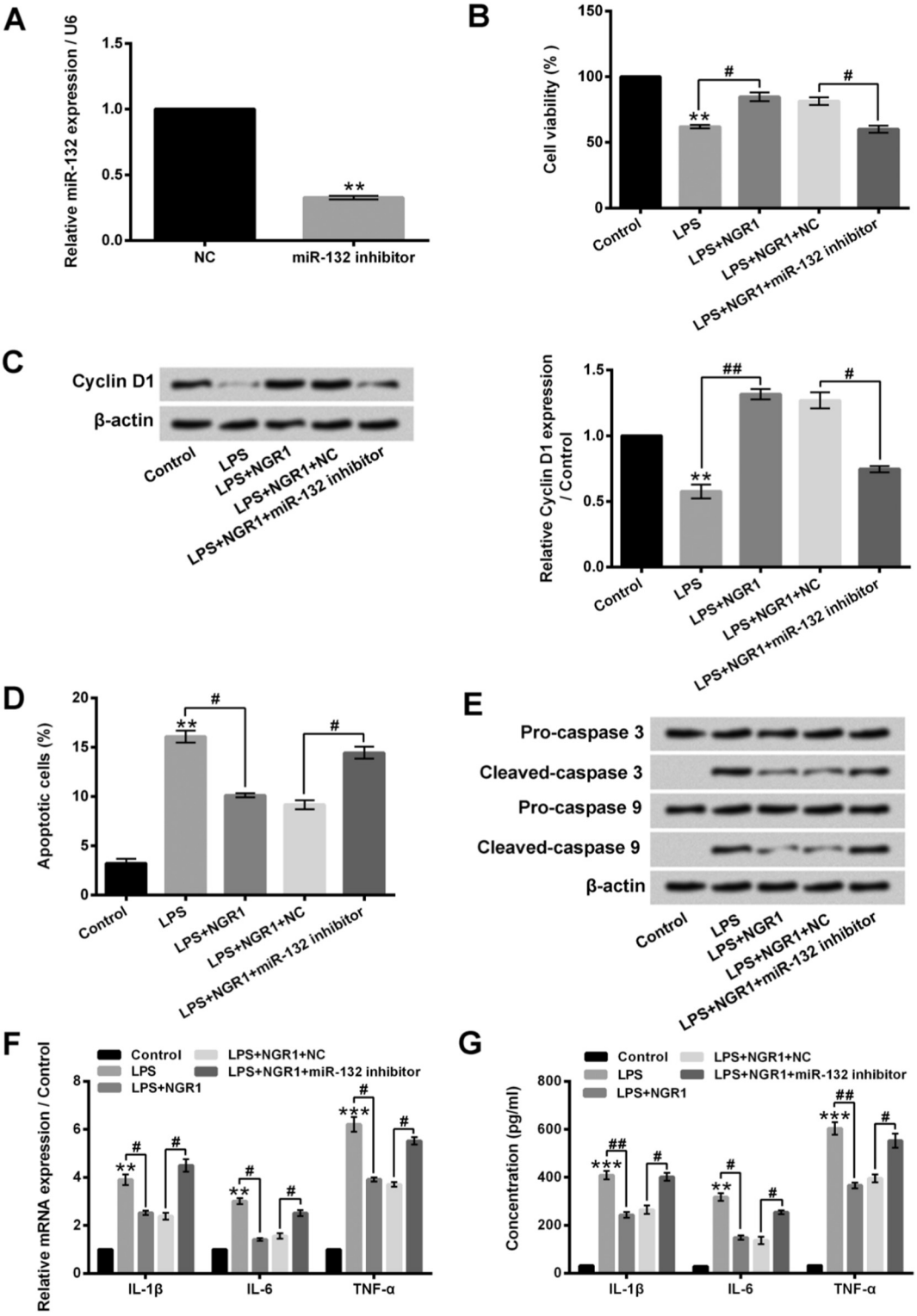

Fig. 6. miR-132 was related to the protective effects of NGR1 on LPS-caused MRC-5 cell inflammatory damage.

3.6. NGR1 alleviated LPS-activated NF-κB and JNK pathways in MRC-5 cells by up-regulating miR-132

Finally, we analyzed the activation of NF-κB and JNK pathways in MRC-5 cells after LPS and/or NGR1 treatment or miR-132 inhibitor transfection. Fig. 7A and B showed that LPS treatment significantly activated NF-κB and JNK pathways in MRC-5 cells via enhancing the expression rates of p/t-p65, p/t-IκBα and p/t-JNK ($P < 0.05$ or $P < 0.01$). NGR1 treatment remarkably alleviated the LPS-activated NF-κB and JNK pathways in MRC-5 cells via reducing the expression rates of p/t-p65, p/t-IκBα and p/t-JNK ($P < 0.05$). More importantly, miR-132 inhibitor transfection notably reversed the effects of NGR1 on LPS-activated NF-κB and JNK pathways in MRC-5 cells ($P < 0.05$). These results indicated that NGR1 alleviated LPS-activated NF-κB and JNK pathways in MRC-5 cells via up-regulating miR-132.

(A and B) After 10 μg/ml LPS and/or 30 μM NGR1 treatment or miR-

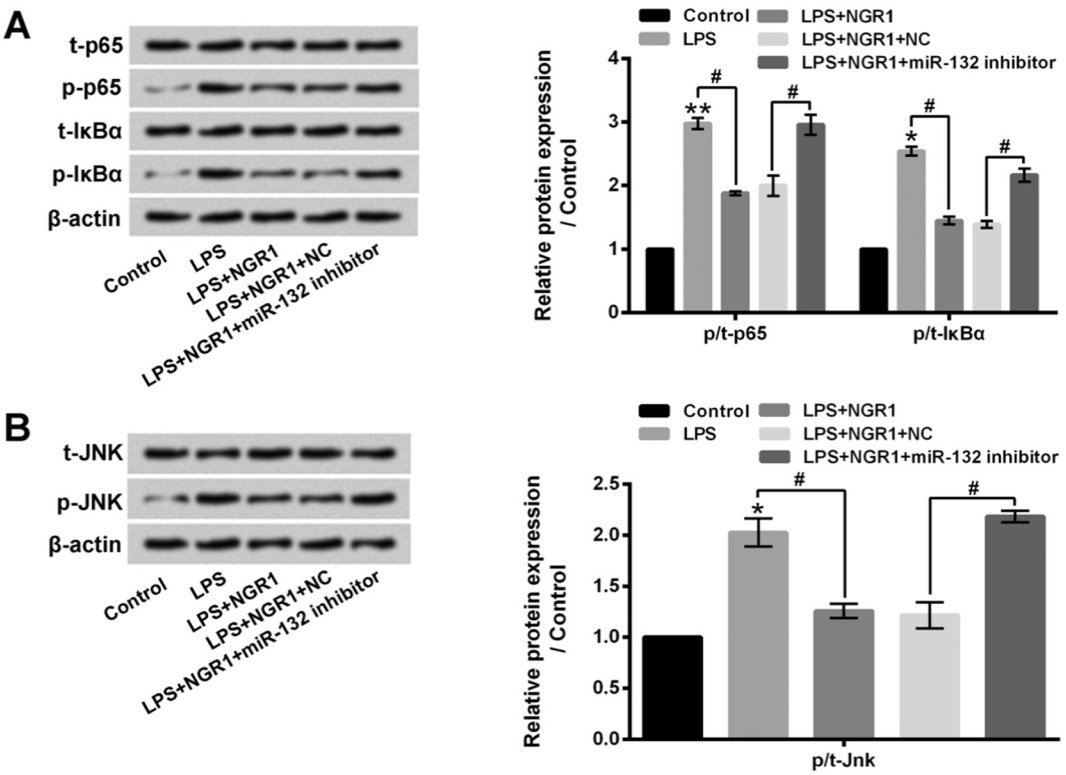

Fig. 7. NGR1 alleviated LPS-activated NF-κB and JNK pathways in MRC-5 cells via up-regulating miR-132.

132 inhibitor transfection, the expression levels of t-p65, p-p65, t-IκBα, p-IκBα, t-JNK and p-JNK in MRC-5 cells were evaluated using western blotting. LPS: Lipopolysaccharide; NGR1: Notoginsenoside R1; miR-132: MicroRNA-132; NF-κB: Nuclear factor kappa B; JNK: c-Jun N-terminal kinase; IκBα: Inhibitor of NF-κB. N = 3. Results were expressed as mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group. $^{\#}P < 0.05$ vs. 10 μg/ml LPS treatment or 10 μg/ml LPS + 30 μM NGR1 treatment group.

4. Discussion

The beneficial roles of plant-derived medicines in disease therapy have aroused wide-spread interest, due to their high efficiency and safety [22]. NGR1 is the main anti-inflammatory compound in the roots of *Panax notoginseng* (Burk.) F.H. Chen (Araliaceae) [10]. In the current study, we revealed that NGR1 could protect human lung fibroblast MRC-5 cells from LPS-caused inflammatory damage. Mechanistically, we found that NGR1 enhanced the expression level of miR-132 in MRC-5 cells. miR-132 was related to the protective effects of NGR1 on LPS-caused MRC-5 cell inflammatory damage. In addition, we disclosed that MGR1 alleviated LPS-activated NF-κB and JNK pathways in MRC-5 cells via up-regulating miR-132.

LPS, also known as endotoxin, is one of the most important cellular wall ingredients of Gram-negative bacteria [23]. LPS-induced pneumonia in vitro and in vivo models were widely used for analyzing the pathogenesis of pneumonia and exploring the new therapeutic medicine for pneumonia [24,25]. MRC-5 is a lung fibroblast cell line derived from normal lung tissue of a 14-week-old male fetus [26]. It can subculture 42 to 46 times with relative stable cell state. There are some literatures that study pneumonia using LPS-stimulated MRC-5 cells [27,28]. In this study, we found that LPS treatment suppressed lung fibroblast MRC-5 cell viability and proliferation, but induced cell apoptosis. The expression levels of IL-1β, IL-6 and TNF-α, which all play key roles in cellular inflammatory response in pneumonia [29], were all increased in MRC-5 cells and in culture supernatant of MRC-5 cells. These results suggested that LPS could cause MRC-5 cell inflammatory damage and implied that LPS-caused MRC-5 cell damage could be used for analyzing the possible anti-inflammatory and protective effects of NGR1 on lung cell injury in pneumonia.

As a naturally anti-inflammatory compound found in *Panax notoginseng* (Burk.) F.H. Chen (Araliaceae), NGR1 has been tested as a potential therapeutic agent for a series of human inflammatory diseases through suppressing inflammatory cytokines production [10,12,30]. In the present research, we revealed that NGR1 treatment had no significant effects on MRC-5 cell proliferation, apoptosis and inflammatory cytokines expression, but notably alleviated LPS-induced MRC-5 cell viability and proliferation inhibition and cell apoptosis. The expression levels of IL-1β, IL-6 and TNF-α were also reduced after NGR1 treatment. These findings indicated that NGR1 could protected MRC-5 cells from LPS-induced inflammatory injury and suggested that NGR1 might be used as an anti-inflammatory and protective medicine for alleviating lung fibroblast cell injury in pneumonia.

Abnormal expression of microRNAs has been found to be involved in the pathogenesis of many human diseases, including pneumonia [31,32]. miR-132 is a well-known anti-inflammatory factor in a number of human cells [19]. Ji et al. reported that miR-132 relieved LPS-induced inflammatory injury in neuronal cells [33]. Liu et al. proved that miR-132 suppressed LPS-induced inflammatory response in alveolar macrophages through cholinergic anti-inflammatory pathway [34]. In the current research, we found that miR-132 was up-regulated in MRC-5 cells after NGR1 treatment. In addition, knockdown of miR-132 reversed the protective effects of NGR1 on LPS-induced MRC-5 cell viability and proliferation inhibition, cell apoptosis, as well as over-production of inflammatory cytokines. These results indicated that miR-132 played critical roles in the protective effects of NGR1 on LPS-induced MRC-5 cell inflammatory injury. NGR1 might exert anti-inflammatory and protective effects on LPS-caused lung fibroblast cell damage via up-regulating miR-132.

NF-κB pathway and JNK pathway both closely related to the cellular inflammatory response [35,36]. They can promote cellular

inflammatory response by enhancing the production of inflammatory cytokines [35,36]. Fowler et al. demonstrated that inhibition of NF-κB pathway improved the outcomes in a mouse model of idiopathic pneumonia syndrome [37]. Tsay et al. reported that pseudomonas aeruginosa colonization promoted ventilator-associated pneumonia via activating JNK pathway [38]. In this research, we found that LPS treatment activated NF-κB and JNK pathway in MRC-5 cells. NGR1 treatment alleviated the LPS-activated NF-κB and JNK pathways in MRC-5 cells. In addition, knockdown of miR-132 reversed the effects of NGR1 on LPS-activated NF-κB and JNK pathways. These findings suggested that NGR1 exerted anti-inflammatory and protective effects on MRC-5 cell injury might through up-regulating miR-132 and then inactivating NF-κB and JNK pathways. To explore the regulatory mechanism of miR-132 on NF-κB and JNK pathways in MRC-5 cells, bioinformatics analysis using TargetScam (www.targetscan.org) was performed. We found that a lot of genes in the human genome were the target genes of miR-132. Considering that microRNAs usually regulate the expression of genes by binding to the 3′-untranslated region (3′UTR) of mRNA [14] and the regulation of NF-κB and JNK pathways are very complex. We guess that miR-132 inactivate the NF-κB and JNK pathways in MRC-5 cells maybe via regulating the up-stream gene expression of NF-κB and JNK pathways.

In conclusion, current work confirmed the protective roles of NGR1 in lung fibroblast cell inflammatory injury. NGR1 protected human lung fibroblast MRC-5 cells from LPS-caused inflammatory damage via up-regulation of miR-132 and then inactivation of NF-κB and JNK pathways.

Declarations of interest

None.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Authorship

Conceives and designed the experiments: Xuehua Zhang, Shan Cong and Longquan Xiang. Performed the experiments: Shan Cong, Longquan Xiang and Xiutai Yuan. Analyzed the data: Shan Cong, Longquan Xiang and Dong Bai. Wrote the paper: Xuehua Zhang.

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