Article

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# Phentolamine Protects against Apoptosis and Inflammation in a Neonatal Pneumonia Cell Model Induced by LPS by Regulating the TrkA/Akt Signaling Pathways

Qian Liu[1,*], Shengwei Wu[1], Zhenlin Chen[1], Jin Cai[1]

[1]Department of Neonatology, The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, 550000 Guiyang, Guizhou, China
*Correspondence: liuqian674888@163.com (Qian Liu)
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**Background: Phentolamine is an $\alpha$-adrenergic receptor blocker that can be used to treat neonatal pneumonia, but its underlying mechanism is unclear. The purpose of this study is to probe the function of phentolamine on lipopolysaccharide (LPS)-induced inflammation and cell death in an *in vitro* model of neonatal pneumonia.**

**Methods: Human MRC-5 cells were incubated with varying doses of phentolamine *in vitro* to evaluate cell viability. Subsequently, LPS was introduced to further investigate the combined effects of phentolamine and LPS on cell viability and apoptosis in MRC-5 cells. The effect of phentolamine/LPS treatment on the Neurotrophic Tyrosine Kinase Receptor A (TrkA)/Protein Kinase B (Akt) signaling pathway and the phosphorylation of pathway proteins in MRC-5 cells was further analyzed via western blot. Additionally, knockout of *TrkA* and *Akt* genes in MRC-5 cells was performed to explore the effects of phentolamine/LPS treatment on cell viability, apoptosis levels, and inflammatory factor levels in MRC-5 cells.**

**Results: Preincubation of MRC-5 cells with a low concentration of phentolamine ($\leq$6 µg/mL) protected against LPS-induced cell inflammatory injury. Phentolamine promoted both TrkA and Akt phosphorylation and Akt activation induced by LPS in MRC-5 cells. The protective effect of phentolamine against LPS-induced apoptosis and inflammation was significantly reduced in response to TrkA or Akt gene knockdown in MRC-5 cells.**

**Conclusions: Phentolamine may protect LPS-induced apoptosis and inflammation by activating the TrkA and Akt signaling pathways.**

**Keywords:** neonatal pneumonia; phentolamine; inflammation; apoptosis

## Introduction

Neonatal pneumonia (NP) is a severe respiratory infectious disease that occurs in the neonatal period [1,2], characterized by sudden onset and rapid changes in the state of an illness. Without timely and effective diagnosis and treatment, NP may induce multi-organ and multi-system dysfunction, including heart and respiratory failure, and even death [3,4]. The incidence of neonatal pneumonia depends on various factors, such as maternal factors, premature birth and low birth weight, duration of membrane rupture, prenatal infections, etc. [5]. Traditional treatment options include antiviral and antimicrobial approaches such as erythromycin or intravenous azithromycin [6]. However, antibiotic abuse significantly increases strain resistance in neonatal pneumonia patients receiving routine treatment [7]. Therefore, exploring the complete molecular mechanism of neonatal pneumonia is essential to develop novel and effective treatment strategies.

Phentolamine is a rapid-onset $\alpha$-adrenoceptor blocker that dilates blood vessels, effectively preventing peripheral vascular resistance, improving cerebral hypoxia, reducing pulmonary artery pressure, correcting the respiratory center, and improving lung function [8]. Phentolamine can effectively reduce bronchial smooth muscle spasms by increasing peripheral venous blood volume, expanding pulmonary arterioles, and reducing cardiac load. This prevents cardiac function damage, enhances myocardial contractility, restores pulmonary gas exchange function, and promotes blood circulation [9]. Nevertheless, the detailed mechanism of phentolamine in NP is still unclear.

The Tyrosine Kinase Receptor A (TrkA)/Protein Kinase B (Akt) signaling pathway has garnered attention for its regulatory functions in cell survival, proliferation, and apoptosis [10]. This pathway, involving the tyrosine kinase receptor TrkA and the serine/threonine kinase Akt, influences critical cellular responses under various pathological conditions [11]. However, the specific involvement of the TrkA/Akt pathway in neonatal pneumonia and its potential modulation by therapeutic agents are subjects of ongoing research.

In this study, we established an inflammatory cell injury model in which newborn embryonic lung fibroblasts (MRC-5) were cultured and treated with lipopolysaccharide (LPS) to induce MRC-5 injury [12–14]. MRC-5 cells were treated with various doses of phentolamine to observe its mitigating effect on LPS-induced cytotoxicity. Moreover, we further investigated the underlying mechanism of TrkA and Akt in the rescue of LPS-induced inflammation and apoptosis by phentolamine.

## Materials and Methods

### *Cell Culture*

We acquired newborn embryonic lung fibroblasts, specifically MRC-5 cells (iCell-h146), from iCell Bioscience Inc in Shanghai, China. Thorough testing, including mycoplasma and Short Tandem Repeat (STR) tests, was conducted on all cell samples used in this study to ensure their quality. The MRC-5 cells were cultured in DMEM medium from Golden Clone Biotechnology Co., LTD., in Beijing, China, supplemented with 10% FBS. Culturing was carried out in an incubator maintained at 37 ℃ with 5% $CO_2$. To induce inflammation, LPS at a concentration of 10 mg/mL was applied to the MRC-5 cells for a duration of 24 hours. Following the LPS treatment, varying concentrations (0.5, 6, 10, 14 µg/mL) of phentolamine (YZ-100110, National Institutes for Food and Drug Control, Beijing, China) were introduced to the MRC-5 cells.

### *Cell Counting Kit-8 (CCK-8) Assay*

MRC-5 cells were cultured in 96-well plates and placed in a 5% $CO_2$ environment at 37 °C. Following this, a CCK-8 solution (CA1210, Solarbio, Beijing, China) was systematically introduced into the 96-well plates, and the MRC-5 cells underwent a 2-hour incubation in darkness. The absorbance of MRC-5 cell viability was subsequently measured at 450 nm using a microplate reader (Cmax plus, Molecular Devices Corporation, Silicon Valley, CA, USA).

### *Apoptosis Assay*

The evaluation of apoptosis in MRC-5 cells was conducted employing the Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) dye assay (T2130, Solarbio, Beijing, China). In parallel, the 4',6-diamidino-2-phenylindole (DAPI) antibody (C0060, Solarbio, Beijing, China) was administered to the MRC-5 cells. Following these steps, MRC-5 cell apoptosis was observed and recorded using a microscope (CX53, Olympus, Tokyo, Japan).

### *Flow Cytometry*

Initially, MRC-5 cells were subjected to trypsin digestion without EDTA after a 24-hour culture period. Subsequently, the cells were transferred to a centrifuge tube and centrifuged at 1000 rpm/min for 5 minutes. Following centrifugation, the cells were carefully washed with PBS and then suspended in a PBS. For staining purposes, 100 µL of the cell suspension was transferred to a 1.5 mL centrifuge tube, and a mixture of 5 µL PI and FITC (CA1040, Solarbio, Beijing, China) was added. The staining process occurred in darkness for 15 minutes. The stained cells were subsequently screened and analyzed using flow cytometry (BECKMAN, Beckman Coulter Co., LTD., Brea, CA, USA).

### *Cell Transfection*

siRNA-negative control (si-NC) (sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense: 5′-ACGUGACACGUUCGGAGAA-3′), small interfering RNA against Tropomyosin receptor kinase A (si-TrkA) (sense: 5′-GAUGGAAUUGGAACUCUAAUU-3′, antisense: 5′-UUAGAGUUCCAAUUCCAUCUU-3′), and small interfering RNA against Akt (si-Akt) (sense: 5′-GAACUUGUCUGGACUCUAGTT-3′, antisense: 5′-CUAGAGUCCAGACAAGUUCTT-3′) were purchased from Greiner (Beijing Dingguo Changsheng Biotechnology Co., LTD., Beijing, China). Cell was transfected with lipofectamine 2000 (Thermo Fisher Scientific, Shanghai, China).

### *RNA Extraction and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)*

Total RNA extraction was accomplished utilizing the TRNzol universal reagent (DP424, TIANGEN BIOTECH (BEIJING) CO., LTD., Beijing, China). Genomic DNA elimination and cDNA synthesis were conducted with the FastQuant cDNA first strand synthesis kit (KR116) and SuperReal fluorescence quantitative premix reagent (FP205, TIANGEN BIOTECH (BEIJING) CO., LTD., Beijing, China). mRNA expression levels were quantified using the PikoRealTM Real-Time PCR system (LightCycler96, Roche, Basel, Switzerland).

The relative quantitative analysis of the samples was conducted using the $2^{-\Delta\Delta Ct}$ method, with all obtained data normalized to the internal reference, $\beta$-Actin. The PCR reaction program included an initial step at 95 °C for 3 minutes, followed by cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 35 minutes. The process concluded with a final extension step at 72 °C for 5 minutes and a final hold at 4 °C. The primer sequences used in this study are listed in Table 1.

### *Western Blot*

Proteins were separated through Sodium Dodecyl Sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Beijing Solaibao Technology Co., LTD., Beijing, China). After a 1-hour blocking period at 25 °C with 5% Bovine Serum Albumin (BSA), immunoblotting was conducted using specific antibodies: Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)

**Table 1. Primer names and primer sequences.**

| Primer name | Primer sequence (5′-3′) |
|---|---|
| *Akt*-F | GCAGCACGTGTACGAGAAGA |
| *Akt*-R | GGTGTCAGTCTCCGACGTG |
| *TrkA*-F | GGTACCAGCTCTCCAACACTGAGG |
| *TrKA*-R | CCAGAACGTCCAGGTAACTCGGTG |
| *β-Actin*-F | CCCGAGCCGTGTTTCCT |
| *β-Actin*-R | GTCCCAGTTGGTGACGATGC |

*Akt*, Protein Kinase B; *TrkA*, Tyrosine Kinase Receptor A.

(1:1000 dilution; Cat No. TA-08, ZSGB-BIO, Beijing, China), TrkA (1:1000 dilution; Cat No. ab302524, Abcam, Cambridge, UK), phosphorylated Tropomyosin receptor kinase A (p-TrkA) (1:1000 dilution; Cat No. PA5-104674, Thermo Fisher Scientific, Wilmington, MA, USA), Akt (1:1000 dilution; Cat No. ab38449, Abcam, Cambridge, UK), and phosphorylated Akt (p-Akt) (1:1000 dilution; Cat No. 44-621G, Thermo Fisher Scientific, Wilmington, MA, USA). Each antibody was incubated separately at 4 °C for 12 hours.

Following incubation, the co-incubation system received antibody labeling with horseradish peroxidase (HRP) (1:2000 dilution; Cat No. ZB-2305, ZB-2301, ZSGB-BIO, Beijing, China). Exposure was carried out using a chemiluminescence instrument (Tanon-4600, Yuanpinghao Biotechnology Co., LTD., Beijing, China). The grey value analysis of the target protein bands was then performed using Image J software (Version 1.5f, National Institutes of Health, Bethesda, MD, USA).

### *Enzyme-linked Immunosorbent Assay (ELISA)*

The assay kits for interleukin (IL)-6 (SEKH-0013), Monocyte Chemoattractant Protein-1 (MCP-1) (SEKH-0236), tumor necrosis factor (TNF)-$\alpha$ (SEKH-0047), and interferon (IFN)-$\gamma$ (SEKH-0046) were procured from Solarbio (Beijing, China). Matrix coating was performed by applying matrix coating buffer to cover the bottom of a 96-well ELISA plate, followed by an overnight incubation at 4 °C. Subsequently, three washes of the well bottoms with wash buffer were conducted. Samples, including positive and negative control samples from the laboratory, were introduced by adding cell culture supernatant into the respective wells. The ELISA plate was incubated, and detection antibodies with specific binding to the target proteins were added to the wells. Additional washing steps were performed, incorporating wash buffer into the wells and removing the liquid. The substrate was applied to initiate the reaction between the substrate and the enzyme-conjugated antibodies. The experiment concluded by measuring the absorbance of each well using an enzyme-linked immunosorbent assay (ELISA) reader (Cmax plus, Molecular Devices Corporation, Silicon Valley, CA, USA). Throughout the experiment, the instructions outlined in the assay kit manual were followed.

### *Statistical Analysis*

The statistical analysis was carried out utilizing GraphPad Prism software (Version 8.0, GraphPad Software Inc, San Diego, CA, USA; https://www.graphpad-prism.cn/). One-way analysis of variance (ANOVA) was used for data analysis, with the results presented as mean ± standard deviation (SD). Statistical significance was considered at a $p$-value $< 0.05$.

## Results

### *Phentolamine Prevented LPS-triggered Cell Death in MRC-5 Cells*

MRC-5 cells were initially treated with varying concentrations of phentolamine for 24 hours to assess its cytotoxic effects. The cell proliferation assay revealed that phentolamine doses equal to or greater than 10 µg/mL result in MRC-5 cell death (Fig. 1A) ($p < 0.05$). Subsequently, after inducing damage to MRC-5 cells by adding LPS (10 mg/mL), different doses of phentolamine were introduced for intervention therapy, and MRC-5 cell activity was measured. The results of the cell viability assay are demonstrated in Fig. 1B. LPS treatment alone significantly enhanced MRC-5 cell death compared to the control ($p < 0.001$). Nevertheless, 6 µg/mL phentolamine significantly prevented cell death in MRC-5 cells ($p < 0.01$) (Fig. 1B). Therefore, we chose a 6 µg/mL concentration of phentolamine for subsequent studies because this concentration exhibited a protective effect against LPS-induced cell death without contributing to cell death.

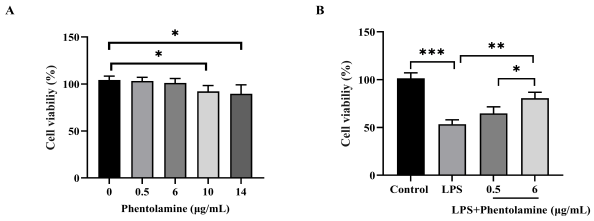


**Fig. 1. Function of phentolamine on lipopolysaccharide (LPS)-triggered cell death in MRC-5 cells.** (A) Cell viability was detected after co-incubation of MRC-5 cells with different phentolamine doses *in vitro* vs 0 group, *$p < 0.05$ (n = 6). (B) MRC-5 cells were incubated with different phentolamine doses *in vitro* and treated with LPS (6 µg/mL) at the same time, and cell viability was detected (n = 6) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

### *Phentolamine Reversed LPS-induced Apoptosis*

TUNEL assay showed that LPS induced clear cell apoptosis compared to the control, while phentolamine significantly reduced LPS-induced apoptosis in MRC-5 cells (Fig. 2A). Analysis of apoptosis rates in MRC-5 cells

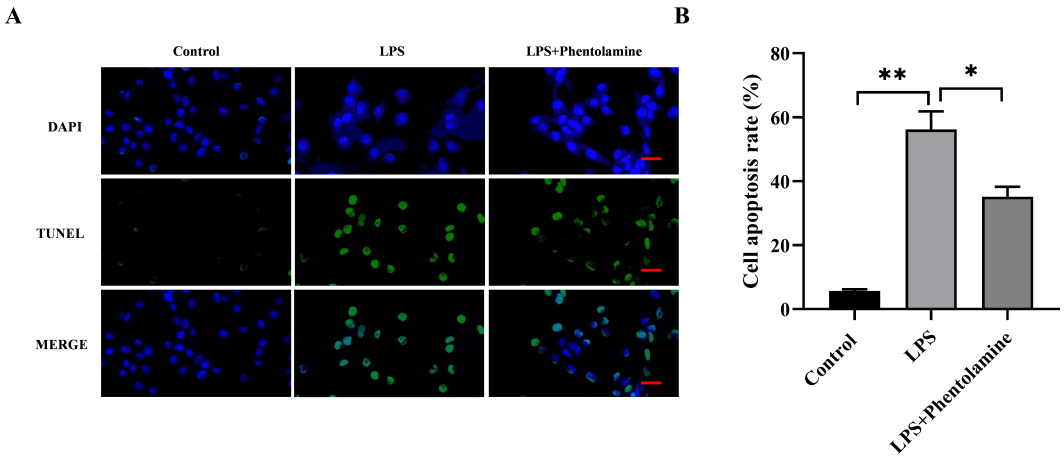


**Fig. 2. Apoptosis response of MRC-5 cells to different treatments as determined by Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) and 4',6-diamidino-2-phenylindole (DAPI).** (A) TUNEL assay was used to detect apoptotic cells, and DAPI immunohistochemistry was used to identify the nucleus of MRC-5 cells (n = 3). Scale bar: 100 µm. (B) Effects of three different treatments on apoptosis rate (n = 3) (*$p < 0.05$, **$p < 0.01$).

showed that LPS triggered severe apoptosis ($p < 0.01$) whereas phentolamine alleviated LPS-induced apoptosis ($p < 0.05$) (Fig. 2B).

### *Phentolamine-activated TrkA/Akt Signaling in LPS-induced MRC-5 Cells*

Next, we aimed to determine the function of phentolamine on signal transduction in LPS-induced MRC-5 cells. Therefore, we investigated the role of phentolamine in TrkA/Akt signaling pathway activation in LPS-injured MRC-5 cells. The TrkA, p-TrkA, Akt, and p-Akt protein levels were significantly decreased after LPS treatment compared to the control ($p < 0.05$) (Fig. 3A–E). No obvious difference was observed in the TrkA and Akt protein levels between the LPS+ phentolamine treatment group and the LPS treatment group ($p > 0.05$) (Fig. 3A,B). However, phentolamine treatment significantly increased the p-TrkA and p-Akt protein expression levels ($p < 0.05$) (Fig. 3A–E).

### *Akt Knockdown Attenuated the Protective Effect of Phentolamine on LPS-triggered Injury in MRC-5 Cells*

We investigated Akt's regulatory role in the rescue effect of phentolamine in LPS-injured MRC-5 cells. Transfection of si-Akt into MRC-5 cells resulted in a significantly lower expression level of *Akt* mRNA and protein compared to the si-NC group ($p < 0.05$) (Fig. 4A,B). Then, we determined the function of siRNA on the activity of MRC-5 cells, revealing that si-Akt transfection caused MRC-5 cell death ($p < 0.05$) and increased the cytotoxic function of LPS of MRC-5 cells ($p < 0.05$) (Fig. 4C). Furthermore, in LPS-triggered MRC-5 cells, cell viability in the si-Akt group was significantly lower than that in the si-NC group ($p < 0.05$) (Fig. 4C). Subsequently, MRC-5 cells transfected with si-RNA were treated with phentolamine for one day before being treated with LPS. Thereafter, flow cytometry exhibited increased apoptosis in the si-Akt group than in the si-NC group ($p < 0.001$) (Fig. 4D,E). Akt knockdown decreased the protective function of phentolamine on LPS-induced cytotoxicity.

### *TrkA Silencing Attenuated the Protective Function of Phentolamine on LPS-induced Injury in MRC-5 Cells*

We explored whether TrkA plays a regulatory effect in the rescue effect of phentolamine on LPS-injured MRC-5 cells. Transfection of si-TrkA into MRC-5 cells resulted in a lower expression of TrkA mRNA than in the si-NC group ($p < 0.05$) (Fig. 5A,B). Cell viability was significantly decreased in the si-TrkA group compared to the control and si-NC groups ($p < 0.05$) (Fig. 5C). Furthermore, following LPS treatment, cell viability was markedly reduced compared to the si-NC group ($p < 0.01$). In the LPS/si-TrkA group, cell viability was lower than that in the LPS/si-NC group ($p < 0.05$). Flow cytometry demonstrated that si-TrkA transfected MRC-5 cells detected more apoptosis than the si-NC group ($p < 0.001$) (Fig. 5D,E). TrkA knockdown decreased the protective response of phentolamine on LPS-induced injury.

### *Akt or TrkA Silencing Decreased the Inflammatory Repair Effect of Phentolamine on LPS-injured MRC-5 Cells*

Phentolamine treatment significantly decreased levels of inflammatory factors in MRC-5 cells ($p < 0.01$) (Fig. 6A–D). MRC-5 cells transfected with si-Akt and si-TrkA were incubated with phentolamine and then treated

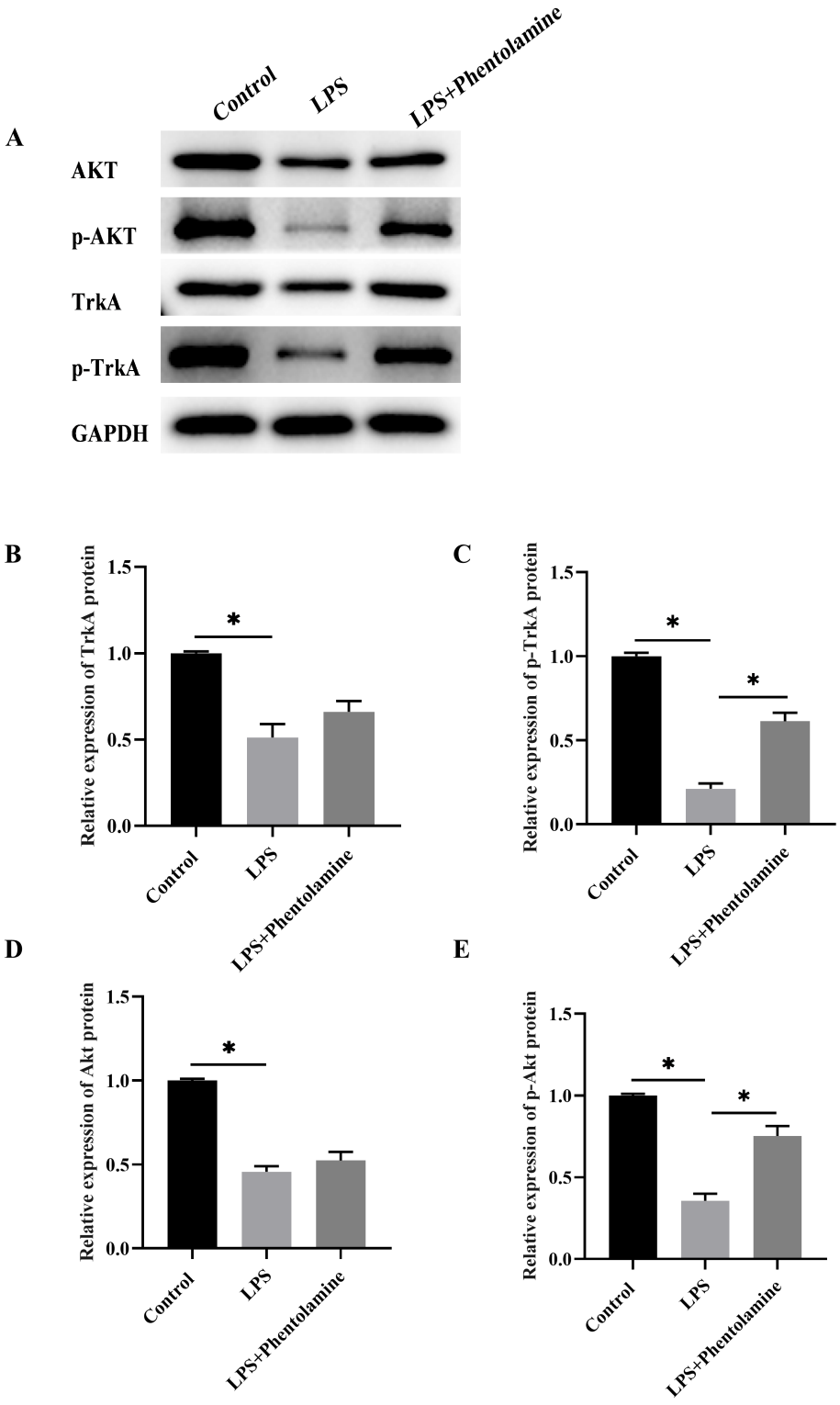


**Fig. 3. Effect of phentolamine on Tyrosine Kinase Receptor A (TrkA)/Protein Kinase B (Akt) induced by LPS in MRC-5 cells.** (A–E) The protein production of TrkA, phosphorylated Tropomyosin receptor kinase A (p-TrkA), Akt, and phosphorylated Akt (p-Akt) was analyzed by western blot analysis (n = 6) (*$p < 0.05$).

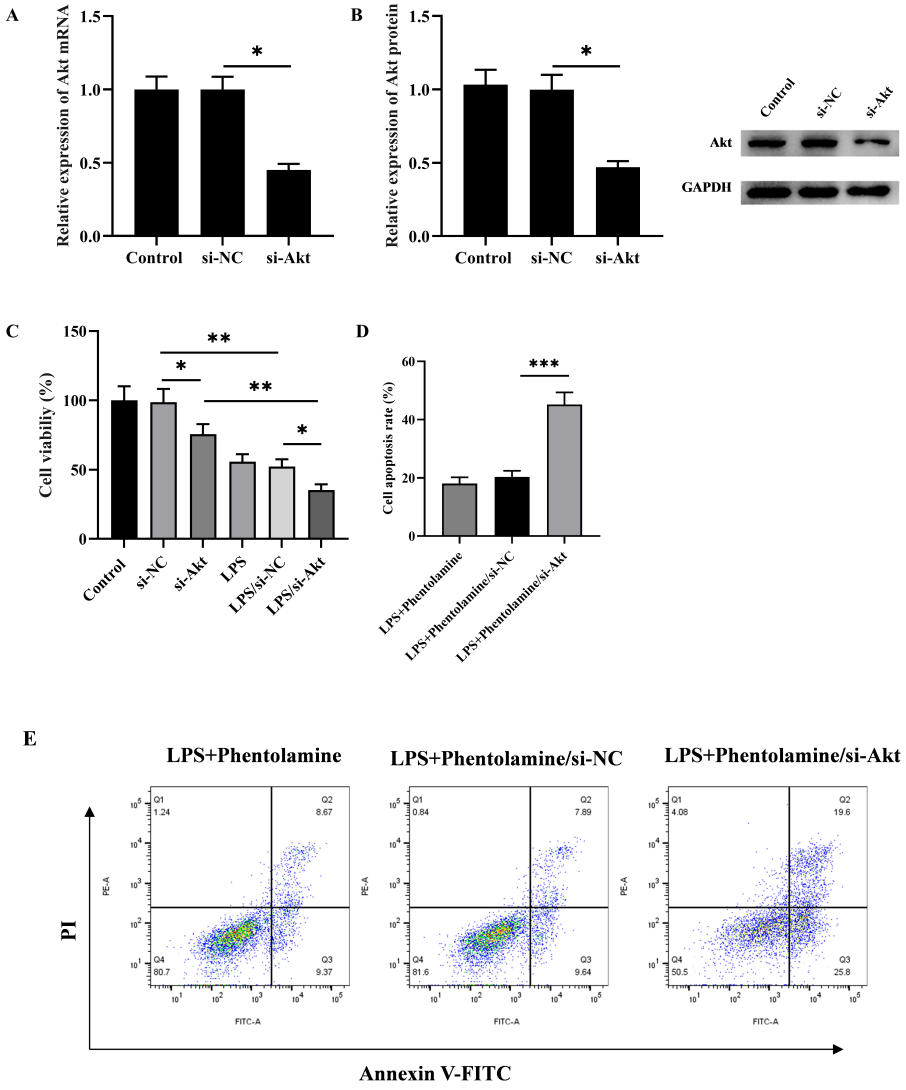


**Fig. 4. Function of Akt knockdown on MRC-5 cells doubly treated with phentolamine and LPS.** (A,B) *Akt* gene expression in MRC-5 cells (n = 3). (C) Effect of small interfering RNA against Akt (si-*Akt*) on the viability of MRC-5 cells treated with LPS (n = 6). (D,E) Effect of small interfering RNA against Akt (si-*Akt)* on MRC-5 cell apoptosis when co-treated with phentolamine and LPS (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

with LPS for an additional 24 hours. ELISA was used to detect the levels of IL-6, MCP-1, TNF-$\alpha$, and IFN-$\gamma$ in MRC-5 cells. The levels of IL-6, MCP-1, TNF-$\alpha$, and IFN-$\gamma$ in the si-Akt and si-TrkA groups were significantly higher than those in the si-NC group ($p < 0.01$) (Fig. 6A–D).

## Discussion

This study found that phentolamine has a clear protective effect against LPS-induced MRC-5 cell inflammation and apoptosis.

In this study, a cell viability assay showed that low to moderate concentrations (≤6 μg/mL) of phentolamine did not induce cell death. However, at higher concentrations (≥10 μg/mL), phentolamine significantly triggered the death of MRC-5 cells. Related studies have demonstrated that high doses of phentolamine cause cytotoxicity both *in vitro* and *in vivo* [15,16]. Additionally, we found that phentolamine significantly ameliorated LPS-induced embryonic lung cell death *in vitro*. To avoid confusion between LPS-induced cell death and the endogenous toxic effects of phentolamine, we chose the maximum safe concentration of phentolamine (6 μg/mL) for subsequent experiments.

In addition, we investigated the effect of phentolamine on LPS-induced apoptosis and inflammatory injury in em-

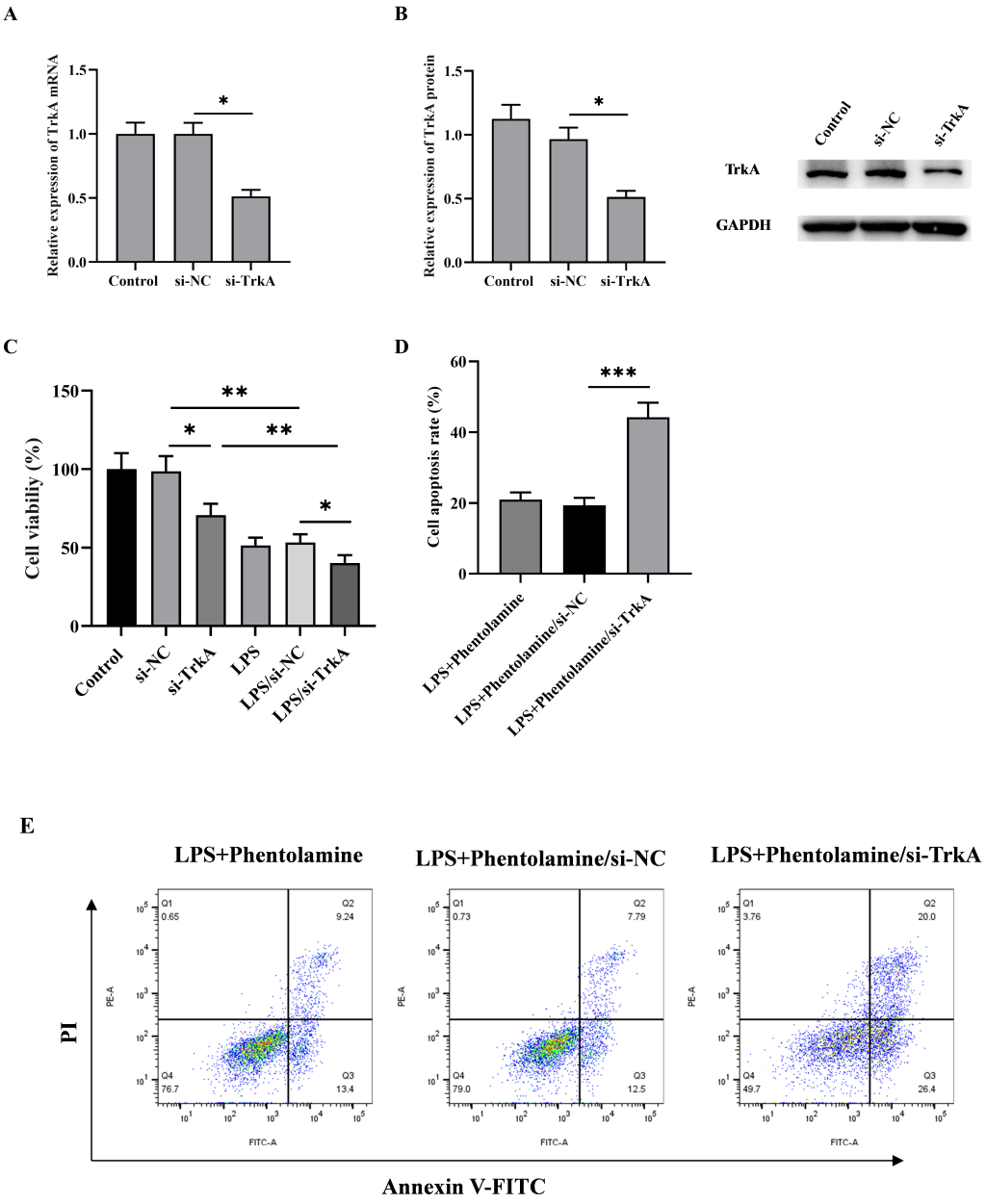


**Fig. 5. Function of TrkA knockdown on MRC-5 cells doubly treated with phentolamine and LPS.** (A,B) *TrkA* gene expression in MRC-5 cells (n = 3). (C) Effect of si-TrkA on the viability of MRC-5 cells treated with LPS (n = 6). (D,E) Effect of si-TrkA on apoptosis of MRC-5 cells co-treated with LPS and phentolamine (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

bryonic lung cells. Our results showed that phentolamine significantly reduced MRC-5 cell apoptosis and inflammatory injury. Similarly, the results of Han *et al*. [17] demonstrated that phentolamine significantly prevents acute kidney injury and reduces inflammation and apoptosis in mice. Previous studies have shown that phentolamine can significantly improve the inflammatory response and lung function in severe neonatal pneumonia [18]. This is the first time we have reported the inhibitory role of phentolamine on inflammation and apoptosis in embryonic lung cells.

In this study, we investigated signaling pathways associated with the protective effect of phentolamine on LPS-injured MRC-5 cells. Our data showed that phentolamine treatment induced upregulation of p-TrkA and p-Akt, although there was no significant change in TrkA and Akt expression. Phosphorylation can either activate or inhibit the function of pathway proteins, thereby modulating the intensity and direction of signal transduction. Conversely, the status of pathway proteins can influence the activity of kinases and phosphatases, thereby impacting the phospho-

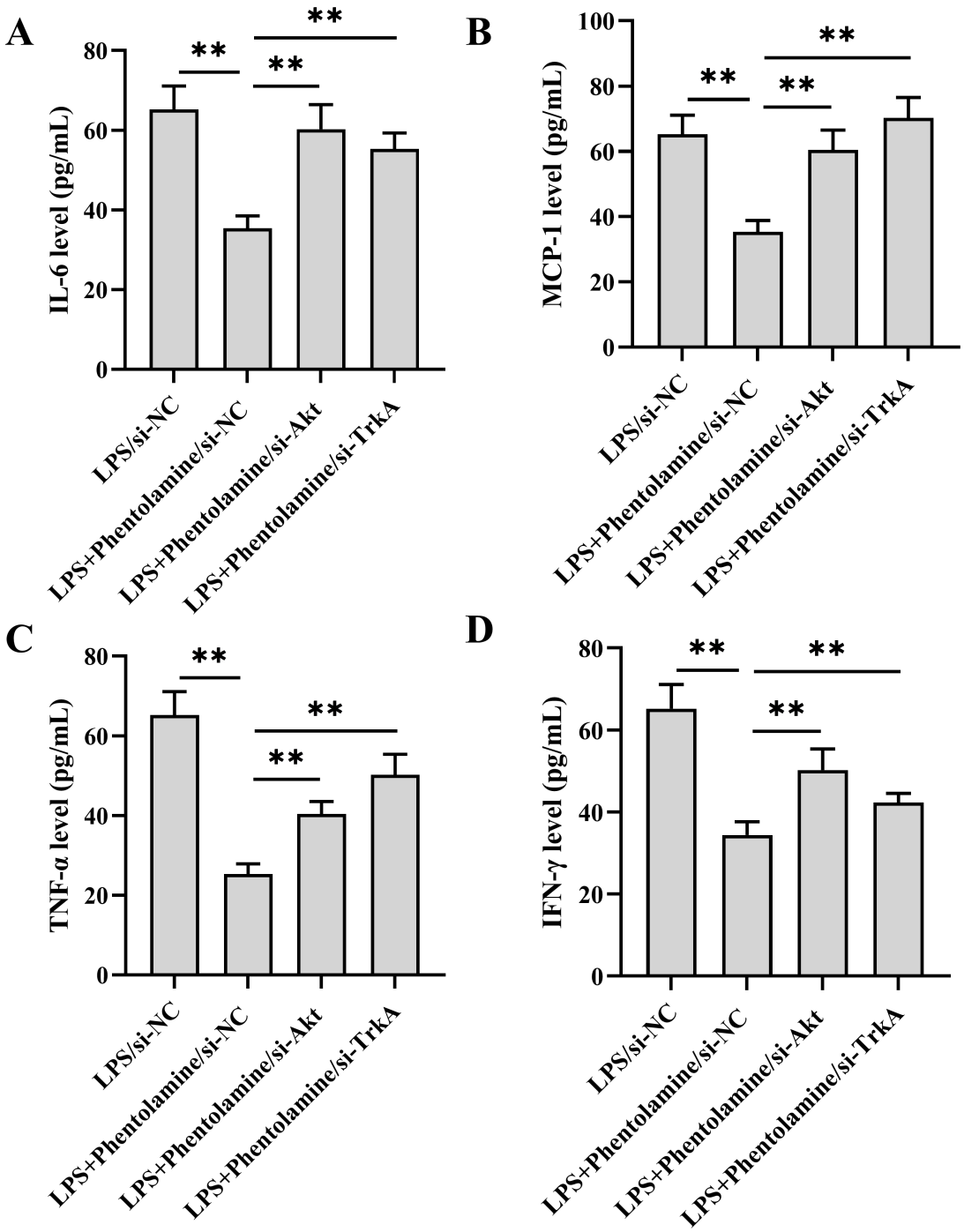


**Fig. 6. Effect of Akt and TrkA knockdown on MRC-5 cells treated with phentolamine and LPS.** (A–D) Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of interleukin (IL)-6, Monocyte Chemoattractant Protein-1 (MCP-1), tumor necrosis factor (TNF)-$\alpha$, and interferon (IFN)-$\gamma$ in LPS+ phentolamine-treated MRC-5 cells transfected with si-Akt and si-TrkA (n = 3) (**$p <$ 0.01).

rylation state of other proteins [19]. Notably, there are no reports that the mechanism of phentolamine on neonatal pneumonia-associated cell injury involves the TrkA/AkT-related pathway. This study provides the first report on this topic.

Previous studies have indicated the relevance of TrkA to cell proliferation and apoptosis, with phosphorylation of TrkA inducing TrK activity [20]. Upon the binding of TrkA to its ligands, the TrkA receptor is phosphorylated, activating the PI3K-Akt pathway [21,22]. Activated Akt signal participates in various activities including glucose metabolism, protein metabolism, fat metabolism, cell cycle regulation, and cell invasion [23,24]. Gao *et al.* [10] clearly established that TrkA/Akt signaling is involved in

the treatment of cellular damage in neonatal pneumonia. Therefore, we hypothesized that the therapeutic mechanism of phentolamine in neonatal pneumonia may occur through the activation of TrkA phosphorylation, followed by further activation of Akt signaling. In addition, phentolamine also exhibited improvement in LPS-induced cell injury, possibly attributable to the anti-inflammatory effects of Akt reported in previous studies [25,26]. Moreover, the anti-cytotoxicity of phentolamine in LPS-triggered MRC-5 cell injury may be mediated through the Akt signaling pathway.

## Conclusions

Phentolamine decisively alleviates inflammatory and apoptotic injuries induced by LPS, demonstrating a clear association with the activation of the TrkA/Akt signaling pathway.

## Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Author Contributions

QL and SW contributed to the concept and designed the research study. ZC and JC performed the research. JC and SW provided help and advice on the experiments. QL and ZC contributed to the analysis and interpretation of the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

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This research received no external funding.

## Conflict of Interest

The authors declare no conflict of interest.

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