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Glycitin alleviates lipopolysaccharide-induced acute lung injury via inhibiting NF-κB and MAPKs pathway activation in mice

Yu Chen, Shuai Guo, Kangfeng Jiang, Ying Wang, Mei Yang, Mengyao Guo*

Department of Clinical Veterinary Medicine, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, People's Republic of China

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ABSTRACT

Acute lung injury (ALI) is a pulmonary diffuse dysfunction disease caused by immoderate inflammatory response breaking the coordination of physiological structures and functions, and there are very few effective treatments to reduce high morbidity of ALI in critical patients. Glycitin is a natural ingredient derived from the seeds of leguminous plants and may have potent anti-inflammation features. The purpose of this study was to investigate the anti-inflammation effect of glycitin on LPS-induced ALI in mice and elucidate its possible anti-inflammatory mechanisms. The results of histopathological changes, the wet/dry weight ratio as well as the myeloperoxidase (MPO) activity indicated that glycitin obviously alleviated the lung injury induced by LPS. In addition, qPCR and ELISA results found that glycitin could dose-dependently decrease the expressions of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. Western blotting was performed to revealed that glycitin inhibited the activation of NF-κB and MAPKs signaling pathways by suppressing the expression of TLR4 protein and the phosphorylation of IKKβ, IκBα, p65, p38, ERK, and JNK. All data indicated that glycitin could protect lung tissues from LPS-induced inflammation via inhibiting TLR4-mediated NF-κB and MAPKs signaling pathways.

1. Introduction

Acute lung injury (ALI) is a clinically common inflammatory injury disease in lung tissues caused by various pathogenic factors in vitro and in vivo [1]. Pulmonary edema and pulmonary hemorrhage is the main pathological feature of ALI because of inflammation-induced damage to alveolar capillary endothelial cells and alveolar epithelial cells [2]. Although the therapies and mechanisms of ALI have been reported so many, 22%–58% mortality of ALI is still frightening [3]. Previous studies had reported that excessive inflammatory response in the lung tissues was the most harmful factor for ALI [4]. At present, it is generally agreed that relieving inflammatory response is the most effective treatment in ALI [5,6].

It is widely believed that the high incidence of ALI is closely related to microbial infection, such as the gram negative bacteria [5]. Lipopolysaccharide (LPS) which is one of immune stimulus component of the gram negative bacteria plays an crucial role in various inflammatory responses [7–9]. It is suggested that LPS is a typical pathogen associated molecular patterns (PAMP) recognized by pathogen-recognition receptors (PRRs) in the innate immune system [10,11]. Toll-like receptors 4 (TLR4) acts as one of PRRs to recognize LPS and arose signal transduction [12]. It has been confirmed that LPS can trigger inflammatory responses via TLR4 mediated-NF-κB and MAPKs signaling pathways [11]. Then activated NF-κB p65 and AP-1 could promote transcription of multiple target genes associating with pro-inflammatory mediators, such as IL-1β, IL-6, and TNF-α [13,14]. It had been verified that obstruction of TLR4-mediated NF-κB and MAPKs pathways could suppress uncontrolled inflammatory responses, and then temperate inflammation had less influence on tissues and organs damage [15].

Glycitin (4′-hydroxy-6-methoxyisoflavone-7-D-glucoside) (Fig. 1A) is a kind of soy isoflavone extracted from soybean and soy food products [16]. It has been reported to have multiply biological functions, such as anti-oxidant, anti-obese, anti-cardiovascular disease and promoting wound healing [17–21]. In addition, most of soy isoflavones have been shown to have anti-inflammatory effects [22–25], which indicates that glycitin may be used as a potential drug for the treatment of ALI. Therefore, this study explored whether glycitin could exert anti-inflammatory effect on LPS-induced ALI in mice and anti-inflammatory molecular mechanism in RAW264.7 cells. More importantly, the results of this study could offer some reference for the treatment of ALI in humans.

* Corresponding author at: College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, People's Republic of China.
E-mail address: guomy1985@sohu.com (M. Guo).

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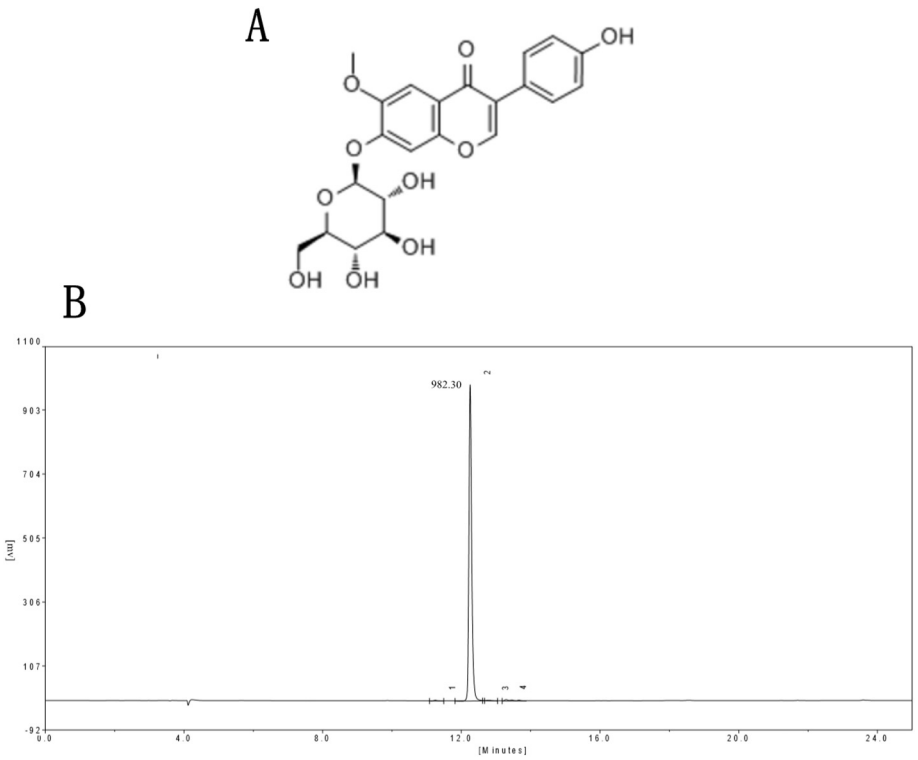

Fig. 1. (A) The chemical structure of glycitin. (B) HPLC of glycitin.

2. Materials and methods

2.1. Reagents

Glycitin was purchased from Shanghai Yuanye BioTechnology Co., Ltd. (Shanghai, China). LPS (*Escherichia coli* 055:B5) was provided by Sigma-Aldrich (St. Louis, MO, USA). RAW264.7 cells and HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The myeloperoxidase (MPO) detection kits were purchased from the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). The reverse transcriptase reagent and the SYBR Green Plus Reagent Kit were bought from Vazyme Biotech Co., Ltd. (Nanjing, Jiangsu, China). The mouse IL-1β, IL-6 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were obtained from BioLegend (Camino Santa Fe, CA, USA). All of the antibodies (TLR4, IKKβ, p-IKKβ, IκBα, p-IκBα, p65, p-p65, p38, p-p38, ERK, p-ERK, JNK, p-JNK, β-actin and the HRP-conjugated goat anti-rabbit antibody) were provided by Cell Signaling Technology (Beverly, MA, USA). All other chemical reagents using in this study were reagent grade.

2.2. High performance liquid chromatography (HPLC) detection

The purity of glycitin was measured by high performance liquid chromatography (HPLC). The experiment was carried out using an EChrom2000 DAD data system (Elite, Dalian, China). Briefly, 20 μL glycitin was separated on a Hyper ODS2 C18 analytical column (5 μm, 250 mm × 4.6 mm). Subsequently, the elution was performed using the acetonitrile/0.1% phosphate water (v/v, 5:95,) mobile phase. The flow rate was 1.0 mL/min, and the detection wavelength was 246 nm.

2.3. Tachypleus amebocyte lysate (TAL) test

The endotoxin content of glycitin was detected with TAL endotoxin test kit (Zhanjiang Bokang Marine Biological Co., Ltd., China) following the instruction manual. Bacterial endotoxin test (BET) water was served as a negative control, and LPS (1 μg/mL) was positive control. Glycitin was diluted with BET water to different concentrations. The reaction results were judged according to the gel state. "+" indicates that the gel is formed, the suspected gel does not deform, does not slip off the tube wall, and the reaction is positive; "–" means that the gel is not formed or the gel formed is not solid, and the deformed well slips off the tube wall, and the reaction is negative.

2.4. Mice and ALI animal model

A total of 60 BALB/c male mice (6–8 weeks old, 18–22 g weight) were purchased from Huazhong Agricultural University Laboratory Animal Research Center. (Wuhan, China). All mice were allowed to obtain food and water ad libitum in the environment maintained at 25 °C ± 1 °C and 65% humidity. All procedures of this study were carried out in accordance with standards provided by the Laboratory Animal Research Center of Hubei province, and approved by the Ethical Committee on Animal Research at Huazhong Agricultural University (HZAUMO-2015-12).

All mice were randomly classified into the following six groups: control group, LPS group, LPS + glycitin (5, 10 and 20 mg/kg) groups, and LPS + dexamethasone (DEX, 5 mg/kg) group. LPS-induced acute lung injury and drugs administration was carried out as following. Briefly, each mouse inhaled 50 μL 1 mg/mL LPS through the nose to induce ALI. The control group was intranasally received 50 μL sterile PBS. After 24 h of the inhalation, glycitin, dissolved by DMSO and diluted with Dulbecco's modified Eagle's medium (DMEM) to 2 mg/mL, was injected into the abdominal cavity at different doses (5, 10 and 20 mg/kg) in glycitin groups three times (once every 8 h). The DEX group was given an intraperitoneal injection of DEX (5 mg/kg). The blank group received equal volume of DMEM. Then the mice were euthanized at 8 h after the last drug treatment, and lung tissues were collected and kept in −80 °C.

2.5. Collection of bronchoalveolar lavage fluid (BALF) and cell counts

Fresh lungs were washed three times with 0.5 mL cold sterile PBS

Table 1
Results of TAL test.

Experimental reaction	Negative control BET water	Different concentration of glycitin (μg/mL)							Positive control LPS (1 μg/mL)
		5	10	15	20	25	50	100	
Test 1	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	+ + +
Test 2	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	+ + +
Test 3	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	+ + +

Note: "+" indicates the degree of positive reaction; "–" indicates the degree of negative reaction.

Table 2
Primers used for qPCR.

Name	Primer sequence (5′–3′)	GenBank accession number	Product size (bp)
TLR4	TTCAGAGCCGTTGGTGTATC CTCCCATTCCAGGTAGGTGT	NM_021297.2	170
IL-1β	CCTGGGCTGTCCTGATGAGAG TCCACGGGAAAGACACAGGTA	NM_008361.4	131
IL-6	GGCGGATCGGATGTTGTGAT GGACCCCAGACAATCGGTTG	NM_031168.1	199
TNF-α	CTTCTCATTCCTGCTTGTG ACTTGGTGGTTTGCTACG	NM_013693.3	198
GAPDH	CAATGTGTCCGTCGTGGATCT GTCCTCAGTGTAGCCCAAGATG	NM_001289726.1	124

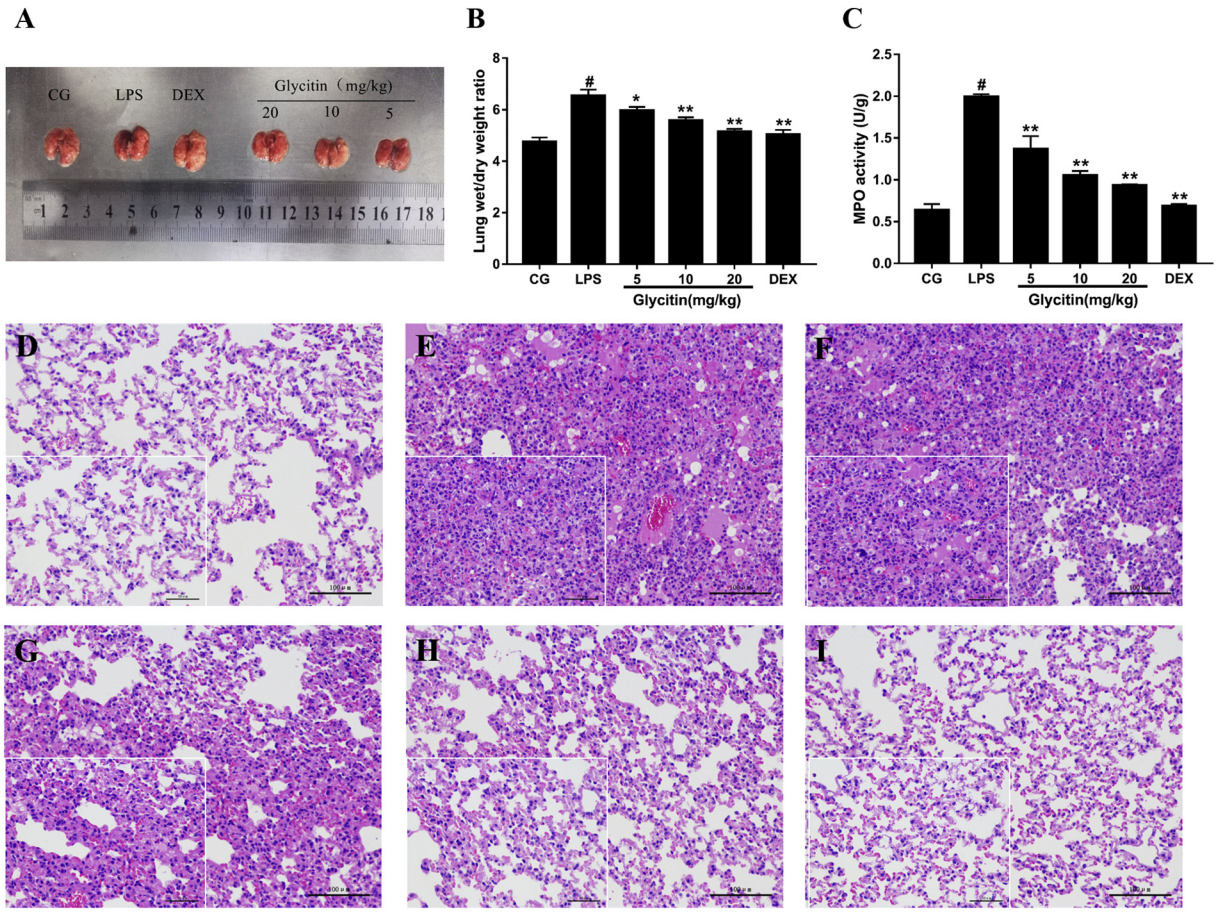

Fig. 2. Effects of glycitin on LPS-induced lung injury. All mice were stimulated with LPS (1 mg/mL) for 24 h, then intraperitoneally injected with different concentrations of glycitin (5, 10, and 20 mg/kg) and DEX (5 mg/kg) three times. (A) Morphology of the lung. (B) Lung wet/dry weight ratio. (C) MPO activity. CG is the control group. LPS is the LPS-stimulated group. DEX is dexamethasone treatment group after LPS stimulation. (D–I) Histopathological analysis of lung tissue with H&E staining. Bottom left images scale bar = 50 μm, large images scale bar = 100 μm (D) Control group. (E) LPS group. (F–H) glycitin (5, 10, and 20 mg/kg) groups. (I) Dexamethasone group. The value is presented as means ± S.E.M. of three independent experiments. #$p < 0.01$ vs. the control group. *$p < 0.05$ vs. the LPS group; **$p < 0.01$ vs. the LPS group.

through the endotracheal intubation, and recovered to a total volume of 1.3 mL. BALF was centrifuged (4 °C, 3000 rpm, 10 min) to obtain pelleted cells. The cell pellets were resuspended in PBS for total cell counts with a hemocytometer, and cell smears were prepared for differential cell counts by the Wright – Giemsa staining method. The supernatant of BALF was detected cytokines using ELISA kit.

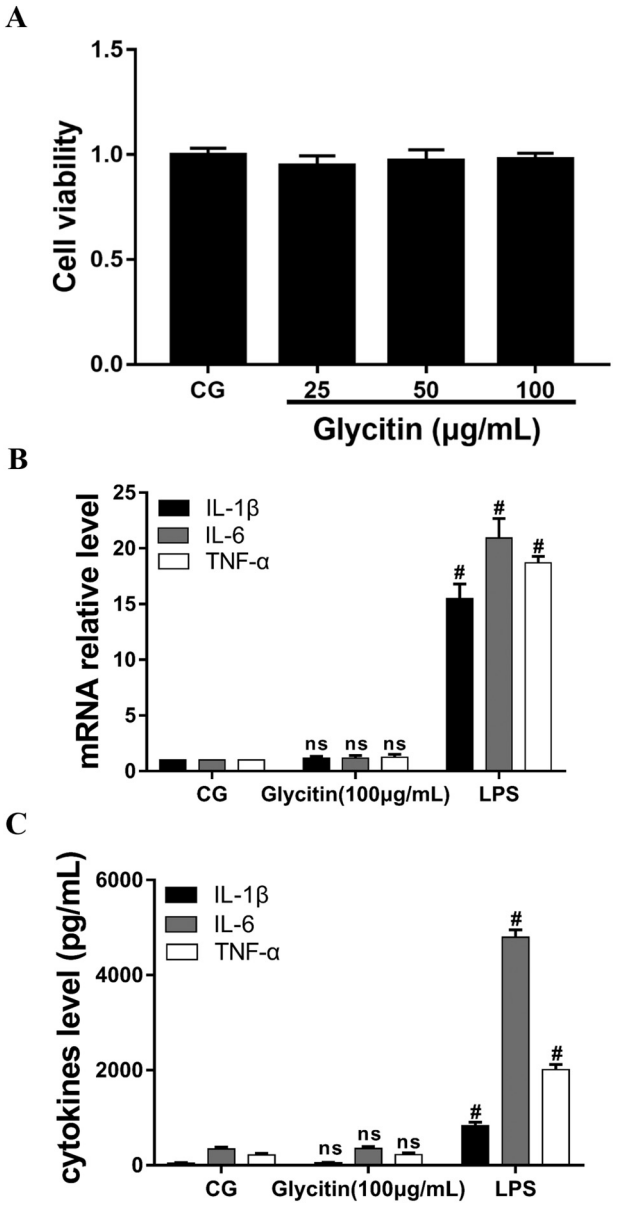

Fig. 3. Effects of glycitin on RAW 264.7 cells. (A) The cells were grown with different concentrations of glycitin (25, 50, and 100 μg/mL) for 24 h, and then the cell viability was measured using the CCK-8 assay. (B&C) The cells were dealt with glycitin alone or LPS, the expression of IL-1β, IL-6, TNF-α were examined by RT-qPCR and ELISA. Data represent the mean ± S.E.M. of three independent experiments. #$p < 0.05$ vs. the control group. ns is no significant difference compared to the control group.

2.6. Lung wet/dry weight ratio assay

The water content of lungs was determined by calculating the wet/dry weight ratio of lung tissues. The fresh lung was cut, washed quickly in PBS, dried and weighed to receive the wet weight. Then the lung was seasoned at 80 °C for 72 h to get the dry weight. The results of wet/dry ratio were represented as the wet weight dividing by the dry weight.

2.7. Myeloperoxidase analysis

Myeloperoxidase (MPO) is mainly found in neutrophils and monocytes, so MPO is regarded as a marker reflecting the degree of inflammation [7]. The lung tissues homogenate was received by using glass homogenizer to grind lung tissue and buffer (w/v, 1:19). The MPO assay kit was used to detect the supernatant of lung tissues homogenate according to the instructions of manufacturer, and the spectrophotometer was performed to obtain absorbance values at 460 nm.

2.8. Histopathological evaluation

Harvested fresh lung tissues were excised, and fixed with 4% formaldehyde solution for subsequent paraffin section making. Briefly, lung tissue blocks were dehydrated in elevated concentrations of alcohol, transparentised with the xylene solution, embedded in paraffin, sliced into 5 μm sections and spreaded out on the slide. Hereafter, hematoxylin and eosin (H&E) staining was carried out to observe the pathological changes of lung tissues with optical microscope (Olympus, Japan).

2.9. Cell culture and treatment

RAW264.7 cells were cultured in DMEM/high glucose medium with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C with 5% CO_2. The cells were stimulated with LPS (1 mg/mL) for 2 h and then dealt with various concentrations of glycitin (25, 50 and 100 μg/mL) or DEX (100 μg/mL) for 24 h. The untreated cells were used as control group. The cell supernatant and cells were harvested for next study.

2.10. Cell viability assay

The effect of glycitin on RAW264.7 cells viability was evaluated with Cell Counting Kit-8 (CCK-8) according to the instructions of manufacturer. RAW264.7 cells were grown in 96 well plates. Then the cells were dealt with different concentrations of glycitin (25, 50, 100 μg/mL). After 24 h of incubation, 10 μL of CCK-8 was added into each well for 4 h at 37 °C. Subsequently, the OD value was measured at 450 nm with a microplate reader (Bio-Rad Instruments, Hercules, CA, USA).

2.11. HEK293 cells transfection

HEK293 cells were cultured in DMEM/F12 (1:1) medium with 5% CO_2 at 37 °C. The cells were co-transfected with pEGFP-N1-mTLR4 and pDsRED-N1-mMD2 plasmids (Shanghai R&S Biotechnology Co., Ltd., Shanghai, China) by using the FuGENE HD transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. HEK293 cells co-transfected with pEGFP-N1 and pDsRED-N1 plasmids were considered as control. Then cells were grown in the condition of present or absent LPS for 2 h, various concentration of glycitin (25, 50 and 100 μg/mL) was added to co-culture for 24 h. The protein samples of cells were harvested to perform western blotting. IL-8 was measured by ELISA kit.

2.12. Quantitative real-time PCR analysis

Total RNA of lung tissues and RAW264.7 cells were extracted with the TRIzol reagent according to the manufacturer's instructions (Invitrogen, China). The concentration and purity of the extracted RNA was measured with Q5000 (Quawell Technology, USA). Then cDNA was obtained by reverse transcriptase reagent according to the instruction manual. The levels of TLR4, IL-1β, IL-6, and TNF-α mRNA were detected by quantitative real-time polymerase chain reaction (RT-qPCR) with the Light Cycler 96 instrument (Roche, Basel, Switzerland). The primers used in qPCR are listed in the Table 2.The qPCR was performed using the SYBR Green Plus Reagent Kit following the instructions of the manufacturer. The mRNA relative expression levels were normalized with GAPDH through the $2^{-\Delta\Delta Ct}$ comparative method.

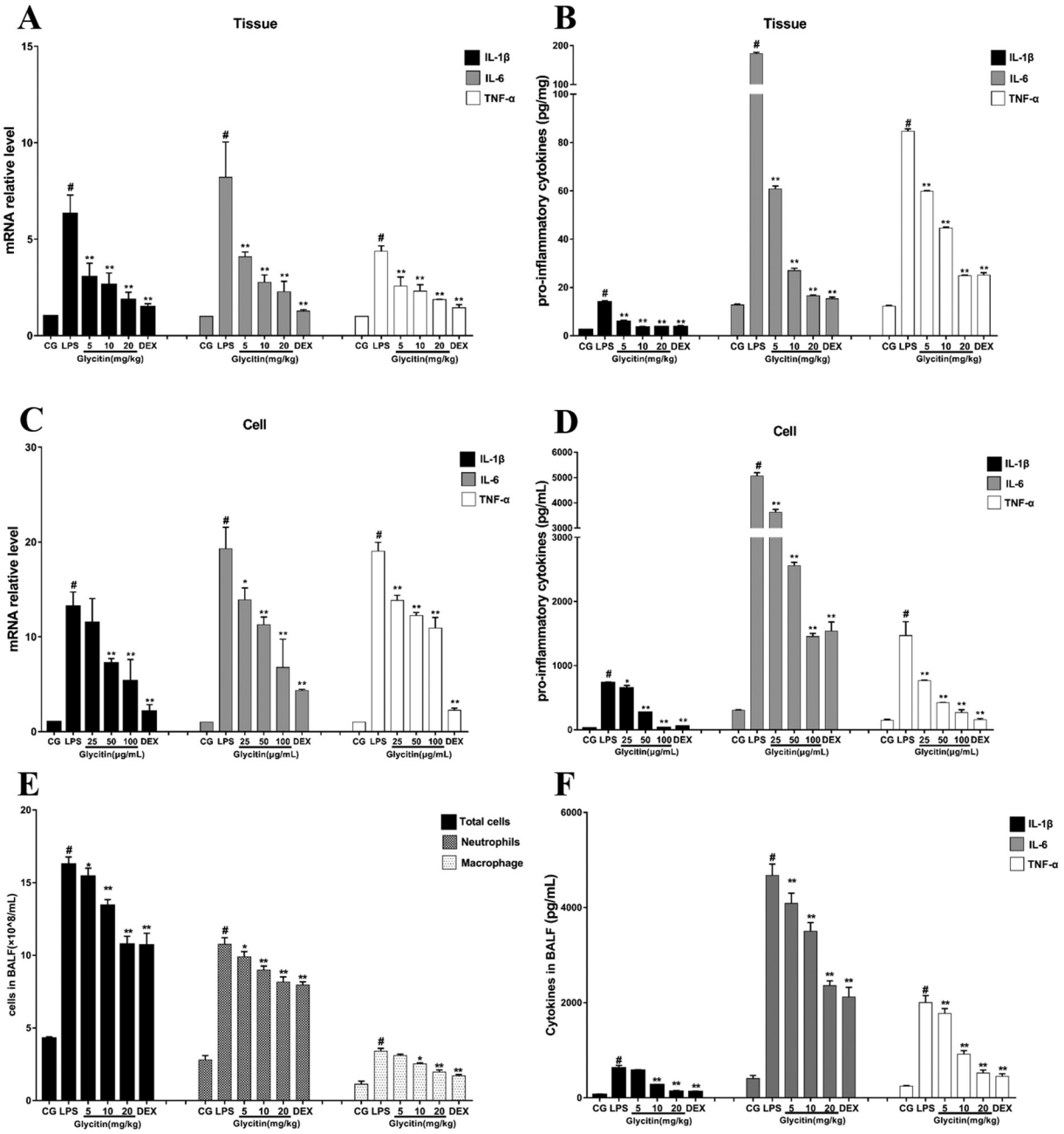

Fig. 4. Effects of glycitin on cytokines in lung tissues and RAW264.7 cells. The cells were stimulated with LPS (1 μg/mL) for 2 h and then treated with different concentrations of glycitin (25, 50, and 100 μg/mL) for 24 h. (A C) The expression of IL-1β, IL-6, TNF-α mRNA in vivo and in vitro was measured by RT-qPCR (n = 6). GAPDH was used as an endogenous control. (B D) The pro-inflammation cytokines IL-1β, IL-6, TNF-α were detected by ELISA (n = 3). (E) The number of inflammatory cells, such as neutrophils and macrophages, in BALF were counted (n = 3). (F) The levels of IL-1β, IL-6, TNF-α in BALF were measured by ELISA (n = 3). CG is the control group. LPS is the LPS-stimulated group. DEX is the dexamethasone group. Data represent the mean ± S.E.M. of three independent experiments. #p < 0.01 vs. the control group. *p < 0.05 vs. the LPS group; **p < 0.05 vs. the LPS group.

2.13. ELISA analysis

The effect of glycitin on cytokines expression after LPS stimulation was determined in lung tissue and RAW264.7 cells. The tissue homogenate and cell supernatants were harvested to detect the concentration of IL-1β, IL-6, and TNF-α using ELISA kits following the manufacturer's instructions.

2.14. Western blotting analysis

The total protein of the lung tissues and cells was isolated by RIPA reagent (Biosharp, China). The concentration of protein was determined by using the BCA Protein Assay Kit (Thermo Scientific, MA). Then equivalent amounts of protein (50 μg) was separated by 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking in 5% BSA blocking buffer for 2 h, the membranes were incubated into primary antibodies (1:1000) at 4 °C overnight and subsequently incubated with the secondary antibodies (1,2000) for 2 h at room temperature. The immunoblot signals on the PVDF membranes were displayed with Enhanced Chemiluminescence Detection System.

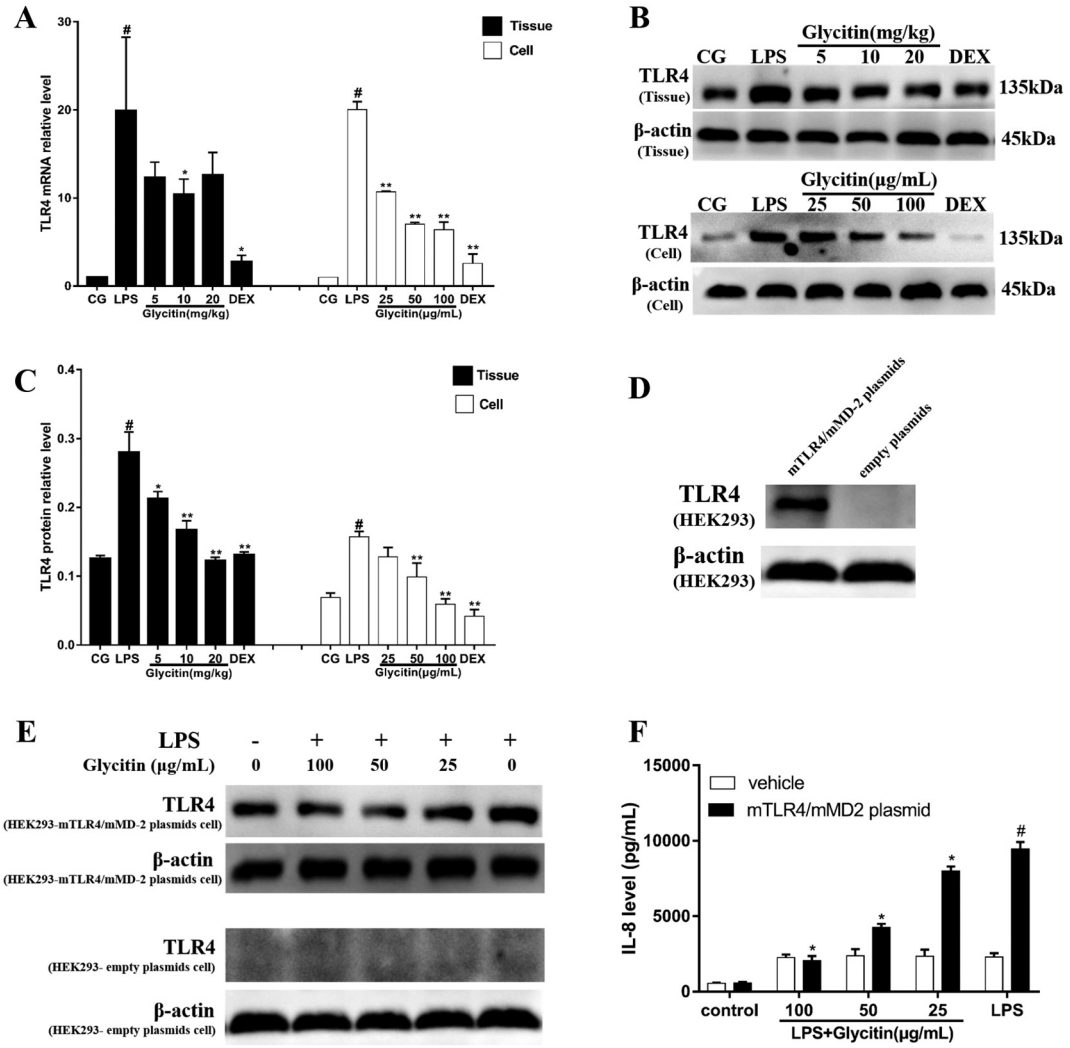

Fig. 5. Effects of glycitin on the expression level of TLR4 protein. (A) The expression of TLR4 mRNA was measured by RT-qPCR in lung tissues and in RAW264.7 (n = 6). GAPDH was used as the control. (B) The level of TLR4 protein was determined by western blotting in lung tissues and cells. β-Actin was used as a control. (C)TLR4 protein level in vivo and in vitro was calculated by IPP 6.0 software (n = 3). (D) HEK293 cells were co-transfected with mTLR4/mMD-2 plasmids compared to empty plasmids-transfected cells. Then HEK293-mTLR4/mMD-2 cells were cultured in the condition of present or absent LPS for 2 h, treatment with various concentration of glycitin (25, 50 and 100 μg/mL) for 24 h. (E&F) The level of TLR4 and IL-8 in HEK293-mTLR4/mMD-2 cells or HEK293-empty plasmids cells. CG is the control group. LPS is the LPS stimulated group. DEX is the dexamethasone group. Data represent the mean ± S.E.M. of three independent experiments. #p < 0.01 vs. the control group. *p < 0.05 vs. the LPS group; **p < 0.01 vs. the LPS group.

2.15. Statistical analysis

The Graphpad Prism 7.00 software was used for the statistical analyses. Statistical data were presented as the mean ± S.E.M. of three individual experiments. Differences between groups were analyzed by one-way ANOVA or Student's *t*-tests. # p < 0.01 vs. the control group, * p < 0.05 vs. the LPS group, ** p < 0.01 vs. the LPS group.

3. Results

3.1. Glycitin contains no endotoxin

HPLC showed that the test drug glycitin only had a single peak (Fig. 1B), proved that glycitin was very pure and contained no impurities. The TAL test results displayed that endotoxin had not been detected in different concentrations glycitin (Table 1).

3.2. Effects of glycitin LPS-induced lung injury in mice

Lung wet/dry weight ratio, MPO activity assay, and histopathological analysis were used to evaluate lung injury. The dark red congestion was obviously visible on surface of lung in LPS-stimulated mice, and the degree of congestion in the glycitin group was reduced with increasing dose (Fig. 2A). Lung wet/dry weight ratio remarkably increased in the mice challenged with LPS, but it evidently reduced after glycitin treatment (Fig. 2B). MPO assay was employed to indirectly analyze the degree of inflammation on LPS-induced lung injury. As shown in Fig. 2C, glycitin could significantly decrease MPO activity, although conversely MPO activity dramatically increased after LPS stimulation. In addition, Pathological sections were also used to examine changes in lung tissue structure. There were no visible histopathological lesions in the control group (Fig. 2D), whereas pathological changes such as infiltration of massive inflammatory cells and severe alveolar hyperemia were clearly observed in the LPS group (Fig. 2E). Conversely, the infiltration of inflammatory cells and the extent of alveolar congestion significantly reduced with increasing doses of glycitin compared with the LPS group (Fig. 2F–H).

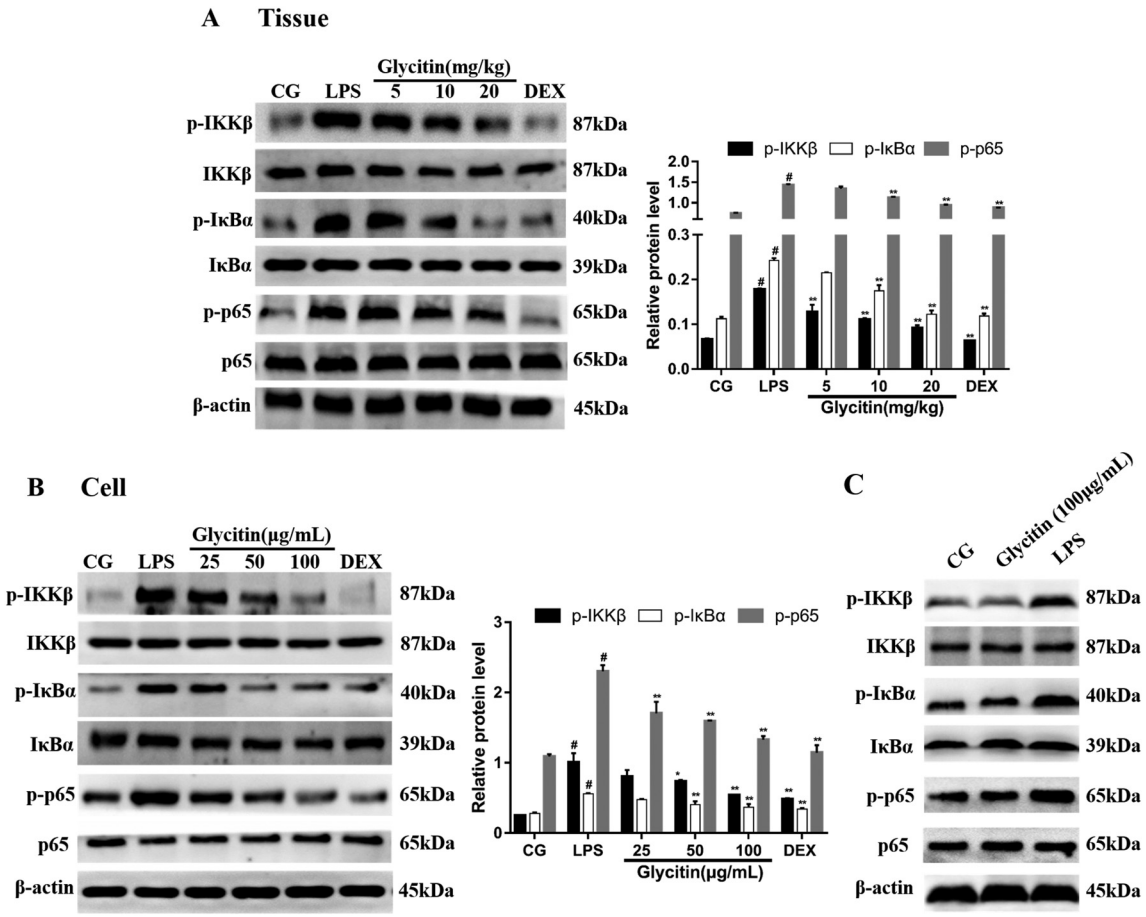

Fig. 6. Effects of glycitin on the NF-κB pathway activation. (A) The protein levels of IKKβ, IκBα and p65 were detected by western blot in lung tissues. (B) The expression levels of IKKβ, IκBα and p65 proteins were analyzed using specific antibodies in RAW264.7 cells. (C) Effects of glycitin alone treatment on NF-κB. β-Actin was used as the control. Gray values of proteins were calculated using IPP 6.0 software (n = 3). CG is the control group. LPS is the LPS-stimulated group. DEX is the dexamethasone group. The data represent the mean ± S.E.M. of three independent experiments. #p < 0.01 vs. the control group. *p < 0.05 vs. the LPS group; **p < 0.01 vs.the LPS group.

3.3. Effects of glycitin on RAW264.7 cells

The possible cytotoxicity of glycitin was assessed with the CCK-8 kit on RAW264.7 cells. The results indicated that glycitin at the concentrations used (25, 50, and 100 μg/mL) had not affected on RAW246.7 cells (Fig. 3A). There was no significant difference in cytokines level between glycitin alone group and the CG group (Fig. 3B&C).

3.4. Effects of glycitin on inflammatory cytokines and inflammatory cells

The pro-inflammatory cytokines levels of lung tissues and RAW264.7 cells were detected by qPCR and ELISA. The qPCR assay in vivo and in vitro found that the levels of IL-1β, IL-6, and TNF-α mRNA in the LPS group greatly increased relative to the control group. However, these increases in the LPS group were dose dependently reduced in the glycitin group (Fig. 4A, C). The results of ELISA are consistent with qPCR results (Fig. 4B, D). Moreover, the trend of inflammatory cytokines in BALF was the same as that of lung tissues (Fig. 4E). The number of neutrophils and macrophages in BALF significantly increased after LPS challenging compared with the control group. Meanwhile, treatment with glycitin (5, 10, and 20 mg/kg) and DEX (5 mg/kg) obviously reduced the number of total cells, neutrophils, and macrophages.

3.5. Effects of glycitin on the expression of TLR4 protein

The expression level of TLR4 mRNA and its protein were determined in vivo and in vitro. We found that the expression of TLR4 mRNA level was greatly increased in the LPS group (Fig. 5A). On the other hand, glycitin significantly suppressed the increase of TLR4 protein relative to the LPS group (Fig. 5B&C). In order to demonstrate that glycitin suppresses LPS-induced inflammatory responses by targeting TLR4, HEK293 cells co-transfected with mTLR4 and mMD2 plasmids showed that the transfection was successful and TLR4 was over-expressed efficiently compared to vehicle-transfected cells (Fig. 5D). We also examined the level of TLR4 in LPS-challenged HEK293-mTLR4/mMD-2 cells after glycitin treatment. The results of western blotting displayed that glycitin dose-dependently decreased the expression of TLR4 in HEK293-mTLR4/mMD-2 cells (Fig. 5E).

3.6. Effects of glycitin on the NF-κB pathway

NF-κB is a nuclear transcription factor that initiates the transcription of inflammatory genes. TLR4 activates NF-κB through a series of signaling [26]. We performed western blotting to investigate the effect of glycitin on the key proteins associated with NF-κB pathway. As shown in Fig. 6A, the levels of p-IKKβ, p-IκBα, and p-p65 proteins in lung tissues significantly increased in the LPS group compared to the control group. In contrast, their levels were greatly reduced in the glycitin group, and inhibition of these proteins phosphorylation was enhanced

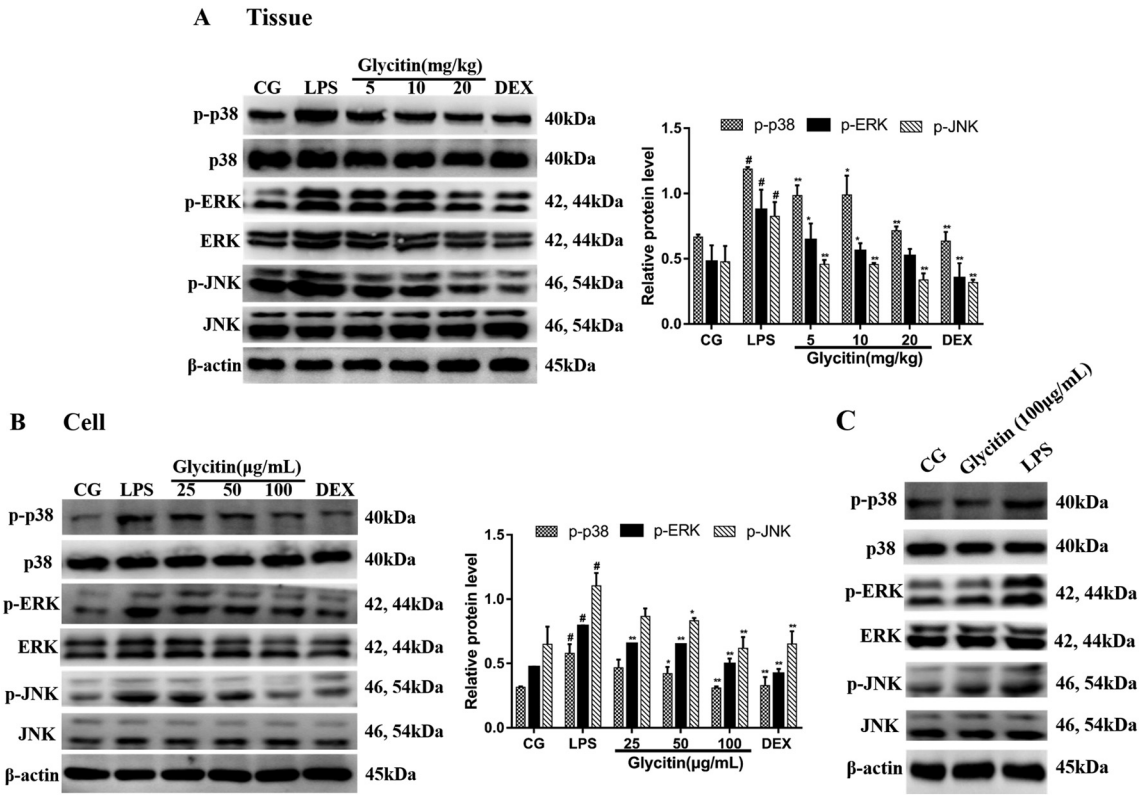

Fig. 7. Effects of glycitin on the MAPKs pathway activation. (A) The expression levels of p38, ERK, and JNK proteins in lung tissues were determined by western blotting. (B) The protein levels of p38, ERK, and JNK in RAW264.7 cells. (C) Effects of glycitin alone treatment on MAPK. β-Actin was used as the control. Gray values of proteins were counted by IPP 6.0 software (n = 3). CG is the control group. LPS is the LPS-stimulated group. DEX is the dexamethasone group. The data represent the mean ± S.E.M. of three independent experiments. #p < 0.01 vs. the control group. *p < 0.05 vs. the LPS group; **p < 0.01 vs. the LPS group.

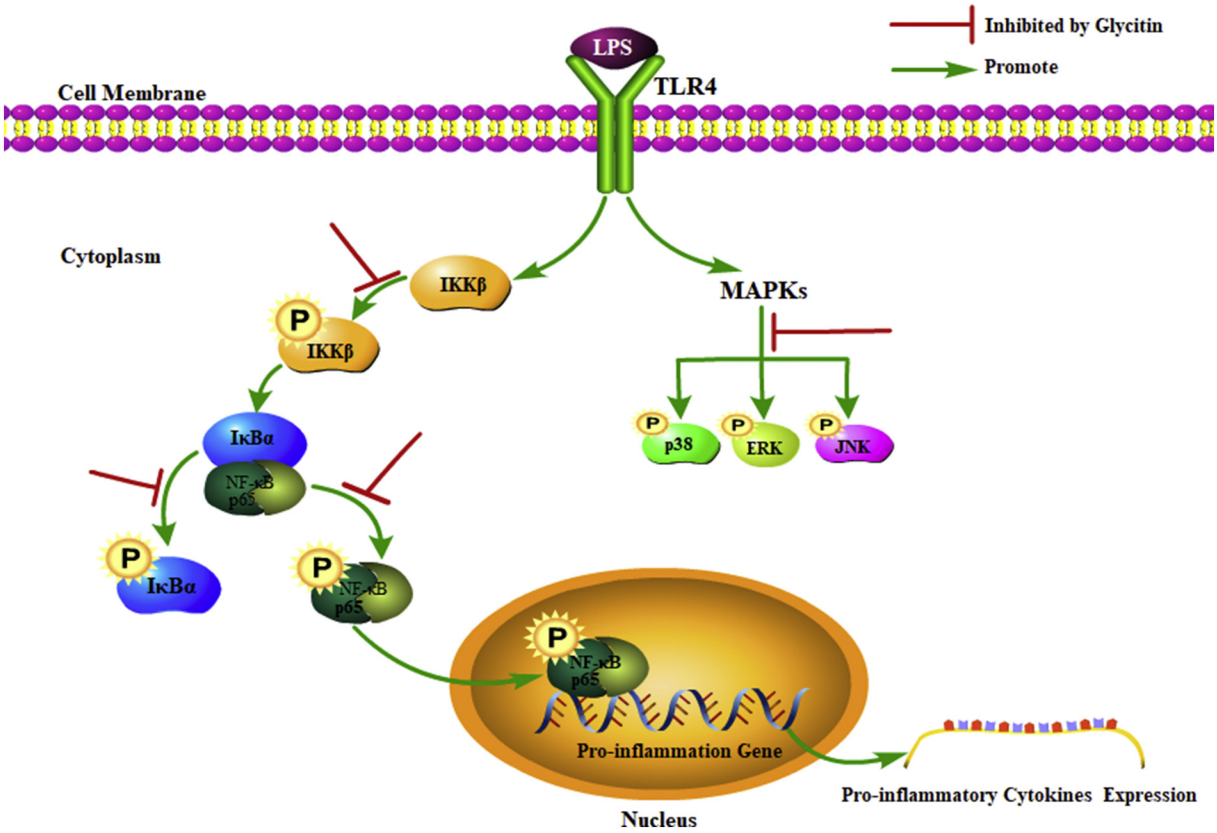

Fig. 8. NF-κB and MAPKs pathways in LPS-stimulated ALI.

with increasing doses of glycitin (Fig. 6A). Moreover, the levels of phosphorylated IKKβ, IκBα, and p65 proteins in RAW264.7 cells were the same as those in lung tissues (Fig. 6B). And glycitin alone treatment had no significant difference compared with CG group on the expression of p-IKKβ, p-IκBα, and p-p65 (Fig. 6C).

3.7. Effects of glycitin on the MAPKs pathway

MAPKs is also of great importance pathway triggered by TLR4 [27]. To investigate the anti-inflammatory effect of glycitin on the MAPKs pathway, the p38, ERK and JNK proteins were detected by western blotting. The results illustrated that the activation of MAPKs proteins expression was evidently rising in mice stimulated with LPS. But the levels of p-p38, p-ERK, and p-JNK proteins in the glycitin groups were greatly decreased in a dose-dependent manner (Fig. 7A). Meanwhile, the levels of phosphorylated MAPKs proteins on RAW264.7 cells are consistent with in vivo (Fig. 7B). As shown in Fig. 7C, glycitin alone treatment group was consistent with the results of the control group on the level of p-p38, p-ERK and p-JNK.

4. Discussion

ALI is one of multiple serious inflammatory diseases that are difficult to cure or have a poor prognosis [3,28]. Although inflammation is usually known as protective effects to abolish non-self-invasion and autologous necrosis, uncontrolled inflammation always leads to tissue or organ injury and even systemic dysfunction [29,30]. Thus, anti-inflammation is essential strategy in the therapy of ALI [31]. In our study, we explored the anti-inflammatory effects of glycitin on LPS-induced ALI and potential molecular mechanism for the first time.

As is known to all, pathological features of ALI are alveolar edema, inflammatory cell infiltration, and the damage of lung structure [32,33]. In the present study, the wet/dry weight ratio was measured to indirectly assess pulmonary edema [22]. The wet/dry weight ratio results showed that lung water content was sharply increased after LPS stimulation, but it significantly reduced by glycitin administration, indicating that glycitin could effectively ameliorate pulmonary edema. Furthermore, MPO serves as a biomarker of neutrophil to indirectly reflect the number of neutrophils in inflammatory tissues [34,35]. MPO activity lessening in LPS-induced ALI after glycitin treatment suggested that glycitin could impede the migration of neutrophils into lung tissue. In addition, H&E stain result confirmed that glycitin could prevent the alveolar structural damage from LPS-induced inflammation. All the above results demonstrated that ALI animal model established successfully, and glycitin had a therapeutic effect on ALI.

Many pro-inflammatory cytokines play a central role in the initiation and propagation of the inflammation [9]. IL-1β and TNF-α were generated by various types of cells, and they could lead to the inflammatory cells recruitment and other pro-inflammatory mediators secretion [7]. IL-6 is also a key pro-inflammatory cytokine and strongly promotes the acute inflammatory injury [8]. In this study, we found that glycitin significantly down-regulated the levels of IL-1β, IL-6, and TNF-α after LPS stimulation in vivo and in vitro, the finding in accord with the MPO results demonstrated that glycitin suppresses LPS-induced inflammation through reducing the production of pro-inflammatory mediators.

Previous research has found that TLR4 activated by LPS contributes to inflammatory responses through TLR4-mediated downstream signaling transmission, primarily including the NF-κB and MAPKs pathways [10,11,36]. To further explore the potential anti-inflammatory mechanisms of glycitin against LPS-induced ALI, we detected the associated proteins of TLR4-mediated NF-κB and MAPKs pathways. We found that the expression of TLR4 protein significantly up-regulated after LPS stimulation, and glycitin suppressed the expression of TLR4 after LPS stimulation. The member of NF-κB family, consist of p50, p52, p65, Rel B, and c-Rel, can modulate the transcription of pro-inflammatory genes whose products of translation level play a critical role in the regulation of inflammatory response [37,38]. The NF-κB proteins are normally sequestered in the cytoplasm by the inhibitory protein IκBα [39]. Upon a series of activation after LPS stimulation, activated IKK triggers IκBα activation and degradation, and then release p65 [40,41]. Subsequently, phosphorylated p65 could rapidly translocate into nucleus, and bind to target genes to promote downstream gene transcription [42]. Phosphorylated IKKβ, IκBα and p65 protein levels were inhibited by glycitin in dose-dependent manner, which could greatly reduce the opportunity of p-p65 coming into nucleus. Moreover, it has also been reported that the p38, ERK and JNK of MAPKs activate AP-1 which promotes to the production of pro-inflammatory cytokines [39,43]. Activated MAPKs proteins were significantly suppressed by glycitin, which meant AP-1 had less chance to be activated. Our results suggested that glycitin exerts protective effect on ALI by obviously inhibiting the phosphorylation of NF-κB and MAPKs in vivo and in vitro.

In conclusion, our studies indicate that glycitin has anti-inflammatory effect to reduce the secretion of pro-inflammatory cytokines in LPS-induced ALI probably due to inhibiting the activation of TLR4-mediated NF-κB and MAPKs signaling pathways (Fig. 8). These results manifest that glycitin may be a potential therapeutic agent against ALI.

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