International Immunopharmacology 76 (2019) 105842

Contents lists available at ScienceDirect

International Immunopharmacology

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

Genipin attenuates mitochondrial-dependent apoptosis, endoplasmic reticulum stress, and inflammation *via* the PI3K/AKT pathway in acute lung injury

Xu Luo[a,1], Bo Lin[b,1], Youguang Gao[b,1], Xianghui Lei[c], Xiang Wang[d], Yunfeng Li[d], Tao Li[d,*]

[a] Department of Critical Care Medicine, The People's Hospital of Longhua, Shenzhen 518109, China
[b] Department of Anesthesiology, The First Affiliated Hospital of Fujian Medical University/The First School of Clinical Medicine, Fujian Medical University, Fuzhou 350005, China
[c] Department of Pathology, The First People's Hospital of Chenzhou/Affiliated Chenzhou Hospital, Southern Mdical University of China, Chenzhou 423000, China
[d] Department of Critical Care Medicine, The First People's Hospital of Chenzhou/Affiliated Chenzhou Hospital, Southern Mdical University of China, Chenzhou 423000, China

ARTICLE INFO

Keywords:
Genipin
Acute lung injury
Apoptosis
Endoplasmic reticulum stress
Inflammation

ABSTRACT

The protective effects of genipin against lipopolysaccharide (LPS)-induced acute lung injury (ALI) have been reported; however, the mechanism is unclear. Genipin performs its pharmacological effects *via* activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway. In the present study, we aimed to determine whether the PI3K/AKT pathway is involved in the protective effects of genipin against mitochondrial-dependent apoptosis, endoplasmic reticulum stress (ERS), and inflammation in ALI. We constructed *in vivo* and *in vitro* models of LPS-induced ALI. PI3K/AKT signaling was inhibited using LY294002. Pretreatment with genipin increased AKT phosphorylation, indicating that PI3K/AKT signaling was upregulated. Genipin pretreatment prevented LPS-induced histopathological deterioration, increased pulmonary edema, and decreased oxygenation index, all of which were inhibited using LY294002. In addition, genipin pretreatment attenuated LPS-mediated mitochondrial apoptosis, as indicated by improved mitochondrial dysfunction, downregulation of BAX (BCL2 associated X, apoptosis regulator), upregulation of BCL2 (BCL2 apoptosis regulator), inhibited the release of cytochrome *c*, activation of caspase-3, and cell apoptosis. Genipin pretreatment inhibited the LPS-induced upregulation of AF4/FMR2 family member 4 (CHOP), glucose-regulated protein, 78 kDa (GRP78), and X-box binding protein 1 (XBP1) levels, indicating ERS suppression. Moreover, genipin pretreatment alleviated LPS-induced inflammation, indicating by blockade of nuclear factor kappa b (NF-κB) signaling activation and reduced tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 levels in the lung and bronchoalveolar lavage fluid. LY294002 could inhibit these genipin-induced protective effects against apoptosis, ERS, and inflammation. Thus, genipin significantly activates PI3K/AKT signaling to ameliorate mitochondria-dependent apoptosis, ERS, and inflammation in LPS-induced ALI.

1. Introduction

Clinical acute lung injury (ALI), a common complication that occurs following sepsis, causes high mortality and morbidity, resulting in a significant burden on society [1]. Currently, it is thought that the multiple organ dysfunctions associated with sepsis can be attributed to a pathochemical and pathophysiological injury cascade, including the inflammatory response, mitochondria dysfunction, endoplasmic reticulum stress (ERS), and apoptosis [2–4].

A recent study demonstrated that genipin pretreatment ameliorates lipopolysaccharide (LPS)-induced ALI by inhibiting nuclear factor kappa B (NF-κB) and NLR family pyrin domain containing 3 (NLRP3) signaling pathways [5]. However, the mechanism of genipin's effects remains unclear. In addition, the role of mitochondrial-dependent apoptosis and ERS in the protection mediated by genipin in ALI has not been described.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway can promote cell survival and growth in several

* Corresponding author at: Department of Critical Care Medicine, The First People's Hospital of Chenzhou/Affiliated Chenzhou Hospital, Southern Mdical University of China, No. 102 Luojiajing, Chenzhou, Hunan Province 423000, China.
E-mail address: litao.7@163.com (T. Li).
[1] Equal contributors.

https://doi.org/10.1016/j.intimp.2019.105842
Received 7 April 2019; Received in revised form 9 August 2019; Accepted 19 August 2019
Available online 26 August 2019

ways, including regulation of apoptosis and ERS [6–8]. Recent studies suggested that activation of PI3K/AKT signaling targets and regulates the activity of B cell lymphoma/leukemia (BCL)2 family members to directly affect apoptosis, resulting in cell survival [9–11]. Regulation of apoptosis to protect against lung injury is also mediated by activation of PI3K/AKT signaling [12,13]. Furthermore, regulation of ERS by the PI3K/AKT signaling pathway has also been demonstrated [7,14].

The results of these previous studies prompted us to hypothesize that upregulation of PI3K/AKT signaling might contribute to the protective effects exhibited by genipin against apoptosis and ERS in LPS-induced ALI.

2. Materials and methods

2.1. Reagents and antibodies

Molecular Probes (Invitrogen, Waltham, MA, USA) supplied 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (MitoProbe™ JC-1). Promega Corp. (Madison, WI, USA) supplied the CellTiter-Glo® assay kit. BestBio Co. (Beijing, China) provided the mitochondrial/cytosolic protein extraction kit. Antibodies recognizing BAX (BCL2 associated X, apoptosis regulator), BCL2 (BCL2 apoptosis regulator), cytochrome *c*, AKT, phosphorylated (p)-AKT, AF4/FMR2 family member 4 (CHOP), glucose-regulated protein, 78 kDa (GRP78), X-box binding protein 1 (XBP1), and β-actin were obtained from Abclonal (Wuhan, China). Promega Corp. supplied the terminal deoxynulceotidyl transferase nick-end-labeling (TUNEL) staining kit. EnVision™ (Dako, Copenhagen, Denmark) provided the immunohistochemical kits. The human lung alveolar epithelial cell line A549 was obtained from Guangzhou Cellcook Biotech Co., Ltd. (Guangzhou, China). Sigma-Aldrich (Saint Louis, MO, USA) provided genipin and other chemicals.

2.2. Cell culture and stimulation

Low glucose Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich; Merck Millipore), with penicillin (100 U/mL, Sigma-Aldrich; Merck Millipore), streptomycin (100 units, Sigma-Aldrich; Merck Millipore), and 10% bovine serum (Sigma-Aldrich; Merck Millipore) was used to culture the A549 cells at 37 °C and 5% CO_2. To establish LPS-induced ALI *in vitro*, LPS at 5 μg/mL was used to stimulate the A549 cells for 6 h.

The cells were randomly divided into five groups using a random number table: The control group (cells were pretreated with the vehicle for 60 min and incubated under normoxic conditions); the genipin control group (cells pretreated with 50 μM genipin for 60 min and incubated under normoxic conditions); the LPS group (cells pretreated for 60 min with the vehicle and then stimulated by LPS); the genipin treatment group (cells pretreated for 60 min with 50 μM genipin and then stimulated by LPS); and the LY294002 group (cells pretreated with for 60 min with 50 μM genipin and 20 μM LY294002 and then stimulated by LPS). The vehicle control comprised dimethyl sulfoxide (DMSO). The dose of genipin pretreatment was based on our published article [15].

2.3. Animals

The Medical Faculty Ethics Committee of the First People's Hospital of Chenzhou, Chenzhou, China, approved all the animal procedures and care. The animal experiments complied with the Guidelines for the Care and Use of Laboratory Animals from the NIH. The Experimental Animal Centre of South Medical University provided the Sprague-Dawley rats (male, weight: 180–220 g), which were housed under temperature- and humidity-controlled conditions on a 12/12 h day/night cycle with unrestricted access to a standard diet and tap water.

2.4. Animal pretreatment

A rat model of ALI was induced by intratracheal administration of LPS. Animals were anesthetized under sodium pentobarbital (30 mg/kg body weight) intramuscularly, and then placed in a supine position. A cervical middle line incision in the skin was used to expose the trachea surgically, after which LPS at 5 mg/kg body weight was injected slowly into each rat's trachea. After treatment, at 12 h after LPS administration, all the rats were sacrificed.

The animals were randomly divided into five groups (n = 6 per group) using a random number table: 1) The control group (rats received pretreatment with vehicle for 60 min and were given 0.5 mL intratracheal normal saline (NS); 2) the genipin control group (rats received pretreatment with genipin (5 mg/kg) for 60 min and then 0.5 mL intratracheal NS; 3) the LPS group (rats received pretreatment with vehicle for 60 min, followed by LPS instillation); 4) the genipin group (rats were pretreated with genipin (5 mg/kg) for 60 min, followed by LPS instillation); and 5) the LY294002 group (rats were pretreated with genipin (5 mg/kg) and LY294002 (0.3 mg/kg) for 60 min, followed by LPS instillation). The vehicle control comprised dimethyl sulfoxide (DMSO). The dose of genipin pretreatment was based on a previous study [15].

2.5. Nuclear extraction

According to a previous study [16], cells were resuspended and swollen in buffer A (0.15% NP-400, 1 mM EDTA,1 0 mM HEPES, 0.1 mM EGTA, 1 mM DTT, 10 mM KCl, and proteinase inhibitor cocktail (1:100 dilution)) for 10 min on ice, then centrifuged at 2000 ×*g* for 10 min at 4 °C. The pellets was washed and resuspended in buffer B (0.5% NP-40, 1 mM EGTA, 20 mM HEPES, 1 mM DTT, 1 mM EDTA, 400 mM NaCl, and proteinase inhibitor cocktail (1:100 dilution)) for 25 min at 4 °C, and then centrifuged at 12,000 ×*g* for 10 min. The supernatant was collected and used as the nuclear extract.

2.6. Western blotting analysis

Isolated lung tissues were homogenized, and the protein levels were assessed using western blotting. An isolation kit was used to obtain cytosolic and mitochondrial proteins, in accordance with the manufacturer's instructions. Proteins were resolved on 10% SDS-PAGE, electroblotted onto a polyvinylidene fluoride (PVDF) membrane, and blocked in 5% skimmed milk. The PVDF membranes were incubated at 4 °C overnight with primary antibodies recognizing BAX, BCL2, cytochrome *c*, AKT, p-AKT, CHOP, GRP-78, XBP1, NF-κB P65, p-P65 and β-actin, separately. Horseradish peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody. The immunoreactive protein bands on the membranes were visualized using an enhanced chemiluminescence reagent (Absin Biotechnology Co., Ltd., Shanghai, China).

2.7. Mitochondrial membrane potential (MMP) determination

The potential-sensitive fluorescent dye JC-1 was used to assess the MMP. Cells treated with different reagents were subjected to LPS. The cells were then incubated with JC-1 (5 μmol/L) for 15 min at 37 °C. An inverted fluorescent microscope (Nikon, Ti-E Live Cell Imaging System, Tokyo, Japan) was used to observe the results.

2.8. Histopathological and immunohistochemical analyses

Twelve hours after LPS administration, the right lobes of the lungs were immersed in 10% neutral buffered formalin, fixed, embedded in paraffin, and sectioned. The sections were stained using hematoxylin and eosin (H&E), followed by observation and evaluation of the pathological changes in the lung tissues using the following criteria: 1) Neutrophil infiltration extent; 2) damage to airway epithelial cells; 3)

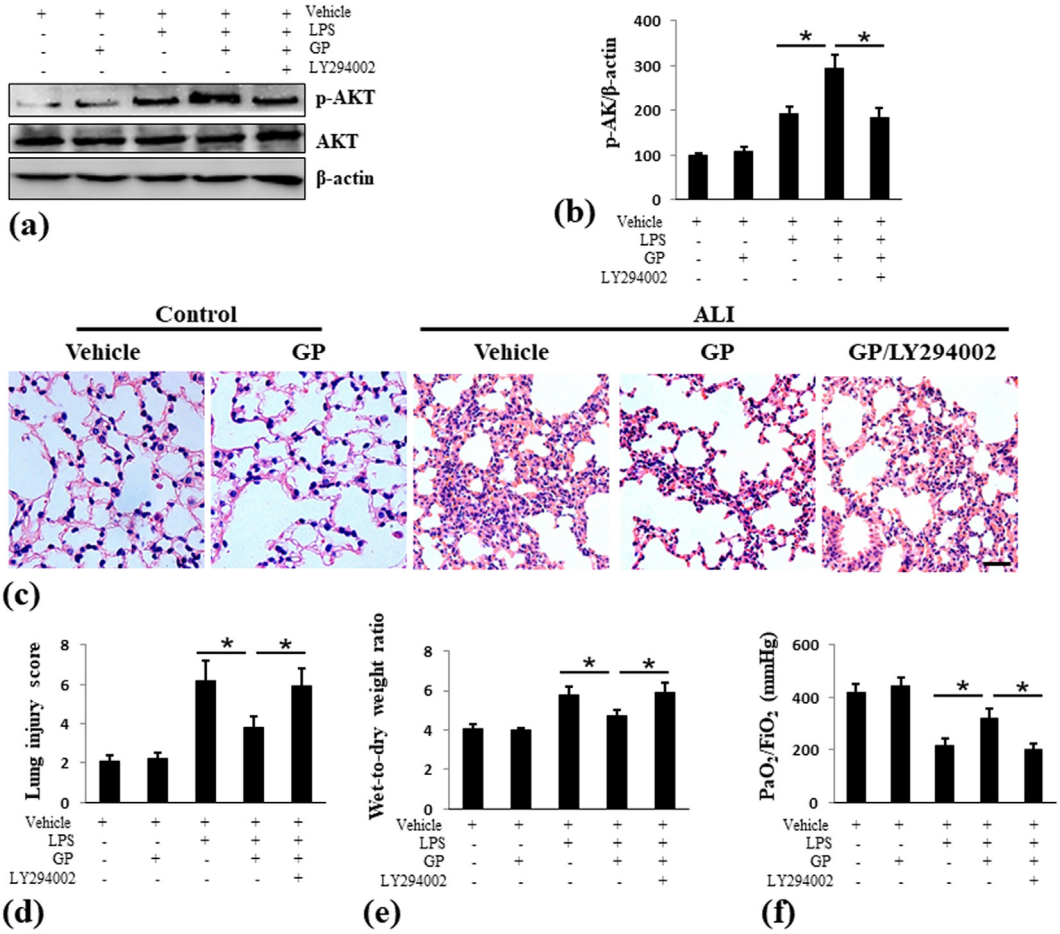

Fig. 1. Genipin pretreatment upregulates PI3K/AKT signaling and attenuates LPS-induced lung injury *in vivo*.
Rats were pretreated with genipin (5 mg/kg) or vehicle for 60 min, followed by LPS at 5 mg/kg delivered intratracheally. Twelve hours later, the rats were sacrificed. To investigate the association of the protective effects of genipin with the PI3K/AKT signaling pathway, LY294002 (0.3 mg/kg) was given simultaneously with genipin. (a) Western blotting was used to assess the levels of phosphorylated (p)-AKT, and AKT. (b) Densitometric quantification of the level of p-AKT. (c) H&E staining for histological determination (scale bar: 500 μm); (d) lung injury score. (e) Weight ratio of the wet lung:dry lung. (f) Blood gas assay for the oxygenation index (PaO_2/FiO_2). Data are shown as the mean ± SD (n = 6 per group). *$P < 0.05$ *vs.* the indicated groups. PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, protein kinase B; LPS, lipopolysaccharide; H&E, hematoxylin and eosin; SD, standard deviation.

the extent of interstitial edema; 4) the formation of hyaline membranes; and 5) the occurrence of hemorrhage. Each section was scored based on five criteria that were determined by the degree of deterioration: normal = 0; minimal alteration = 1; mild alteration = 2; moderate alteration = 3; and severe alteration = 4. We recorded the lung injury score for each criterion.

Immunohistochemical staining of the paraffin-embedded sections was carried out using anti-CHOP (1:200 dilution), anti-GRP-78 (1:200 dilution), and anti-XBP1 (1:200 dilution) antibodies overnight at 4 °C. The bound antibodies were detected using an avidin-biotin-peroxidase complex kit and counterstained with hematoxylin.

2.9. Ratio of wet to dry lung (lung wet/dry (W/D)

After LPS administration, we measured the water content of the lungs. The right lungs were excised, blotted, and weighed to determine the wet weight. The lungs were then desiccated at 80 °C for 48 h and weighed to provide the dry weight. We then calculated the wet/dry ratio to assess tissue edema.

2.10. Oxygenation index (PaO_2/FiO_2) analysis

Twenty-four hours after the administration of LPS, the rats were anesthetized and intubated endotracheally using a 20-gauge catheter, with mechanical ventilation using pure oxygen at 7 mL/kg (100 breaths/min) for 20 min. Arterial blood samples from the carotid artery were obtained and a commercial blood gas analyzer (model ABL8000; Radiometer Copenhagen, Westlake, Ohio) was used to analyze the blood samples. The ratio of arterial oxygen partial pressure/fractional inspired oxygen (PaO_2/FiO_2) was used to determine the oxygenation index.

2.11. Assessments of cell apoptosis

In vivo and *in vitro* apoptosis was measured using TUNEL staining. Hoechst staining was used to indicate the cell nucleus. Cells showing green fluorescence were apoptotic cells, which were counted in 10 visual fields at 200× magnification. The apoptotic index *in vivo* was defined as the average number of TUNEL-positive cells in each section. The apoptotic index *in vitro* was defined as the proportion of TUNEL-positive cells in 10 random visual fields.

2.12. Inflammatory mediators determination

In the lung and bronchoalveolar lavage fluid (BALF), the concentrations of IL-6, IL-1β, and TNF-α were measured using a commercial enzyme linked immunosorbent assay kit. The results were expressed as pg/mL of BALF or μg/mg of tissue.

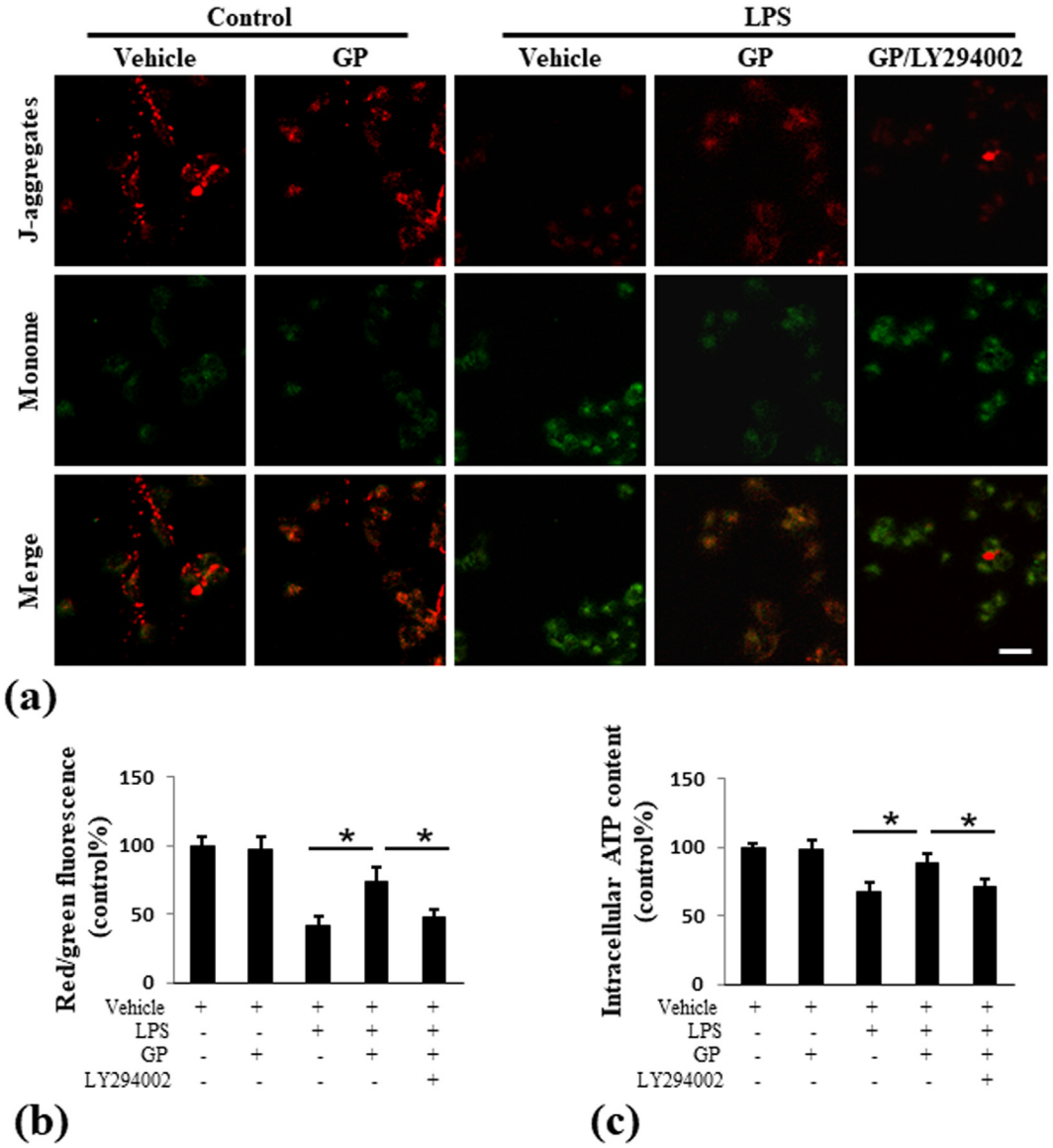

Fig. 2. Genipin attenuates LPS-induced mitochondrial dysfunction *via* the PI3K/AKT pathway *in vitro*.
A549 cells pretreated with genipin (50 μM) or vehicle for 60 min were incubated with LPS (5 μg/ml) for 6 h. LY294002 (20 μM) was administered simultaneously with genipin to test the association of the protective effects of genipin with the PI3K/AKT signaling pathway. (a) Laser confocal-scanning microscopy analysis of cells stained with MMP and JC-1 (scale bar: 50 μm). (b) Intracellular red and green JC-1 fluorescence quantification. (c) Luciferase-based assay of cellular ATP levels. Data are shown as the mean ± SD (n = 6 per group). *$P < 0.05$ *vs.* the indicated groups. PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, protein kinase B; LPS, lipopolysaccharide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; MMP, mitochondrial membrane potential; ATP, Adenosine triphosphate; SD, standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A tracheal cannula was placed into each mouse under anesthesia and used to wash the lung three times using 4 mL of phosphate-buffered saline (PBS) to collect BALF samples. Briefly, the rats were sacrificed, a median sternotomy was performed, and blunt dissection was used to isolate the trachea. Next, we inserted a suitable small-caliber tube into the airway and secured it. The lungs were slowly infused with PBS, and then the BALF was withdrawn into the tube. Approximately 80% of the fluid was recovered.

2.13. Statistical analysis

The experimental results are presented as the mean ± the standard deviation (SD). The data were analyzed statistically using one-way analysis of variance (ANOVA), followed by a least significant difference (LSD) multiple comparison test or Student's *t*-test where appropriate. In the results, n represents the number of animals and statistical significance was accepted at a P value < 0.05.

3. Results

3.1. Genipin pretreatment upregulates PI3K/AKT signaling and attenuates LPS-induced lung injury

To investigate whether genipin pretreatment activates PI3K/AKT signaling, the level of p-AKT was assessed *in vitro*. Genipin pretreatment upregulated the p-AKT level in LPS-challenged rats compared with those treated with the vehicle, indicating that genipin pretreatment activated the PI3K/AKT signaling pathway (Fig. 1). Treatment with LY294002, a specific inhibitor of PI3K, inhibited the genipin-induced activation of PI3K/AKT signaling.

It is well established that LPS administration causes injury to the lung. Twelve hours after LPS administration, the histopathology, lung

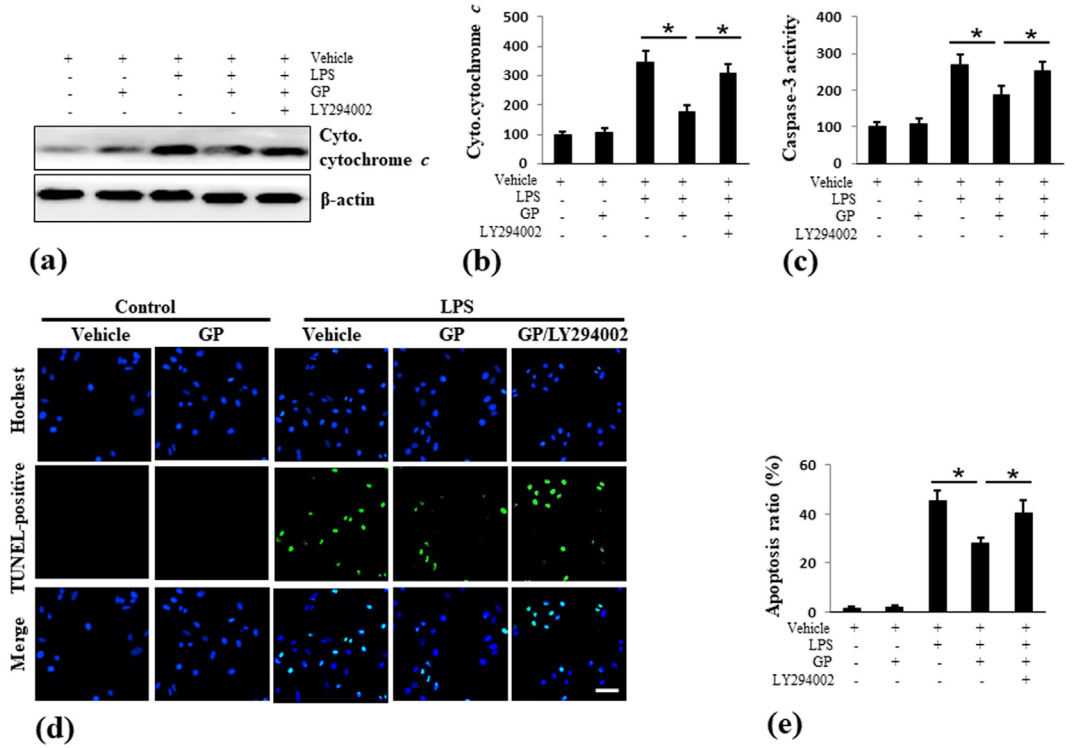

Fig. 3. Genipin attenuates LPS-induced apoptosis *via* the PI3K/AKT pathway *in vitro*.
A549 cells pretreated with genipin (50 μM) or vehicle for 60 min were incubated with LPS (5 μg/ml) for 6 h. LY294002 (20 μM) was administered simultaneously with genipin to test the association of the protective effects of genipin with the PI3K/AKT signaling pathway. (a) Western blotting was used to assess the levels of cytoplasmic cytochrome *c*. (b) Densitometric quantification of the level of cytochrome *c*. (c) The activity of caspase-3. (d) TUNEL staining was used to assess cell apoptosis (scale bar: 100 μm). (e) Quantification of the rate of cell apoptosis. Data are shown as the mean ± SD (n = 6 per group). $^*P < 0.05$ *vs.* the indicated groups. PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, protein kinase B; LPS, lipopolysaccharide; TUNEL, terminal deoxynulceotidyl transferase nick-end-labeling; SD, standard deviation.

edema, and oxygenation index were detected in lung tissues to observe the extent of lung injury. As shown in Fig. 1, ALI-challenged rats displayed severe inflammatory cell infiltration, thickened alveolar walls, diffuse edema, decreased alveolar space, and enhanced interstitial congestion compared with those in the control rats. However, these histological changes were dramatically reduced by pretreatment with genipin. In addition, pretreatment with genipin significantly alleviated LPS-induced lung injury, as evidenced by decreased lung edema and oxygenation index compared with the ALI-challenged rats that did not receive genipin pretreatment (Fig. 1). However, the genipin-induced protection against lung injury was blocked by treatment with LY294002. These results indicated that genipin provides a protective role against LPS-induced acute lung injury in rats by activating the PI3K/AKT pathway.

3.2. Genipin pretreatment inhibits LPS-induced mitochondria-dependent apoptosis via the PI3K/AKT pathway

To investigate whether the PI3K/AKT pathway participate the protective effects of genipin on LPS-induced mitochondria-dependent apoptosis *in vitro*, the MMP was measured using the potential-sensitive fluorescent dye, JC-1, to evaluate mitochondrial dysfunction. The control cells showed red fluorescence (Fig. 2a and b). However, LPS exposure caused the rapid disappearance of red fluorescence and the appearance of green fluorescence, indicating that the MMP was dissipated. Pretreatment with genipin attenuated the changes in MMP significantly, as shown by the repression of green fluorescence and restoration of red fluorescence. However, the increase in green fluorescence and decrease in red fluorescence in the cells of the LY294002 group suggested that LY294002 treatment blocked the protective effects of genipin against MMP dissipation. Mitochondrial dysfunction results in decreased levels of ATP. We found that LPS stimulation decreased the cellular ATP level compared with that in the control group, which was reversed by genipin pretreatment. However, the cellular ATP level declined in the cells of the LY294002 group, indicating that LY294002 treatment blocked the protective effects of genipin against ATP decrease (Fig. 2c). The regulation of mitochondrial apoptosis is mediated by cytochrome *c* release, followed by caspase activation. We found that genipin pretreatment prevented LPS-induced upregulation of cytosolic cytochrome *c*, caspase-3 activation, and cell apoptosis (Fig. 3). However, these protective effects induced by genipin pretreatment were inhibited by LY294002 treatment.

BCL2 family proteins also regulate mitochondrial-dependent apoptotic signaling.. Therefore, to confirm the protective effects of genipin in rats, the levels of BAX, BCL2, and cell apoptosis in the lung were evaluated. As shown in Fig. 4, upregulation of BAX, downregulation of BCL2, and increased numbers of TUNEL-positive cell were observed in the LPS group compared with those seen in the control group, indicating apoptotic signaling activation. Subsequently, we found that the levels of BAX and lung cell apoptosis decreased and the level of BCL2 increased in ALI-challenged rats pretreated with genipin compared with those treated with the vehicle. However, LY294002 treatment blocked the genipin-induced protection against apoptosis activation in the lung.

These results reveal that genipin inhibits mitochondria-dependent apoptosis *via* the PI3K/AKT pathway in LPS-induced ALI.

3.3. Genipin pretreatment inhibits LPS-induced ERS via PI3K/AKT pathway

To investigate the influence of genipin on the LPS-induced ERS, the levels of CHOP, GRP-78, and XBP1 were measured using western blotting and immunohistochemical analysis *in vitro* and *in vivo*. The levels of CHOP, GRP-78 and XBP1 increased in the LPS group compared

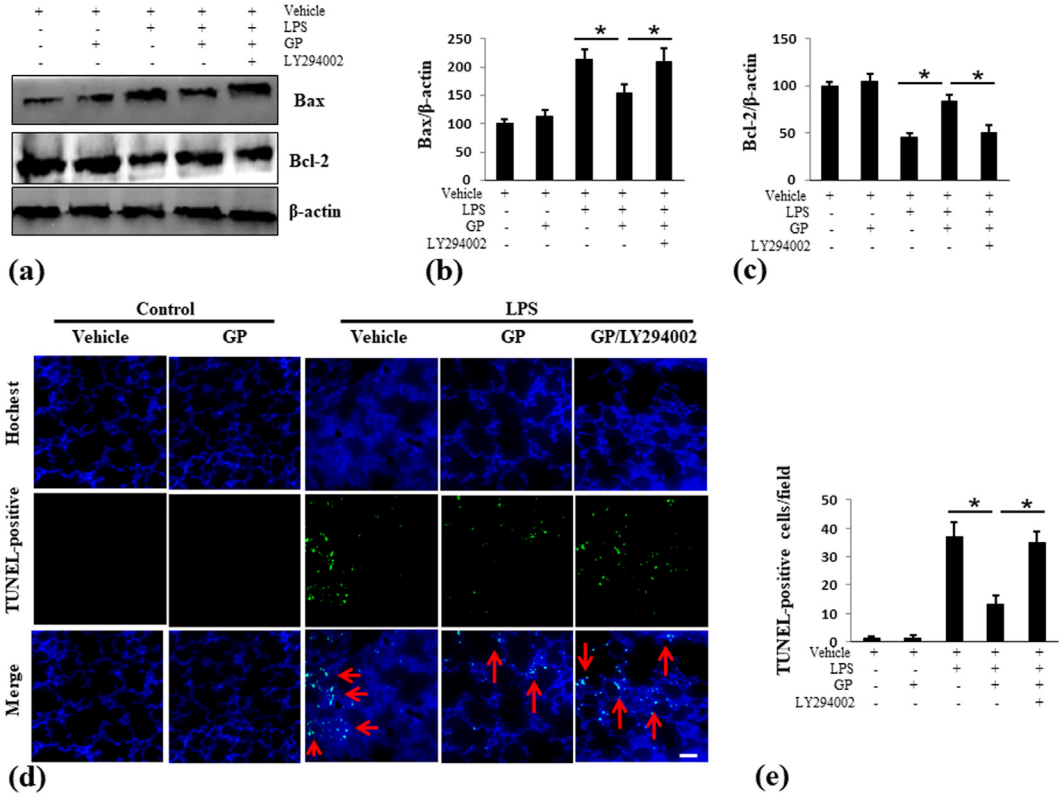

Fig. 4. Genipin attenuates LPS-induced activation of apoptosis by PI3K/Akt pathway *in vivo*.
Rats were pretreated with genipin (5 mg/kg) or vehicle for 60 min, followed by LPS at 5 mg/kg delivered intratracheally. Twelve hours later, the rats were sacrificed. To investigate the association of the protective effects of genipin with the PI3K/AKT signaling pathway, LY294002 (0.3 mg/kg) was given simultaneously with genipin. (a) Western blotting was used to assess the levels of BAX and BCL2. (b) Densitometric quantification of the level of BAX. (c) Densitometric quantification of the level of BCL2. (d) TUNEL staining was used to assess the apoptosis of rat pulmonary cells (scale bar: 500 μm); (e) In 10 microscopic fields per animal, the numbers of TUNEL-positive cells were. Data are shown as the mean ± SD (n = 6 per group). *$P < 0.05$ *vs.* the indicated groups. PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, protein kinase B; LPS, lipopolysaccharide; TUNEL, terminal deoxynulceotidyl transferase nick-end-labeling; BAX, BCL2 associated X, apoptosis regulator; BCL2, BCL2 apoptosis regulator; SD, standard deviation.

with that in the control group *in vitro* and *in vivo*, indicating activation of ERS (Fig. 5). Genipin pretreatment significantly inhibited LPS-induced ERS, as reflected by downregulated levels of CHOP, GRP-78 and XBP1 *in vitro* and *in vivo*. However, increased levels of CHOP, GRP-78 and XBP-1 were detected in the LY294002 group, suggesting that the genipin-induced protective effect against ERS was blocked by LY294002 treatment. These results revealed that genipin inhibits ERS through the PI3K/AKT pathway in LPS-induced ALI.

3.4. Genipin pretreatment inhibits LPS-induced inflammation via PI3K/AKT pathway

Inflammatory mediators have key functions in ALI; therefore, we determined the level of inflammatory mediators in the lung tissue and BALF. We detected substantially increased levels of IL-1β, TNF-α, and IL-6 in the BALF and lungs of ALI-challenged rats compared with those in the control group. By contrast, genipin pretreatment significantly suppressed the levels of IL-1β, TNF-α, and IL-6 in the BALF and lungs in the ALI-challenged rats. Interestingly, compared with those that received genipin treatment alone, in the lungs of ALI-challenged rats that were treated simultaneously with genipin and LY294002, increased levels of IL-1β, TNF-α, and IL-6 were detected. These results suggest that genipin pretreatment inhibits ALI-associated inflammation *via* the PI3K/AKT pathway (Fig. 6a–f).

LPS promotes nuclear factor kappa B (NF-κB) translocation into the nucleus and regulates the expression of related inflammatory genes [17]. In the present study, we measured NF-κB signaling *in vitro* (Fig. 6g–j). We found that LPS induced the upregulation of NF-κB P65 levels and its phosphorylated version, which were inhibited by genipin pretreatment. Interestingly, LY294002 treatment inhibited the preventive effect of genipin against the LPS-induced increase in P65 and p-P65. NF-κB signaling activation depends on the translocation of NF-κB P65 into the nucleus. We found that genipin pretreatment inhibited the upregulation of P65 in the nucleus of LPS-treated cells. Furthermore, LY294002 treatment blocked the preventive effect of genipin against the LPS-induced upregulation of P65 in the nucleus. These data indicated that genipin pretreatment inhibits LPS-induced activation of NF-κB signaling.

4. Discussion

The lipid kinase PI3K generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which is a second messenger that facilitates the translocation of AKT to the plasma membrane. At the membrane, AKT is phosphorylated and plays a pivotal role in important cellular functions, such as cell survival, by phosphorylating various substrates [13,18,19]. It was reported that genipin modulates PI3K/AKT pathways to exert an anti-inflammatory effect to treat neurodegenerative diseases [20]. Genipin stimulated constitutive nitrous oxide synthase (cNOS) activity *via* activating PI3K/AKT signaling and exerted antioxidant effects in ischemia-reperfusion of old rat hearts [21]. However, whether genipin activates PI3K/AKT signaling in ALI has not been studied. In the present study, we found that genipin pretreatment upregulated the phosphorylation of AKT, suggesting activation of the PI3K/AKT pathway. The role of PI3K/AKT signaling in the development of ALI has been studied extensively [22,23]. In addition, genipin could attenuate

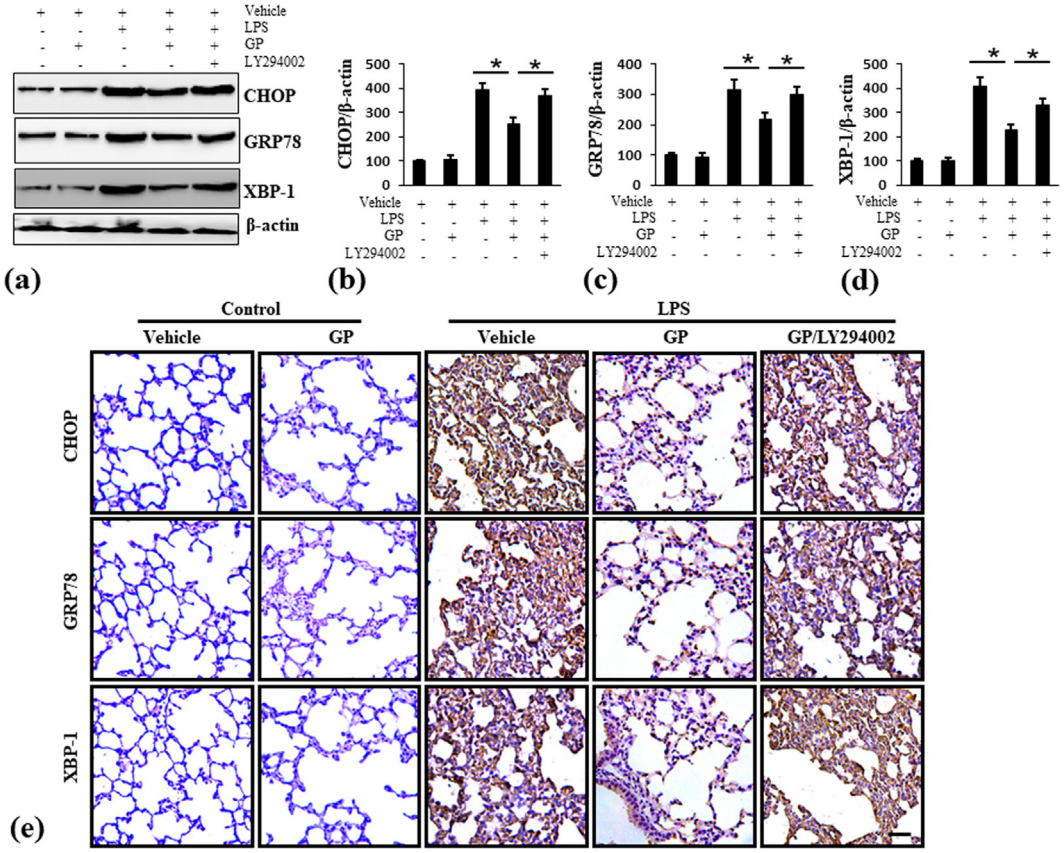

Fig. 5. Genipin attenuates LPS-induced ERS by PI3K/Akt pathway *in vitro* and *in vivo*.
(a) A549 cells pretreated with genipin (50 μM) or vehicle for 60 min were incubated with LPS (5 μg/ml) for 6 h. LY294002 (20 μM) was administered simultaneously with genipin to investigate the association of the protective effects of genipin with the PI3K/AKT signaling pathway. Western blotting was used to assess the levels of CHOP, GRP78, and XBP1. (b) Densitometric quantification of the level of CHOP. (c) Densitometric quantification of the level of GRP78. (d) Densitometric quantification of the level of XBP1. (e) Rats were pretreated with genipin (5 mg/kg) or vehicle for 60 min, followed by LPS at 5 mg/kg delivered intratracheally. Twelve hours later, the rats were sacrificed. To investigate the association of the protective effects of genipin with the PI3K/AKT signaling pathway, LY294002 (0.3 mg/kg) was given simultaneously with genipin. CHOP, GRP-78, and XBP-1 levels in the lung were determined using immunohistochemistry (scale bar: 500 μm). Data are shown as the mean ± SD ($n = 6$ per group). *$P < 0.05$ *vs.* the indicated groups. PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, protein kinase B; LPS, lipopolysaccharide; ERS, endoplasmic reticulum stress; CHOP, AF4/FMR2 family member 4; GRP78, glucose-regulated protein, 78 kDa; XBP1, X-box binding protein 1; SD, standard deviation.

LPS-induced lung injury; however, the mechanism was unclear [5]. In present study, we found that LY294002-induced inhibition of PI3K/Akt signaling reversed genipin's protective effects against LPS-induced histopathological deterioration, lung edema, and decreased oxygenation index. This suggested the involvement of the PI3K/AKT signaling pathway in the genipin-mediated protective effects against lung injury. We then investigated the mechanism of genipin-induced activation of PI3K/AKT signaling. Alveolar type II cell injury is a key factor in the pathogenesis of ALI [24]. Therefore, A549 cells, which are widely used in the study of ALI, were used in the *in vitro* experiments in the present study [1].

Apoptosis plays an important role in the pathogenesis of ALI [2,3]. Accumulating evidence suggests that PI3K/AKT signaling plays an important role in mediating apoptosis [25,26]. For example, the PI3K/AKT pathway could regulate the activity of BCL2 family members, which serve as regulators of mitochondrial-related apoptosis [9,27]. Although its protective effects against apoptosis has been studied in several animal models [28,29], the effects of genipin on apoptosis in ALI is unknown. The *in vitro* and *in vivo* results of the present study showed that genipin significantly prevented LPS-induced apoptosis *via* the PI3K/AKT pathway. Apoptosis can be activated by two main pathways: The extrinsic pathway and the intrinsic pathway [30]. The intrinsic apoptotic signaling pathway is mediated by activation of mitochondria. The release of cytochrome *c* and apoptosis-inducing factors, followed by caspase activation, mediate mitochondrial regulation of apoptosis. All of which are regulated by members of the BCL2 family of proteins. In the present study, we found that genipin inhibited LPS-induced mitochondrial dysfunction *via* upregulation of BAX, and downregulation of BCL2, caspase activation and cytochrome *c* release, indicating that genipin significantly inhibited LPS-induced mitochondria-dependent apoptotic signaling. However, the PI3K inhibitor LY249002 blocked these protective effects of genipin. Thus, the results suggested that genipin attenuates LPS-mediated mitochondria-dependent apoptosis *via* the PI3K/AKT pathway.

The endoplasmic reticulum (ER) is an important subcellular organelle that is responsible for the correct folding and sorting of proteins [31,32]. Endoplasmic reticulum stress (ERS) causes a pathological imbalance in ER homeostasis and induces physical dysfunction [29]. During ERS, the markers GRP78, CHOP, and XBP1 are activated [31,32]. Increasing evidence points to ERS being implicated in ALI pathogenesis [33,34]. ERS inhibition alleviates LPS-induced lung inflammation [35]. In addition, genipin protects Neuro2a cells from A23187 (a calcium ionophore)-induced and tunicamycin-induced cytotoxicity by mediating ERS [36,37]. However, the mechanism of action of genipin in ERS is unknown. In the present study, we found that genipin pretreatment significantly reduced LPS-induced ERS *via* the PI3K/AKT pathway. Our findings are in accordance with previous studies in which the role of PI3K in ERS modulation was fully clarified

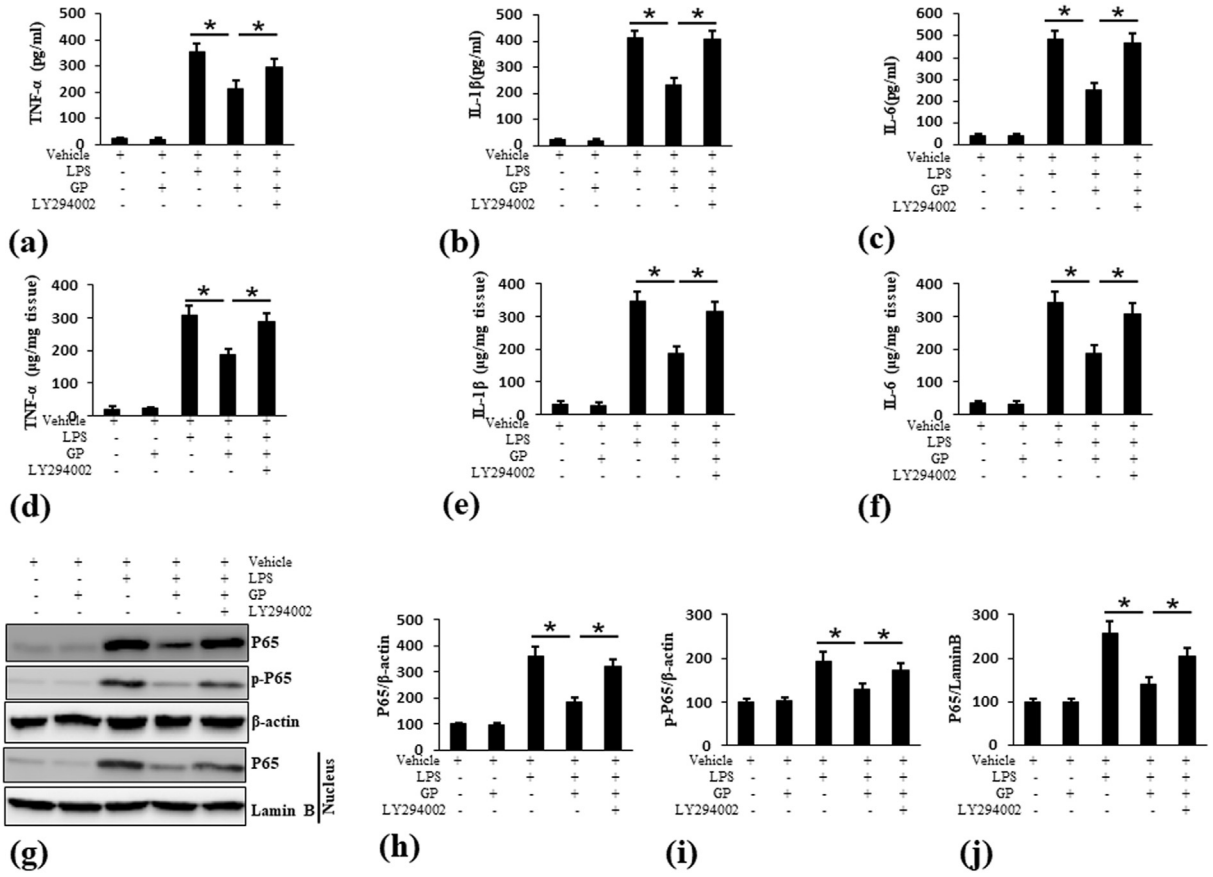

Fig. 6. Genipin attenuates LPS-induced inflammation by PI3K/Akt pathway.
(a) Rats were pretreated with genipin (5 mg/kg) or vehicle for 60 min, followed by LPS at 5 mg/kg delivered intratracheally. Twelve hours later, the rats were sacrificed. To investigate the association of the protective effects of genipin with the PI3K/AKT signaling pathway, LY294002 (0.3 mg/kg) was given simultaneously with genipin. BALF contents of TNFα, IL1β, and IL6 (a–c). Lung tissue contents of TNFα, IL1β, and IL-6 (e–f). (g) A549 cells pretreated with genipin (50 μM) or vehicle for 60 min were incubated with LPS (5 μg/ml) for 6 h. LY294002 (20 μM) simultaneously with genipin were given to investigate the association of the protective effects of genipin with PI3K/AKT signaling. Western blotting was used to assess the levels of NF-κB P65 and phosphorylated P65. (h) Densitometric quantification of the level of P65. (i) Densitometric quantification of the level of phosphorylated P65. (j) Densitometric quantification of the level of P65 in the nucleus. Data are shown as the mean ± SD (n = 6 per group). *$P < 0.05$ *vs.* the indicated groups. PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, protein kinase B; LPS, lipopolysaccharide; TNFα, tumor necrosis factor alpha; IL1β, interleukin 1 beta; IL6, interleukin 6; BALF; bronchoalveolar lavage fluid; NF-κB P65, RELA proto-oncogene, NF-KB subunit; SD, standard deviation.

[38,39].

The inflammatory response, which includes the release of proinflammatory cytokines, is an important characteristic of ALI. Our previous study demonstrated that the inhibition of these cytokines, including TNF-α, IL-1β, and IL-6, prevents the development of ALI [1]. In addition, genipin protects against LPS-induced acute systemic inflammation and cecal ligation and puncture (CLP)-induced sepsis [40]. Although genipin inhibits inflammation in LPS-induced ALI [5], the precise mechanism remains unclear. Previous studies reported that activation of PI3K/AKT signaling inhibits the LPS-induced inflammation response in ALI [41,42]. In the present study, we found that an inhibitor of PI3K reversed the protective effects of genipin against inflammation in ALI, indicating that genipin prevents LPS-induced inflammation *via* the PI3K/AKT pathway.

As a potent stimulus, LPS causes cell damage by activating toll-like receptor 4 (TLR-4), after which nuclear factor kappa B (NF-κB) translocates into the nucleus to regulate the expression of related inflammatory genes [17]. PI3K/AKT signaling regulated NF-κB pathways negatively and reduced the inflammatory response in LPS-stimulated cells [43]. Thus, PI3K signaling might play a pivotal role in the maintenance of homeostasis and the integrity of the immune response during sepsis [44]. Correspondingly, PI3K-AKT signaling inhibition enhanced the mortality and circulating proinflammatory cytokine levels in mice with endotoxemia [45]. Activation of the PI3K pathway negatively regulates potentially pathogenic excessive TLR responses *via* B-cell adaptor for PI3K (BCAP) [46]. The preventive effect of genipin against LPS-induced activation of NF-κB signaling has been demonstrated in a previous study [7]. In the present study, we found that LY294002 inhibited the preventive effect of genipin against LPS-induced activation of NF-κB signaling, suggesting that genipin inhibits LPS-induced activation of NF-κB signaling *via* PI3K/AKT signaling.

5. Conclusion

The results of the present study show convincing evidence that genipin activates the PI3K/AKT signaling pathway significantly, inducing protection against mitochondria-dependent apoptosis, ERS, and inflammation during LPS-induced ALI.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 81500066]; and the Natural Science Foundation of Hunan Province [grant numbers: 2018JJ6004, 2018JJ3015, 2019JJ40010]; and the Startup Fund for Scientific Research, Fujian Medical University [grant numbers: 2018QH1083];

and the Joint Funds for the innovation of Science and Technology of Fujian Province [grant number: 2016Y9012].

Declaration of competing interest

None.

References

[1] X. Luo, R. Liu, Z. Zhang, Z. Chen, J. He, Y. Liu, Mitochondrial division inhibitor 1 attenuates mitophagy in a rat model of acute lung injury, Biomed. Res. Int. 2019 (2019) 1–11.

[2] T. Li, Y. Liu, G. Li, X. Wang, Z. Zeng, S. Cai, et al., Polydatin attenuates ipopolysaccharide-induced acute lung injury in rats, Int. J. Clin. Exp. Pathol. 7 (2014) 8401–8410.

[3] T. Li, S. Cai, Z. Zeng, J. Zhang, Y. Gao, X. Wang, et al., Protective effect of polydatin against burn-induced lung injury in rats, Respir. Care 59 (2014) 1412–1421.

[4] X. Tian, Z. Liu, T. Yu, H. Yang, L. Feng, Ghrelin ameliorates acute lung injury induced by oleic acid via inhibition of endoplasmic reticulum stress, Life Sci. 196 (2018) 1–8.

[5] A. Zhang, S. Wang, J. Zhang, H. Wu, Genipin alleviates LPS-induced acute lung injury by inhibiting NF-kappaB and NLRP3 signaling pathways, Int. Immunopharmacol. 38 (2016) 115–119.

[6] D. Xu, H. Jin, J. Wen, J. Chen, D. Chen, N. Cai, et al., Hydrogen sulfide protects against endoplasmic reticulum stress and mitochondrial injury in nucleus pulposus cells and ameliorates intervertebral disc degeneration, Pharmacol. Res. 117 (2017) 357–369.

[7] Y. Tang, J. Li, C. Gao, Y. Xu, Y. Li, X. Yu, et al., Hepatoprotective effect of quercetin on endoplasmic reticulum stress and inflammation after intense exercise in mice through phosphoinositide 3-kinase and nuclear factor-kappa B, Oxidative Med. Cell. Longev. 2016 (2016) 8696587.

[8] L. Tu, Y. Wang, D. Chen, P. Xiang, J. Shen, Y. Li, et al., Protective effects of notoginsenoside R1 via regulation of the PI3K-Akt-mTOR/JNK pathway in neonatal cerebral hypoxic-ischemic brain injury, Neurochem. Res. 43 (2018) 1210–1226.

[9] N. Torrealba, G. Rodriguez-Berriguete, B. Fraile, G. Olmedilla, P. Martinez-Onsurbe, M. Sanchez-Chapado, et al., PI3K pathway and Bcl-2 family. Clinicopathological features in prostate cancer, Aging Male 21 (2018) 211–222.

[10] A.R. Shenoy, S. Kirschnek, G. Hacker, IL-15 regulates Bcl-2 family members Bim and Mcl-1 through JAK/STAT and PI3K/AKT pathways in T cells, Eur. J. Immunol. 44 (2014) 2500–2507.

[11] R.K. Bao, S.F. Zheng, X.Y. Wang, Selenium protects against cadmium-induced kidney apoptosis in chickens by activating the PI3K/AKT/Bcl-2 signaling pathway, Environ. Sci. Pollut. Res. Int. 24 (2017) 20342–20353.

[12] J. Li, Q. Chen, X. He, A. Alam, J. Ning, B. Yi, et al., Dexmedetomidine attenuates lung apoptosis induced by renal ischemia-reperfusion injury through alpha2AR/PI3K/Akt pathway, J. Transl. Med. 16 (2018) 78.

[13] W. Zhang, J.Q. Zhang, F.M. Meng, F.S. Xue, Dexmedetomidine protects against lung ischemia-reperfusion injury by the PI3K/Akt/HIF-1alpha signaling pathway, J. Anesth. 30 (2016) 826–833.

[14] Q. Song, C.C. Han, X.P. Xiong, F. He, W. Gan, S.H. Wei, et al., PI3K-Akt-mTOR signal inhibition affects expression of genes related to endoplasmic reticulum stress, Genet. Mol. Res. 15 (2016).

[15] Z. Zhang, X. Wang, C. Ma, Z. Li, H. Chen, Z. Zhang, et al., Genipin protects rats against lipopolysaccharide-induced acute lung injury by reinforcing autophagy, Int. Immunopharmacol. 72 (2019) 21–30.

[16] Y. Wang, Y. Liu, M. Zhang, L. Lv, X. Zhang, P. Zhang, et al., Inhibition of PTGS1 promotes osteogenic differentiation of adipose-derived stem cells by suppressing NF-kB signaling, Stem Cell Res Ther 10 (2019) 57.

[17] S.R. Oliveira, P.A. Dionisio, H. Brito, L. Franco, C. Rodrigues, R.C. Guedes, et al., Phenotypic screening identifies a new oxazolone inhibitor of necroptosis and neuroinflammation, Cell Death Discov 4 (2018) 10.

[18] T. Hou, L. Zhou, L. Wang, G. Kazobinka, Y. Chen, X. Zhang, et al., Leupaxin promotes bladder cancer proliferation, metastasis, and angiogenesis through the PI3K/AKT pathway, Cell. Physiol. Biochem. 47 (2018) 2250–2260.

[19] Y. He, Y. Ge, M. Jiang, J. Zhou, D. Luo, H. Fan, et al., MiR-592 promotes gastric cancer proliferation, migration, and invasion through the PI3K/AKT and MAPK/ERK signaling pathways by targeting Spry2, Cell. Physiol. Biochem. 47 (2018) 1465–1481.

[20] D. Luo, T.C. Or, C.L. Yang, A.S. Lau, Anti-inflammatory activity of iridoid and catechol derivatives from Eucommia ulmoides Oliver, ACS Chem. Neurosci. 5 (2014) 855–866.

[21] I. Hoshovs'Ka, I. Korkach, T.V. Shymans'Ka, A.V. Kotsiuruba, V.F. Sahach, Effects of uncoupling proteins on nitric oxide synthesis and oxidative stress development in ischemia-reperfusion of old rat hearts, Fiziol. Zh. 55 (2009) 3–11.

[22] D. Qi, J. He, D. Wang, W. Deng, Y. Zhao, Y. Ye, et al., 17beta-estradiol suppresses lipopolysaccharide-induced acute lung injury through PI3K/Akt/SGK1 mediated up-regulation of epithelial sodium channel (ENaC) in vivo and in vitro, Respir. Res. 15 (2014) 159.

[23] J. Yang, S. Li, L. Wang, F. Du, X. Zhou, Q. Song, et al., Ginsenoside Rg3 attenuates lipopolysaccharide-induced acute lung injury via MerTK-dependent activation of the PI3K/AKT/mTOR pathway, Front. Pharmacol. 9 (2018) 850.

[24] H.B. Suliman, B. Kraft, R. Bartz, L. Chen, K.E. Welty-Wolf, C.A. Piantadosi, Mitochondrial quality control in alveolar epithelial cells damaged by S. aureus pneumonia in mice, Am J Physiol Lung Cell Mol Physiol 313 (2017) L699–L709.

[25] F. Li, D. Jin, C. Tang, D. Gao, CEP55 promotes cell proliferation and inhibits apoptosis via the PI3K/Akt/p21 signaling pathway in human glioma U251 cells, Oncol. Lett. 15 (2018) 4789–4796.

[26] P. Li, Z. Liang, G. Hou, L. Song, R. Zhang, Y. Gan, et al., N-cadherin-mediated activation of PI3K/Akt-GSK-3beta signaling attenuates nucleus pulposus cell apoptosis under high-magnitude compression, Cell. Physiol. Biochem. 44 (2017) 229–239.

[27] Y.G. Lai, M.S. Hou, A. Lo, S.T. Huang, Y.W. Huang, H.F. Yang-Yen, et al., IL-15 modulates the balance between Bcl-2 and Bim via a Jak3/1-PI3K-Akt-ERK pathway to promote CD8alphaalpha+ intestinal intraepithelial lymphocyte survival, Eur. J. Immunol. 43 (2013) 2305–2316.

[28] E. Mahgoub, S.M. Kumaraswamy, K.H. Kader, B. Venkataraman, S. Ojha, E. Adeghate, et al., Genipin attenuates cisplatin-induced nephrotoxicity by counteracting oxidative stress, inflammation, and apoptosis, Biomed. Pharmacother. 93 (2017) 1083–1097.

[29] C. Shumin, X. Wei, L. Yunfeng, L. Jiangshui, G. Youguang, C. Zhongqing, et al., Genipin alleviates vascular hyperpermeability following hemorrhagic shock by up-regulation of SIRT3/autophagy, Cell Death Discov 4 (2018) 52.

[30] E.W. Childs, B. Tharakan, F.A. Hunter, J.H. Tinsley, X. Cao, Apoptotic signaling induces hyperpermeability following hemorrhagic shock, Am. J. Physiol. Heart Circ. Physiol. 292 (2007) H3179–H3189.

[31] Z. He, Y. Zhou, Y. Huang, Q. Wang, B. Zheng, H. Zhang, et al., Dl-3-n-butylphthalide improves functional recovery in rats with spinal cord injury by inhibiting endoplasmic reticulum stress-induced apoptosis, Am. J. Transl. Res. 9 (2017) 1075–1087.

[32] S. Wang, J. Wu, Y.Z. Zeng, S.S. Wu, G.R. Deng, Z.D. Chen, et al., Necrostatin-1 mitigates endoplasmic reticulum stress after spinal cord injury, Neurochem. Res. 42 (2017) 3548–3558.

[33] M.M. Khan, W.L. Yang, M. Brenner, A.C. Bolognese, P. Wang, Cold-inducible RNA-binding protein (CIRP) causes sepsis-associated acute lung injury via induction of endoplasmic reticulum stress, Sci. Rep. 7 (2017) 41363.

[34] R. Hu, Z.F. Chen, J. Yan, Q.F. Li, Y. Huang, H. Xu, et al., Endoplasmic reticulum stress of neutrophils is required for ischemia/reperfusion-induced acute lung injury, J. Immunol. 195 (2015) 4802–4809.

[35] Blockade of Interplay between IL-17A and Endoplasmic Reticulum Stress Attenuates LPS-induced Lung Injury.

[36] M. Yamazaki, K. Chiba, C. Yoshikawa, Genipin suppresses A23187-induced cytotoxicity in neuro2a cells, Biol. Pharm. Bull. 32 (2009) 1043–1046.

[37] M. Tanaka, M. Yamazaki, K. Chiba, Neuroprotective action of genipin on tunicamycin-induced cytotoxicity in neuro2a cells, Biol. Pharm. Bull. 32 (2009) 1220–1223.

[38] Z.R. Li, L. Yang, J. Zhen, Y. Zhao, Z.N. Lu, Nobiletin protects PC12 cells from ERS-induced apoptosis in OGD/R injury via activation of the PI3K/AKT pathway, Exp Ther Med 16 (2018) 1470–1476.

[39] A. Burban, A. Sharanek, C. Guguen-Guillouzo, A. Guillouzo, Endoplasmic reticulum stress precedes oxidative stress in antibiotic-induced cholestasis and cytotoxicity in human hepatocytes, Free Radic. Biol. Med. 115 (2018) 166–178.

[40] H.I. Cho, S.J. Kim, J.W. Choi, S.M. Lee, Genipin alleviates sepsis-induced liver injury by restoring autophagy, Br. J. Pharmacol. 173 (2016) 980–991.

[41] X. Wang, C. Zhang, C. Chen, Y. Guo, X. Meng, C. Kan, Allicin attenuates lipopolysaccharide induced acute lung injury in neonatal rats via the PI3K/Akt pathway, Mol. Med. Rep. 17 (2018) 6777–6783.

[42] J. Yang, S. Li, L. Wang, F. Du, X. Zhou, Q. Song, et al., Ginsenoside Rg3 attenuates lipopolysaccharide-induced acute lung injury via MerTK-dependent activation of the PI3K/AKT/mTOR pathway, Front. Pharmacol. 9 (2018) 850.

[43] P. Tongaonkar, K.K. Trinh, J.B. Schaal, D. Tran, P.S. Gulko, A.J. Ouellette, et al., Rhesus macaque θ-defensin RTD-1 inhibits proinflammatory cytokine secretion and gene expression by inhibiting the activation of NF-κB and MAPK pathways, J. Leukoc. Biol. 98 (2015) 1061–1070.

[44] D.L. Williams, C. Li, T. Ha, T. Ozment-Skelton, J.H. Kalbfleisch, J. Preiszner, et al., Modulation of the phosphoinositide 3-kinase pathway alters innate resistance to polymicrobial sepsis, J. Immunol. 172 (2004) 449–456.

[45] G. Schabbauer, M. Tencati, B. Pedersen, R. Pawlinski, N. Mackman, PI3K-Akt pathway suppresses coagulation and inflammation in endotoxemic mice, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1963–1969.

[46] M. Ni, A.T. MacFarlane, M. Toft, C.A. Lowell, K.S. Campbell, J.A. Hamerman, B-cell adaptor for PI3K (BCAP) negatively regulates toll-like receptor signaling through activation of PI3K, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 267–272.