



Genipin protects rats against lipopolysaccharide-induced acute lung injury by reinforcing autophagy[☆]



Zhijie Zhang^{a,1}, Xue Wang^{a,1}, Chengzhou Ma^b, Zhiwang Li^c, Huayong Chen^a, Zhiming Zhang^{c,*}, Tao Li^{d,**}

^a Department of Anesthesiology, Yidu Central Hospital of Weifang, Weifang, Shandong Province, China

^b Department of Anesthesiology, Dongcheng Street Hospital of Linqu, Weifang, Shandong Province, China

^c Department of Anesthesiology, The First People's Hospital of Chenzhou/Institute of Translation Medicine, University of South China, Chenzhou 423000, China

^d Department of Critical Care Medicine, The First People's Hospital of Chenzhou/Institute of Translation Medicine, University of South China, Chenzhou 423000, China

ARTICLE INFO

Keywords:

Genipin
Acute lung injury
Autophagy
Apoptosis
Inflammation
Mitochondria

ABSTRACT

Although the protective effects of genipin against acute lung injury (ALI) have been described previously, the associated mechanism remains unclear. We have previously reported that genipin exerts its pharmacological effects by regulating autophagy. Here, we hypothesized that the up-regulation of autophagy may contribute to the protective effects exhibited by genipin against ALI. In the present study, ALI was induced by intratracheal LPS administration in rats. Genipin treatment significantly reduced LPS-induced lung injury as evidenced by improved histopathology, decreased lung edema, total cells, and protein concentration in the bronchoalveolar lavage fluid (BALF). This protection was inhibited by 3-methyladenine (3-MA), an inhibitor of autophagy. Genipin treatment reduced the expression of P62 and increased the expression of Beclin-1 and LC3II, indicating increased autophagy. Genipin treatment also alleviated LPS-induced cell apoptosis (down-regulation of Bax, up-regulation of Bcl-2, and decreased number of terminal deoxynucleotidyl transferase dUTP nick end label-positive cells) and oxidative stress (increased SOD and decreased MDA content) in the lung. Furthermore, genipin attenuated LPS-induced production of TNF- α , IL-1 β , and IL-6 in the lung and BALF. These protective effects induced by genipin were reversed by 3-MA treatment, indicating that autophagy was involved in the protective effects exerted by genipin against inflammation and apoptosis in ALI. In A549 cells incubated with LPS for 6 h, genipin treatment increased the number of GFP-LC3 punctae. 3-MA prevented the protective effects of genipin against mitochondrial dysfunction and cell death. These findings suggest that genipin protects against apoptosis and inflammation in LPS-induced ALI by promoting autophagy.

1. Introduction

Clinical acute lung injury (ALI) is a common complication that occurs following sepsis and is associated with high morbidity and mortality [1–3]. Lipopolysaccharide (LPS) is a major endotoxin component of gram-negative bacteria and is thought to be the most important pathogen leading to the development of ALI in sepsis [4,5]. Moreover, the pathophysiology of ALI involves highly complex mechanisms, and recent studies have shown that a systemic inflammatory response and apoptosis are key to this process [6,7].

Although the pathophysiology of ALI has been extensively studied, effective clinical therapy against ALI is currently limited. Therefore, effective agents for ALI treatment are required. Previous studies have demonstrated that genipin (Fig. 1) has various pharmacological effects, including antioxidative, anticancer, and antifungal activities [8,9]. In addition, recent studies have reported that genipin can reduce the LPS-induced inflammatory response in a murine model of ALI [10,11]; however the associated mechanism remains unknown.

Autophagy is a highly controlled process that facilitates the turnover of cellular organelles and proteins, as well as providing protection

[☆] Disclosure: The authors declared no conflict of interest.

^{*} Correspondence to: Z. Zhang, Department of Anesthesiology, The First People's Hospital of Chenzhou/Institute of Translation Medicine, University of South China, NO. 102 Luojiajing, Chenzhou, Hunan Province 423000, China.

^{**} Correspondence to: T. Li, Department of Critical Care Medicine, The First People's Hospital of Chenzhou/Institute of Translation Medicine, University of South China, NO. 102 Luojiajing, Chenzhou, Hunan Province 423000, China.

E-mail addresses: zzm_edu@163.com (Z. Zhang), lita0.7@163.com (T. Li).

¹ Equal contributors.

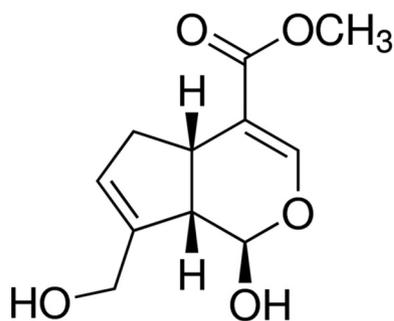


Fig. 1. Structure of genipin.

against conditions of stress [9,12]. An increasing number of studies have reported the importance of autophagy in ALI [13,14]. In addition, it has been demonstrated that enhancing autophagy with activated protein C and rapamycin attenuates systemic inflammation and protects against sepsis-induced ALI [15]. Furthermore, the modulation of autophagy appears to be protective against multiple organ injury in murine models of sepsis [16,17]. This is partially achieved by preventing apoptosis, maintaining a balance between the production of pro- and anti-inflammatory cytokines, and preserving mitochondrial functionality [18].

We have previously demonstrated that genipin attenuates endothelial dysfunction through the up-regulation of autophagy [19]. Moreover, it has been reported that genipin protects against sepsis-induced liver injury by restoring autophagic flux [8]. Therefore, in the present study, we investigated the protective mechanisms of genipin in ALI, with a particular focus on autophagy.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies against Beclin-1, P62, LC3, and GAPDH were obtained from Abclonal (Wuhan, China). A terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining kit was supplied by Promega Corp. (Madison, WI). Immunohistochemical kits were provided by EnVision™ (Dako, Copenhagen, Denmark). MitoProbe™ JC-1 (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide) was purchased from Molecular Probes (Invitrogen, CA, USA). A CellTiter-Glo® assay was supplied from Promega Corp. (Madison, WI, USA). GFP-LC3 plasmids were obtained from Addgene (Watertown, MA, USA). Superoxide dismutase (SOD) and malondialdehyde (MDA) commercial assay kits were obtained from Jiancheng Co. (Nanjing, China). The assay kits used to measure caspase-3 activity, TNF- α , IL-1 β , and IL-6 were purchased from BestBio Co. (Beijing, China). Genipin and other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Animals

Procedures involving animals and their care were approved by the Medical Faculty Ethics Committee of University of South China (Hengyang, China) and complied with the NIH Guidelines for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (weight: 180 g–220 g) were acquired from the Experimental Animal Centre of University of South China and housed under temperature- and humidity-controlled conditions on a 12/12 h day/night cycle with unrestricted access to standard diet and tap water.

2.3. Cell culture, stimulation, and cell viability

A549 cells were grown at 37 °C in 5% CO₂ in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich; Merck Millipore), containing

low glucose, penicillin (100 U/mL, Sigma-Aldrich; Merck Millipore), streptomycin (100 units, Sigma-Aldrich; Merck Millipore), and 10% bovine serum (Sigma-Aldrich; Merck Millipore). The cells were stimulated in 0.5 mM LPS for 6 h to establish LPS-induced ALI in vitro.

Cell viability was determined using a 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay after 6 h post LPS stimulation. The cells were incubated with 0.5 mg/mL MTT at 37 °C for 4 h. Absorbance at 570 nm was determined using an automatic microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA).

To investigate the required dosage for genipin treatment, the cells were respectively pretreated with 20 μ M or 50 μ M of genipin for 60 min, followed by LPS exposure for 6 h and an assessment of cell viability. The results indicated that genipin treatment with dose of 50 μ M, but not 20 μ M, significantly improved the cell viability. Therefore, the dose of 50 μ M of genipin was chosen in the present study.

2.4. Detection of autophagy using GFP-LC3

The cells were transfected with GFP-LC3 (Addgene, Plasmid #21074), a highly specific fluorescent marker of autophagy, to measure the level of autophagy. Lipofectamine® 3000 Reagent (Thermo-Fisher Scientific, USA) was used to transfect the cells. After treatment and LPS exposure, the cellular localization of GFP-LC3 was visualized using a confocal microscope (LSM780; Zeiss Microsystems, Jena, Germany).

2.5. Measurement of the mitochondrial membrane potential (MMP)

The mitochondrial membrane potential (MMP) was determined using the potential-sensitive fluorescent dye, JC-1. Cells were treated and subjected to LPS. JC-1 (5 μ mol/L) was loaded onto the cells for 15 min at 37 °C. The results were visualized using an inverted fluorescent microscope (Nikon, Ti-E Live Cell Imaging System, Japan).

2.6. Measurement of cellular ATP

Intracellular ATP was determined using a luciferase-based assay (CellTiter-Glo®, Promega, Madison, WI), according to the manufacturer's instructions. After LPS exposure, 100 μ L CellTiter-Glo® reagent was added to 100 μ L of the cell suspension containing 10,000 cells per well of a standard opaque-walled 96-well plate. After 10 min, the luminescence was recorded using an automatic microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA).

2.7. Animal model and treatment

A rat model of ALI was induced by an intratracheal administration of LPS. Animals were anesthetized and placed in the supine position. The trachea was surgically exposed using a cervical middle line incision in the skin, after which LPS (5 mg/kg body weight, Sigma) was slowly injected into the trachea of each rat. There was a total of six rats per group. All of the rats were sacrificed for measurement at 12 h after the administration of LPS.

To investigate the protective effects of genipin against ALI, the rats were pre-treated with 2 mg/kg or 5 mg/kg of genipin for 60 min, followed by an intratracheal administration of LPS. The results indicated that treatment with dose of 5 mg/kg, not 2 mg/kg, of genipin significantly attenuated the extent of lung injury. Therefore, the dose of 5 mg/kg of genipin was selected in the present study.

2.8. Cell counts and protein concentration in the bronchoalveolar lavage fluid

BALF samples were collected by washing the lung three times with 4 mL PBS through a tracheal cannula placed into each mouse under anesthesia as described previously [1]. Briefly, after the rats were sacrificed, a median sternotomy was performed and the trachea was

isolated using a blunt dissection. Next, a suitable small-caliber tube was inserted into the airway and secured. Phosphate-buffered saline solution was infused slowly into the lungs, and the bronchoalveolar lavage fluid (BALF) was withdrawn into the tube. The fluid recovery rate was >80%. Lavage samples were centrifuged at $1500 \times g$ for 10 min at 4 °C. The sedimented cells were resuspended in a phosphate-buffered saline solution and subjected to cell counting. The slides were visualized using Wright-Giemsa staining (Sigma), and polymorphonuclear leukocytes (PMNs) were counted in a double-blind fashion. The protein concentration of the BALF supernatants was assessed using a BCA protein assay kit.

2.9. Measurement of inflammatory mediators

The concentration of TNF- α , IL-1 β , and IL-6 in the lung and BALF was measured using a commercial enzyme linked immunosorbent assay kit. The results were expressed as $\mu\text{g}/\text{mg}$ of tissue or pg/mL of BALF.

2.10. Histopathological and immunohistochemical analyses

After 12 h following the administration of LPS, the right lobes of the lungs were immersed in 10% neutral buffered formalin, fixed, paraffin-embedded, and sliced. Following H&E staining, pathological changes in the lung tissues were observed and evaluated in accordance with the following criteria: 1) degree of neutrophil infiltration; 2) airway epithelial cell damage; 3) level of interstitial edema; 4) hyaline membrane formation; and 5) presence of hemorrhage. Each of the sections were scored based on the following five criteria as determined by the degree of deterioration: normal = 0; minimal alteration = 1; mild alteration = 2; moderate alteration = 3; and severe alteration = 4. The lung injury score for each criterion was recorded.

The paraffin-embedded sections were also subjected to immunohistochemical staining with anti-Bcl-2 (1:200 dilution) and anti-Bax antibodies (1:200 dilution) overnight at 4 °C. Immunostaining was carried out with an avidin-biotin-peroxidase complex kit and counterstained with hematoxylin. The integrated optical density (IOD) of the immunostained samples was evaluated using image processing software (Image-Pro Plus version 6; Media Cybernetics, Silver Spring, Maryland, USA).

2.11. Lung wet/dry (W/D) ratio

Following the administration of LPS, the water content of the lungs was measured. The right lungs were excised, blotted, and weighed to obtain the wet weight. Subsequently, the lungs were dried in an incubator at 80 °C for 48 h until its weight was stabilized. The lungs were measured again to obtain the dry weight. The wet/dry weight ratio was calculated by dividing the wet weight by the dry weight.

2.12. Lung microvascular permeability

The permeability assay was performed as previously described [1]. A sample of the lung tissue was weighed, homogenized, and immersed in *N,N*-dimethylformamide (Sigma, USA). Eluted EB was measured at 620 nm using an automatic microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA) and the amount was expressed as micrograms per 100 mg of dry tissue.

2.13. Terminal deoxyribonucleotidyl transferase-mediated deoxyuridine 5-Triphosphate-Digoxigenin nick end labeling (TUNEL) assay

A commercial TUNEL assay kit (Boster Biotech) was used to detect the presence of apoptotic cells in accordance with the manufacturer's instructions. Quantification of the number of apoptotic cells was achieved using a double-blind approach. TUNEL-positive neurons were enumerated in five randomly selected areas surrounding the injury site

under an inverted fluorescence microscope (HB050; Zeiss, Hamburg, Germany).

2.14. Western blot for Beclin-1, P62, and LC3II/I

Homogenized lung tissues were analyzed by a Western blot. The concentration of protein in each sample was determined using a BCA protein assay. An equal amount of protein was loaded into 10% sodium dodecyl sulphate-polyacrylamide gels for electrophoresis. The separated proteins were subsequently electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked in 5% skim milk diluted in PBS for 2 h at room temperature, followed by an overnight incubation with primary antibodies against β -actin (42KD, 1:5000 dilution; Abcam, Cambridge, UK), Beclin-1 (60KD, 1:1000 dilution; Abcam, UK), P62 (62KD, 1:1000 dilution; Abcam, UK), and LC 3 (15/18KD, 1:1000 dilution; Abcam, UK) at 4 °C. The membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (42KD, 1:5000 dilution; Abcam, UK), and the level of protein expression was detected using an enhanced chemiluminescence reagent (Absin Biotechnology Co., Ltd., Shanghai, China).

2.15. Measurement of superoxide dismutase (SOD), and malondialdehyde (MDA)

The level of SOD and MDA in the lung was determined using commercial assay kits (an LPO kit was obtained from Cayman Chemical Co., Ann Arbor, Michigan, USA; SOD and MDA kits were obtained from Jiancheng, Nanjing, China) in accordance with the respective manufacturer's guidelines.

2.16. Statistical analysis

All variables are presented as the means \pm standard deviation (SD). Differences between groups were analyzed using a one-way ANOVA with an LSD multiple-comparison test or a Student's *t*-test, where appropriate. Values were considered significant at a threshold of $P < 0.05$.

3. Results

3.1. Genipin treatment attenuates LPS-induced lung injury, which was prevented by inhibiting autophagy

To explore whether genipin activates autophagy in ALI, rats were pre-treated with genipin (2 mg/kg or 5 mg/kg) or vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats were sacrificed 12 h following LPS administration. To investigate the role of autophagy on the protective effects of genipin, 3-methyladenine (3-MA, 10 mg/kg), a specific inhibitor of autophagy, was administered simultaneously with genipin (5 mg/kg).

As shown in Fig. 2, ALI-challenged rats displayed severe inflammatory cell infiltration, thickened alveolar walls, diffuse edema, decreased alveolar space, and enhanced interstitial congestion compared to the control rats. However, these histological changes were dramatically reduced by treatment with genipin at a dose of 5 mg/kg, but not a dose of 2 mg/kg. In addition, treatment with genipin (5 mg/kg but not 2 mg/kg) significantly alleviated LPS-induced lung injury, as evidenced by decreased lung edema, total cells, PMNs, and protein concentration in the BALF compared to the ALI-challenged rats that did not receive genipin treatment (Fig. 3). These data suggest that genipin treatment attenuates LPS-induced ALI in a dose-dependent manner. Thus, a dose of 5 mg/kg genipin was used to study the mechanism in the present study. These findings indicate that since genipin-mediated protection against lung injury was inhibited by 3-MA treatment, autophagy is involved.

Using in vitro experiments, A549 cells pre-treated with genipin

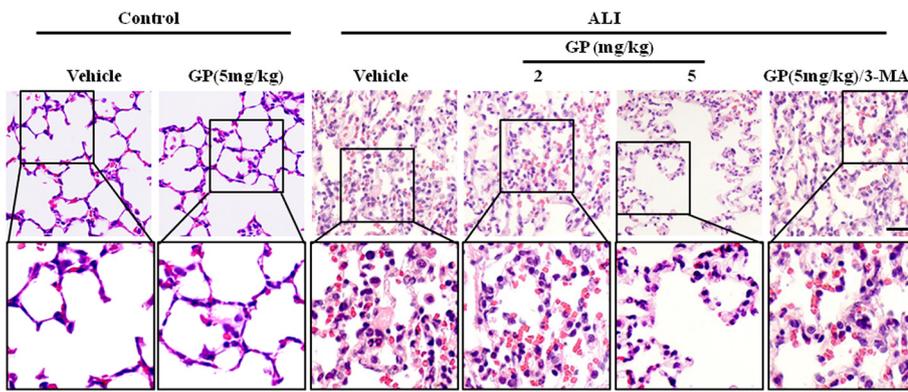


Fig. 2. Genipin treatment attenuates LPS-induced lung injury via autophagy. Rats were pre-treated with genipin (2 mg/kg or 5 mg/kg) or a vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats were sacrificed 12 h following the administration of LPS. To investigate the role of autophagy on the protective effects of genipin, 3-MA (10 mg/kg), a specific inhibitor of autophagy, was administered simultaneously with genipin (5 mg/kg). A histological examination was evaluated by HE-staining (scale bar: 500 μ m).

(20 μ M or 50 μ M) or a vehicle (DMSO) for 60 min were incubated with LPS (0.5 mM) for 6 h. To investigate the role of autophagy on the protective effects of genipin, 3-MA (5 mM) was administered simultaneously with genipin (50 μ M). The level of cell viability was evaluated. We found that treatment with 50 μ M of genipin, but not 20 μ M, significantly enhanced the survival rate of the cells exposed to LPS (Fig. 4a). However, this protective effect was inhibited by treatment with 3-MA, confirming that genipin plays a protective role by regulating autophagy.

3.2. Genipin treatment enhances LPS-induced autophagy

To investigate the effects of genipin on autophagy, A549 cells pre-

treated with genipin (50 μ M) or a vehicle (DMSO) for 60 min were incubated with LPS (0.5 mM) for 6 h. To prevent genipin-induced autophagy 3-MA (5 mM) was administered simultaneously with genipin (50 μ M). GFP-LC3 provides a useful in vitro indicator of autophagy through the evaluation of LC3 punctae. In the event of autophagy, GFP-LC3 foci redistribute from a diffuse pattern to a punctate cytoplasmic pattern. We found that genipin treatment increased the LPS-induced formation of GFP-LC3 punctae, which was reduced following treatment with 3MA (Fig. 4b and c). These data indicate that genipin up-regulates autophagy in vitro in response to LPS exposure.

To assess the effect of genipin on autophagy in vivo, rats were pre-treated with genipin (5 mg/kg) or a vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats

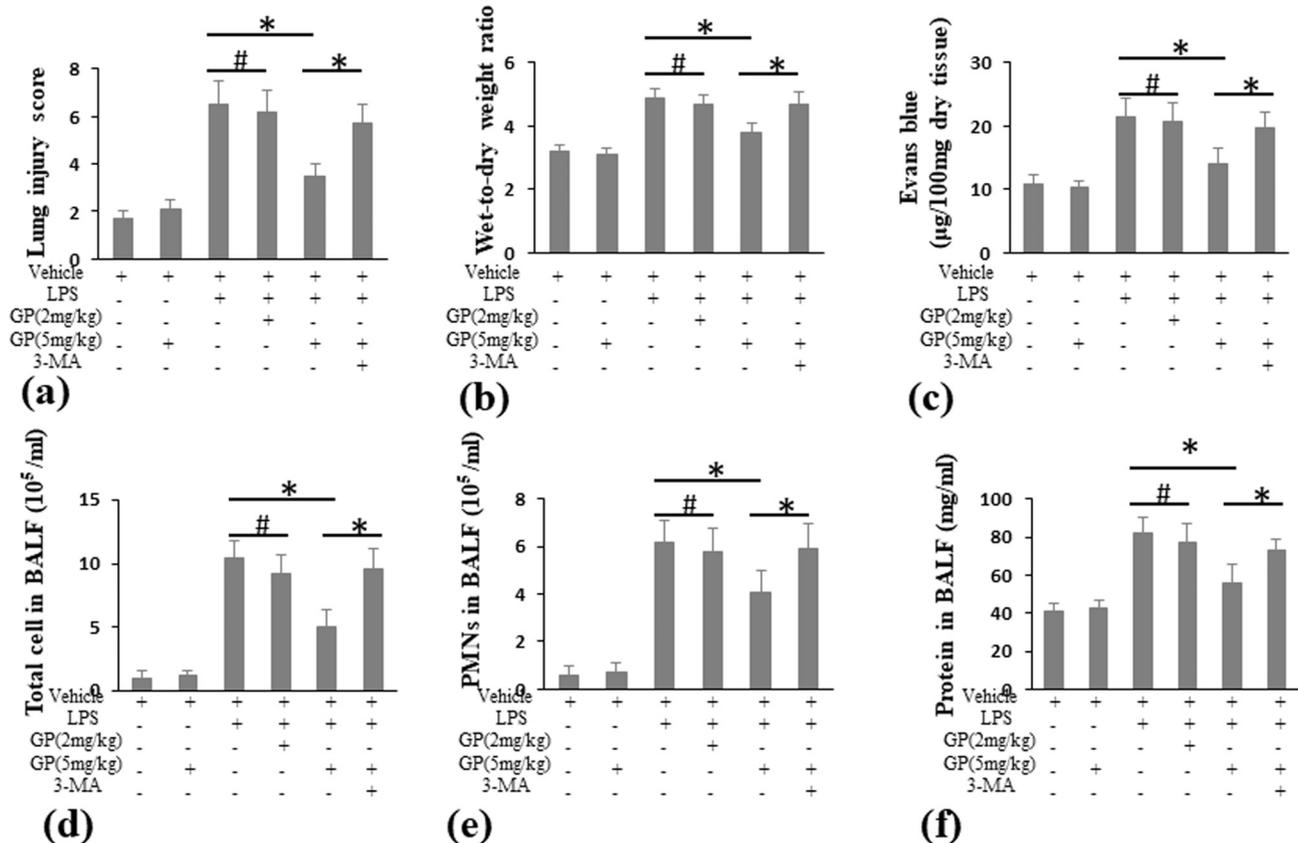


Fig. 3. Genipin treatment attenuates LPS-induced lung injury via autophagy.

Rats were pre-treated with genipin (2 mg/kg or 5 mg/kg) or a vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats were sacrificed 12 h following the administration of LPS. To investigate the role of autophagy on the protective effects of genipin, 3-MA (10 mg/kg), a specific inhibitor of autophagy, was administered simultaneously with genipin (5 mg/kg). (a) Lung injury score. (b) Wet lung/dry lung weight ratio. (c) Evans blue content in the lung. (d) Total cells in the BALF. (e) PMNs in the BALF. (f) Concentration of protein in the BALF. Data are presented as the mean \pm SD (n = 6 per group). *P < 0.05 vs. the indicated groups; #P > 0.05 vs. the indicated groups.

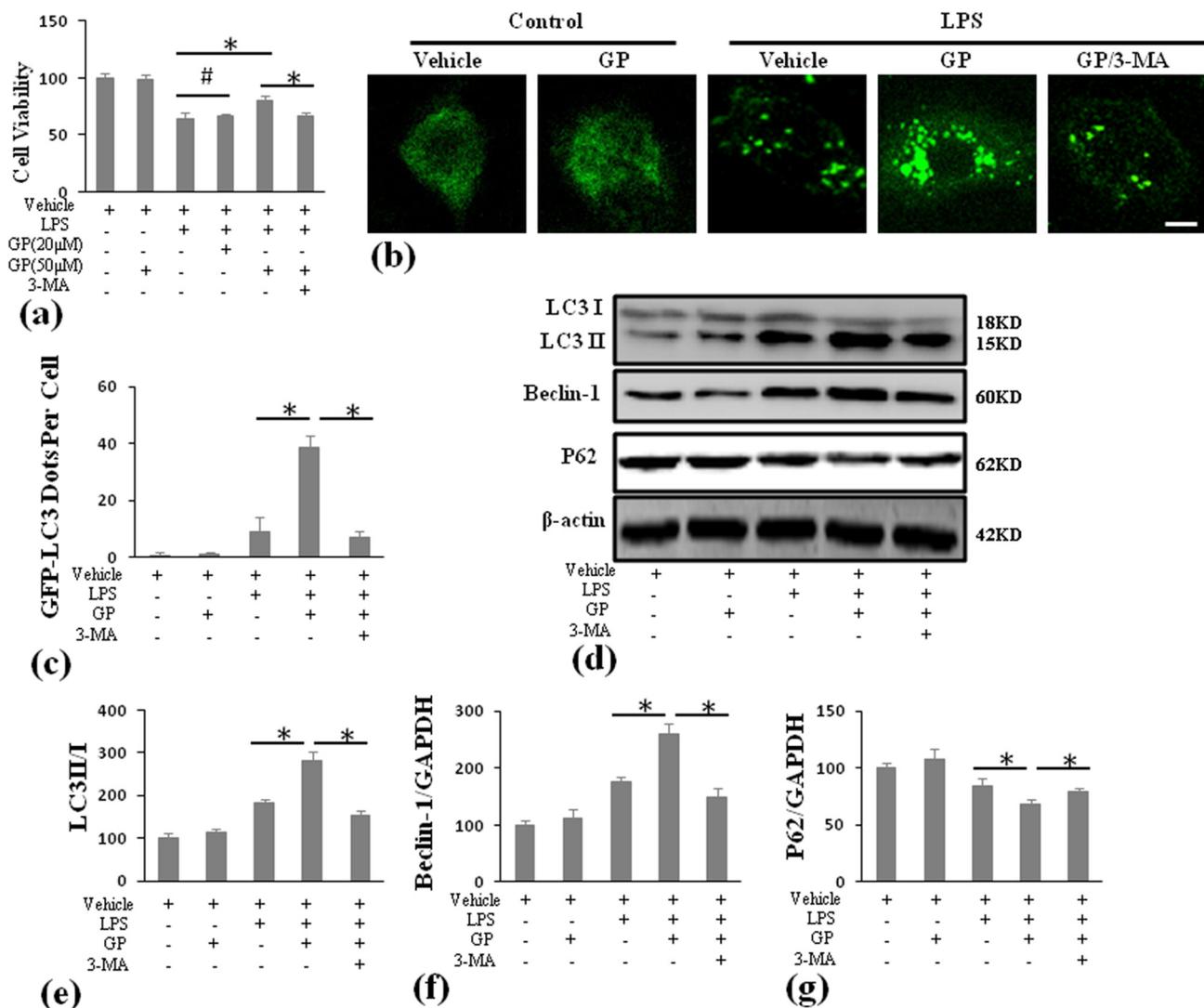


Fig. 4. Genipin treatment up-regulates autophagy.

(a) A549 cells pre-treated with either genipin (20 μ M or 50 μ M) or a vehicle (DMSO) for 60 min were incubated with LPS (0.5 mM) for 6 h. To test the role of autophagy on the genipin-induced protective effects on cell viability, 3-MA (5 mM) simultaneously with genipin (50 μ M) was administered. The cell viability was detected with an MTT assay. (b) A549 cells pre-treated with genipin (50 μ M) or a vehicle (DMSO) for 60 min were incubated with LPS (0.5 mM) for 6 h. To prevent genipin-induced autophagy, 3-MA (5 mM) was administered simultaneously with genipin (50 μ M). GFP-LC3 puncta formation was visualized by confocal microscopy (scale bars: 10 μ m). (c) Quantitative analysis of the number of GFP-LC3 puncta per cell. (d) Rats were pre-treated with genipin (5 mg/kg) or a vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats were sacrificed 12 h following the administration of LPS. To prevent genipin-induced autophagy, 3-MA (10 mg/kg) was administered simultaneously with genipin (5 mg/kg). The expression of P62, LC3, and Beclin-1 protein in the lung was measured by Western blot. (e) Quantification of Beclin-1 protein expression by densitometry. (f) Quantification of LC3II/I protein expression by densitometry. (g) Quantification of P62 protein expression by densitometry. Data are presented as the mean \pm SD (n = 6 per group). *P < 0.05 vs. the indicated groups.

were sacrificed 12 h following the administration of LPS. To prevent genipin-induced autophagy, 3-MA (10 mg/kg) was administered simultaneously with genipin (5 mg/kg). The level of Beclin-1, P62, and LC3 expression in the lung was determined *in vivo*. The level of Beclin-1 and LC3-II expression was increased and P62 expression was decreased in the lungs of the ALI-challenged rats that received genipin treatment compared to those without genipin treatment. These findings indicate that genipin treatment enhanced the autophagy response in rats with LPS-induced ALI (Fig. 4d–g). Genipin-induced autophagy was substantially inhibited following treatment with 3-MA.

3.3. Genipin treatment prevents LPS-induced apoptosis and inflammation by up-regulating autophagy

To investigate the protective effects of genipin *in vitro*, A549 cells pre-treated with genipin (50 μ M) or a vehicle (DMSO) for 60 min were

incubated with LPS (0.5 mM) for 6 h. To prevent genipin-induced autophagy, 3-MA (5 mM) was administered simultaneously with genipin (50 μ M). The mitochondrial membrane potential (MMP) and level of cellular ATP were measured to reflect the degree of mitochondrial function. A potential-sensitive fluorescent dye, JC-1, was used to detect the MMP. The color of this dual-emission probe changes from red-orange to green as the mitochondrial membrane is depolarized. We found that LPS exposure rapidly caused MMP dissipation, as indicated by increased green fluorescence and the simultaneous disappearance of red fluorescence. Genipin treatment significantly improved the LPS-induced changes in MMP, as evidenced by the suppression of green fluorescence and retention of red fluorescence (Fig. 5a and b). Moreover, the level of intracellular ATP was decreased in the LPS-treated cells compared to the control cells (Fig. 5c), whereas treatment with genipin resulted in increased levels of ATP. These data suggest that genipin prevents LPS-induced mitochondrial dysfunction. However,

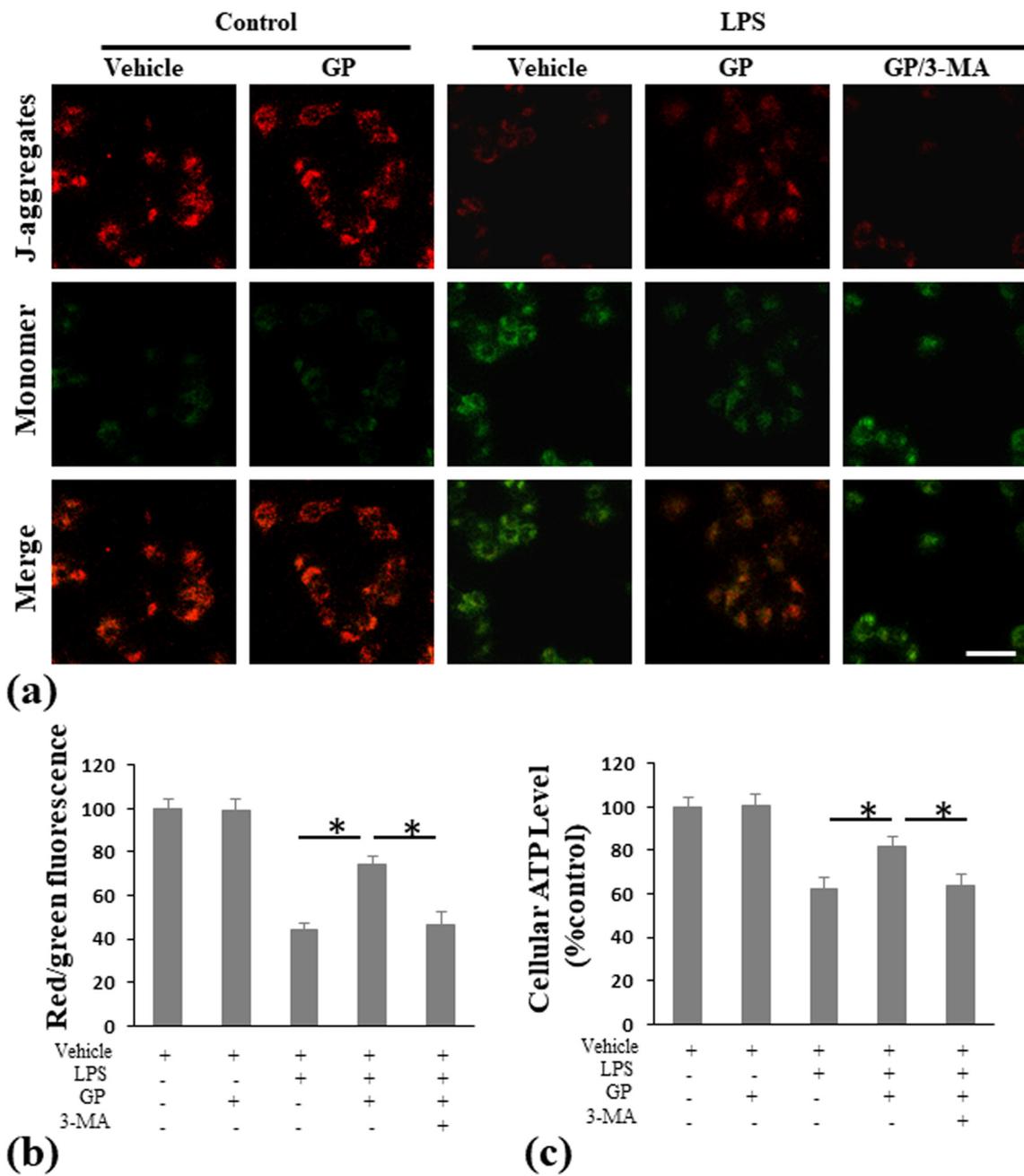


Fig. 5. Genipin attenuates LPS-induced mitochondrial dysfunction via autophagy.

A549 cells pre-treated with genipin (50 μM) or a vehicle (DMSO) for 60 min were incubated with LPS (0.5 mM) for 6 h. To prevent genipin-induced autophagy, 3-MA (5 mM) was administered simultaneously with genipin (50 μM). (a) The cells were stained with JC-1 and the MMP was observed using laser confocal-scanning microscopy (scale bars: 50 μm). (b) Quantification of the intracellular red and green fluorescence of JC-1. (c) The level of cellular ATP was measured using a luciferase-based assay kit. Data are presented as the mean ± SD (n = 6 per group). *P < 0.05 vs. the indicated groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

these protective effects were prevented by treatment with 3-MA.

To investigate the protective effects of genipin on ALI, rats were pre-treated with genipin (5 mg/kg) or a vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats were sacrificed 12 h following LPS administration. To prevent genipin-induced autophagy, 3-MA (10 mg/kg) was administered simultaneously with genipin (5 mg/kg). The importance of apoptosis in the pathogenesis of ALI has been widely studied [20]. In the present study, Bax was up-regulated, Bcl-2 was down-regulated, and TUNEL-positive cells were increased in the lungs of the ALI-treated rats, indicating that apoptosis was up-regulated in ALI. Genipin treatment substantially mitigated the LPS-induced apoptosis, as evidenced by decreased Bax expression,

increased Bcl-2 expression, and decreased number of TUNEL-positive cells in the lung compared to ALI-challenged rats not treated with genipin. However, apoptosis was up-regulated in the rats that simultaneously received genipin and 3-MA treatment. These findings suggest that inhibiting autophagy prevents genipin-mediated protection against apoptosis in ALI (Fig. 6a and b; Fig. 7).

Oxidative stress is one of the most important mediators of mitochondrial dysfunction and apoptotic signaling [21]. We found that genipin treatment remarkably attenuated LPS-induced oxidative stress as increased SOD content and decreased MDA content was observed in the lung. These indicators of oxidative stress were improved following genipin treatment; however, 3-MA treatment reversed the genipin-

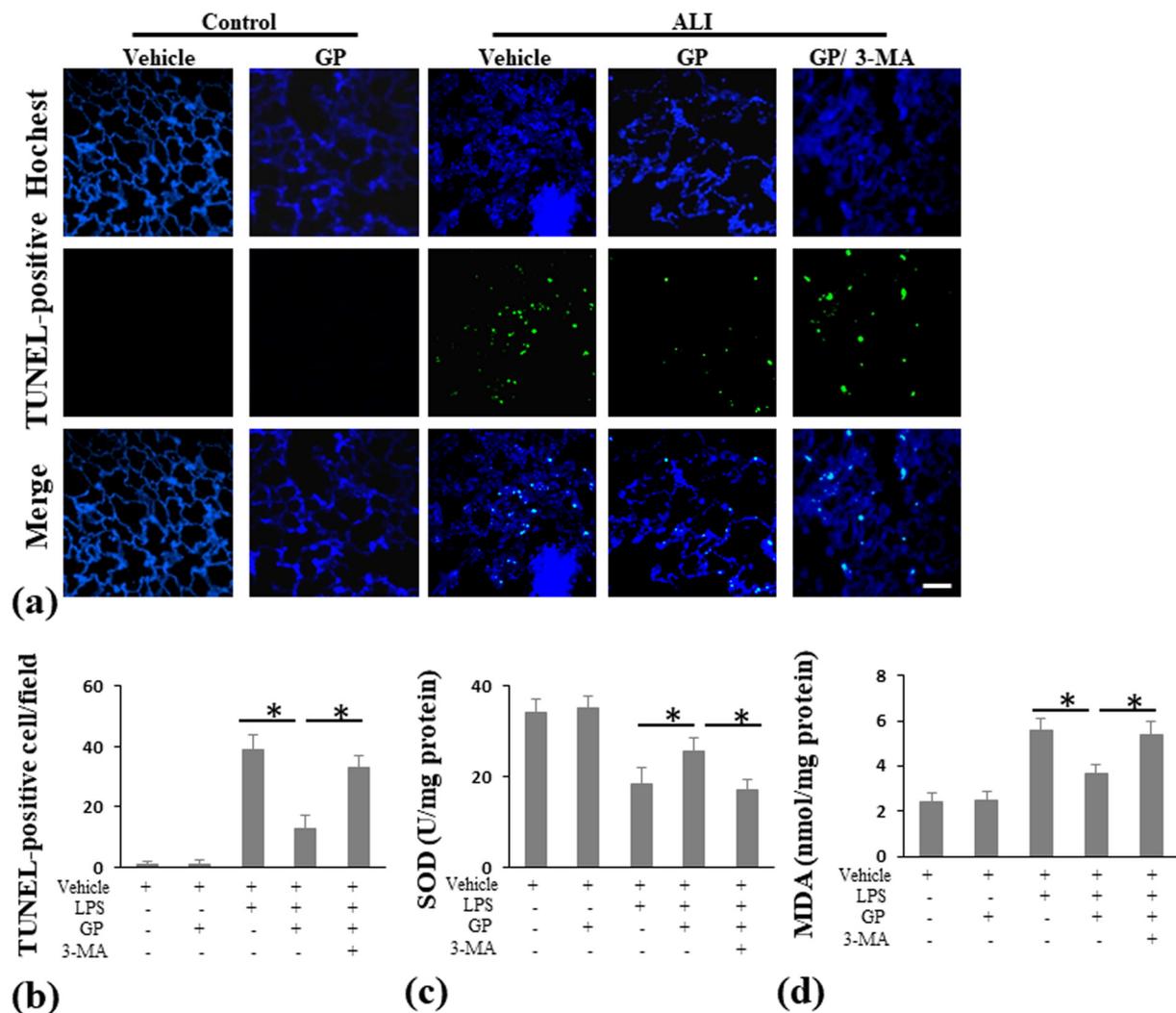


Fig. 6. Genipin attenuates LPS-induced lung cell apoptosis and oxidative stress via autophagy.

Rats were pre-treated with genipin (5 mg/kg) or a vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats were sacrificed 12 h following the administration of LPS. To prevent genipin-induced autophagy, 3-MA (10 mg/kg) was administered simultaneously with genipin (5 mg/kg). (a) Pulmonary cell apoptosis in the lung were measured by TUNEL staining (scale bar: 500 μ m); (b) Quantification of TUNEL-positive cells per field. (c) The SOD content in the lung. (d) The MDA content in the lung. Data are presented as the mean \pm SD (n = 6 per group). *P < 0.05 vs. the indicated groups.

mediated protective effects against LPS-induced oxidative stress (Fig. 6c and d).

Considering the key role of inflammatory mediators in ALI [22], we next measured the level of inflammatory mediators in the lung tissue and BALF. We detected substantially increased levels of TNF- α , IL-1 β , and IL-6 in the lungs and BALF of ALI-challenged rats compared to those in the control group. However, genipin treatment significantly suppressed the levels of TNF- α , IL-1 β , and IL-6 in the lungs and BALF in the ALI-challenged rats. Interestingly, increased levels of TNF- α , IL-1 β , and IL-6 were detected in the lungs and BALF of ALI-challenged rats that were simultaneously treated with genipin and 3-MA compared with those that received genipin treatment alone. These results suggest that inhibiting autophagy prevents the protective effects of genipin against ALI-associated inflammation (Fig. 8).

4. Discussion

Autophagy is a highly conserved catabolic process involving the autolysosomal degradation of cellular components, including protein aggregates, damaged organelles, as well as various pathogens [23,24]. Accumulating evidence suggests that autophagy is stimulated in response to a diverse range of stimuli associated with ALI, including

bacterial infection, lipopolysaccharide (LPS), sepsis, hyperoxia, and chlorine [14]. In the present study, we demonstrate that LPS stimulation activates the autophagic response. Recent studies have reported that the downregulation of autophagy may lead to ALI deterioration following sepsis, and enhancing or restoring autophagy after sepsis results in improved survival in rats [15,25]. Thus, the role of autophagy in the dysfunction of multiple organs during sepsis makes autophagy an attractive therapeutic target for sepsis. Genipin, an aglycone derived from an iridoid glycoside called geniposide, is a major component of the fruit of *Gardenia jasminoides*, which has been widely used for the treatment of inflammatory diseases and hepatic disorders in traditional medicine [8,26,27]. In addition, it has been previously reported that genipin modulates NLRP3 inflammasome activation and IL-1 β release in human macrophages [8]. Moreover, the protective effects of genipin against LPS-induced inflammation in ALI has also been demonstrated [10]. Furthermore, our previous study demonstrated that genipin inhibited vascular hyperpermeability by up-regulating autophagy [19]. However, the effect of genipin on autophagy in ALI remains poorly understood. In the present study, we found that treatment with genipin up-regulates autophagy-related proteins, including the up-regulation of Beclin-1 and LC3-II, down-regulates P62 in vivo, and increases GFP-LC3 punctae formation in vitro, indicating enhanced autophagy. Although

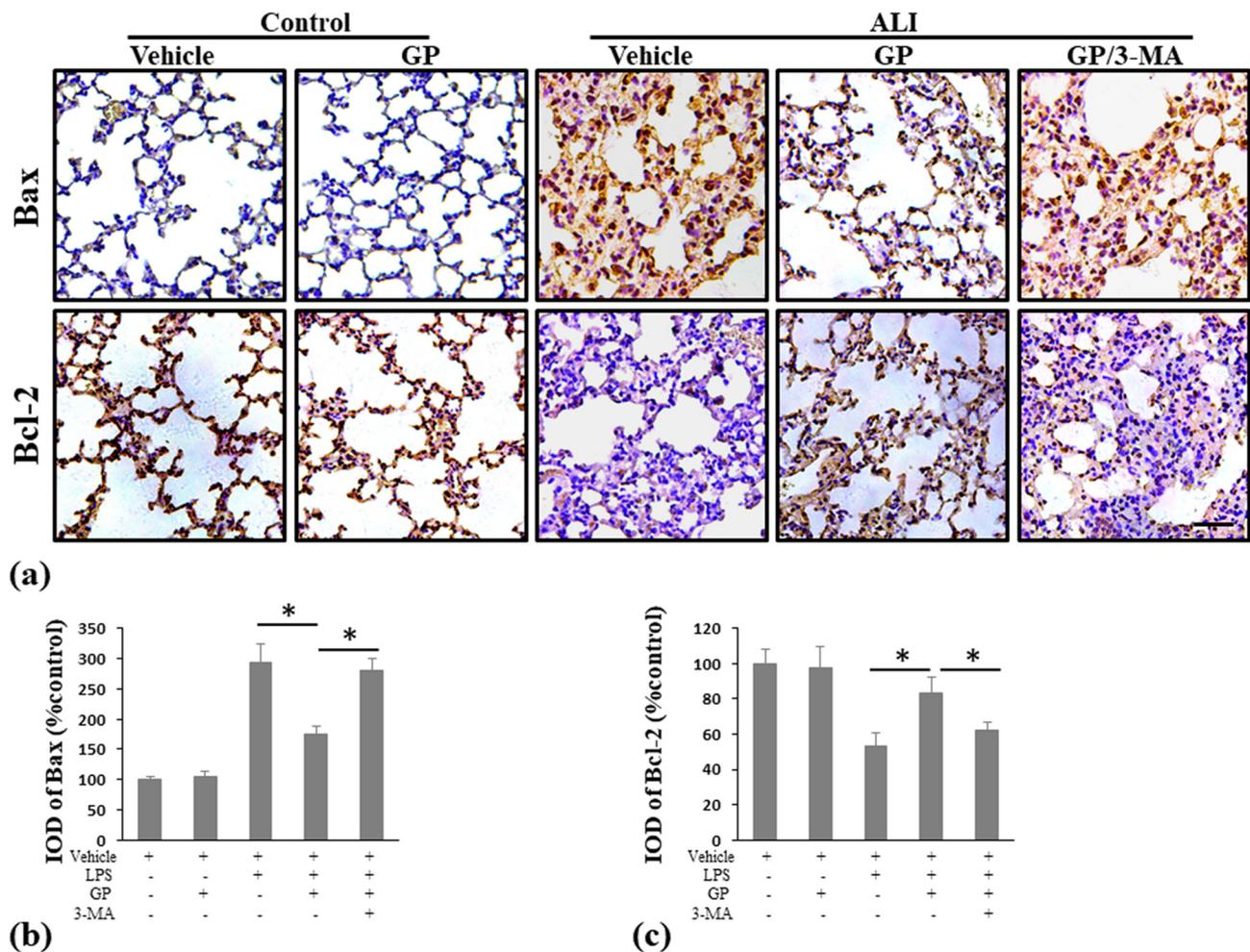


Fig. 7. Genipin prevents the LPS-induced up-regulation of Bax and down-regulation of Bcl-2 via autophagy.

Rats were pre-treated with genipin (5 mg/kg) or a vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats were sacrificed 12 h following the administration of LPS. To prevent genipin-induced autophagy, 3-MA (10 mg/kg) was administered simultaneously with genipin (5 mg/kg). (a) The level of Bax and Bcl-2 protein expression was determined by immunohistochemistry (scale bar: 500 μm); (b) The IOD of Bax; (c) The IOD of Bcl-2. Data are presented as the mean ± SD (n = 6 per group). *P < 0.05 vs. the indicated groups.

our previous study demonstrated that genipin-mediated up-regulation of autophagy was achieved via Sirtuin-3 activation [19], the mechanism by which genipin promotes autophagy in ALI requires further investigation.

An increasing number of studies suggest that autophagy may exert protective effects on the initiation and progression of ALI [14]. Moreover, previous studies have reported that the loss of autophagy-related (ATG) genes (e.g., Atg7, Atg5, and Atg4b) significantly aggravate the development of ALI in mice [13,28]. It has been reported that rapamycin-mediated activation of autophagy exerts a protective effect against LPS-induced ALI by restoring autophagy [15]. However, whether genipin protects against ALI by mediating autophagy remains unclear. In the present study, we found that 3-MA, a specific autophagy inhibitor, markedly reversed the protective effects of genipin, suggesting the involvement of autophagy in the mechanism of genipin-mediated ALI.

In our past studies, we have demonstrated an important role of apoptosis in the pathogenesis of ALI [1,4]. Although the protective effect of genipin against ALI-associated inflammation in ALI has been previously studied, the effects of genipin on apoptosis remains unknown. According to one theory that links autophagy to apoptosis, autophagy is thought to eliminate aged and dysfunctional cellular organelles or denatured proteins to prevent cellular apoptosis [29–31]. In the present study, we found that treatment with genipin reduced LPS-

induced apoptosis in ALI, which was inhibited by 3-MA. These findings indicate that genipin alleviates apoptosis through autophagy in LPS-induced ALI. It has been well-established that oxidative stress plays a vital role in mediating programmed cell death (apoptosis or even necrosis) [21,32]. We also found that genipin significantly reduced LPS-induced oxidative stress via autophagy in ALI.

Mitochondria are pivotal for maintaining the health and proper function of the lung and the importance of mitochondria in the pathogenesis of ALI has been widely studied [33–35]. However, the effects of genipin on mitochondrial dysfunction in ALI remains unclear. Aged and dysfunctional cellular organelles, including dysfunctional mitochondria, can be removed by autophagy, which is necessary for maintaining the quality control of mitochondria and cellular homeostasis [13]. An increasing number of studies have demonstrated that removing dysfunctional or damaged mitochondria by mitochondrial autophagy maintains a healthy population of mitochondria, and largely plays a cytoprotective role in the context of disease pathogenesis [36,37]. In the present study, we found that genipin prevented LPS-induced mitochondrial dysfunction by enhancing autophagic flux.

Numerous studies have reported that an intrapulmonary inflammatory response that includes the release of proinflammatory cytokines is an important characteristic in the process of ALI [38,39]. Our previous study reported that the inhibition of these factors, including TNF-α, IL-1β, and IL-6, prevents the development of ALI [1]. In

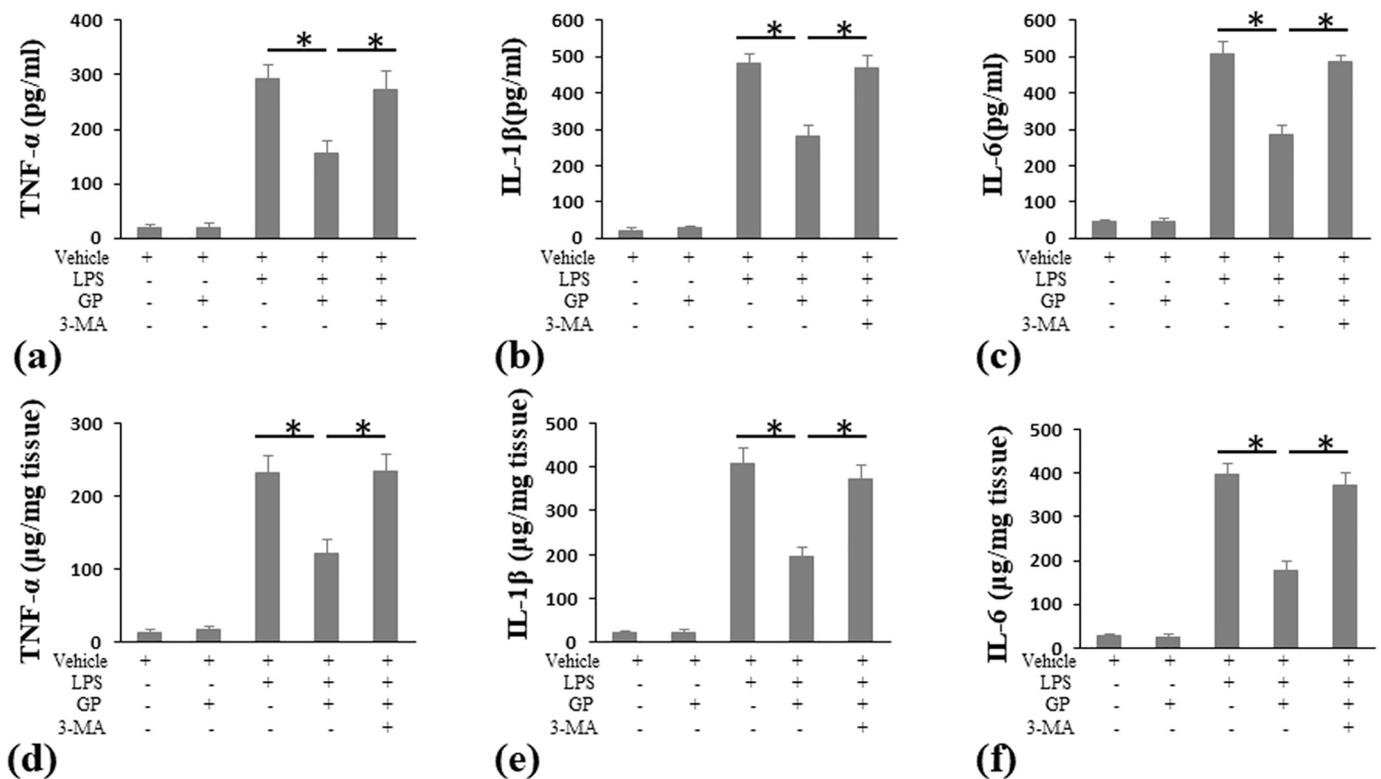


Fig. 8. Genipin attenuates LPS-induced lung inflammation via autophagy.

Rats were pre-treated with genipin (5 mg/kg) or a vehicle (DMSO) for 60 min, followed by an intratracheal administration of LPS (5 mg/kg). The rats were sacrificed 12 h following the administration of LPS. To prevent genipin-induced autophagy, 3-MA (10 mg/kg) was administered simultaneously with genipin (5 mg/kg). (a) The level of TNF- α in the BALF. (b) The level of IL-1 β in the BALF. (c) The level of IL-6 in the BALF. (d) The level of TNF- α in the lung tissue. (e) The level of IL-1 β in the lung tissue. (f) The level of IL-6 in the lung tissue. Data are presented as the mean \pm SD (n = 6 per group). *P < 0.05 vs. the indicated groups.

addition, it has been shown that genipin protects against LPS-induced acute systemic inflammation and CLP-induced sepsis [8]. While it has been reported that genipin inhibits inflammation in LPS-induced ALI [10], the precise mechanism remains unclear. Previous studies have reported that autophagy can also regulate the inflammasome during sepsis [14]. Moreover, an Atg7 deficiency has been found to significantly intensify inflammasome activation, leading to impaired pathogen clearance and aggravated lung injury [40]. Thus, enhancing autophagy could inhibit the inflammatory reaction and attenuate myocardial ischemia reperfusion-induced ALI in diabetic rats [41]. In the present study, we demonstrated that genipin mitigates the LPS-induced inflammatory response in ALI via autophagy.

In summary, we have demonstrated that genipin attenuated LPS-induced ALI using a rat model. Moreover, we demonstrated that such protection afforded by genipin was mediated by ameliorating apoptosis and inflammation via the up-regulation of autophagy. However, the precise mechanism by which genipin mediates autophagy requires further investigation.

Acknowledgements

This study was supported by the Natural Science Foundation of Hunan Province (grant numbers: 2018JJ6004, 2018JJ3015) and the National Natural Science Foundation of China (grant numbers: 81500066, 81601708).

Conflict of interest

None.

References

- [1] 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.
- [2] N.S. Sharma, C.V. Lal, J.D. Li, X.Y. Lou, L. Viera, T. Abdallah, et al., The neutrophil chemoattractant peptide proline-glycine-proline is associated with acute respiratory distress syndrome, *Am. J. Physiol. Lung Cell. Mol. Phys.* 315 (2018) L653–L661.
- [3] C. Brandenberger, K.M. Kling, M. Vital, M. Christian, The role of pulmonary and systemic immunosenescence in acute lung injury, *Aging Dis.* 9 (2018) 553–565.
- [4] 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.
- [5] J. Park, Y. Chen, M. Zheng, J. Ryu, G.J. Cho, Y.J. Surh, et al., Pterostilbene 4'-beta-glucoside attenuates LPS-induced acute lung injury via induction of heme oxygenase-1, *Oxidative Med. Cell. Longev.* 2018 (2018) 2747018.
- [6] W. Xie, Q. Lu, K. Wang, J. Lu, X. Gu, D. Zhu, et al., miR-34b-5p inhibition attenuates lung inflammation and apoptosis in an LPS-induced acute lung injury mouse model by targeting progranulin, *J. Cell. Physiol.* 233 (2018) 6615–6631.
- [7] L. Shao, D. Meng, F. Yang, H. Song, D. Tang, Irisin-mediated protective effect on LPS-induced acute lung injury via suppressing inflammation and apoptosis of alveolar epithelial cells, *Biochem. Biophys. Res. Commun.* 487 (2017) 194–200.
- [8] 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.
- [9] A.M. Leidal, B. Levine, J. Debnath, Autophagy and the cell biology of age-related disease, *Nat. Cell Biol.* 20 (2018) 1338–1348.
- [10] 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.
- [11] V. Rajanbabu, L. Galam, J. Fukumoto, J. Enciso, P. Tadikonda, T.N. Lane, et al., Genipin suppresses NLRP3 inflammasome activation through uncoupling protein-2, *Cell. Immunol.* 297 (2015) 40–45.
- [12] Y.C. Chung, J.H. Lim, H.M. Oh, H.W. Kim, M.Y. Kim, E.N. Kim, et al., Calcimimetic restores diabetic peripheral neuropathy by ameliorating apoptosis and improving autophagy, *Cell Death Dis.* 9 (2018) 1163.
- [13] S. Aggarwal, P. Mannam, J. Zhang, Differential regulation of autophagy and mitophagy in pulmonary diseases, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 311 (2016) L433–L452.
- [14] K. Wang, Y. Chen, P. Zhang, P. Lin, N. Xie, M. Wu, Protective features of autophagy in pulmonary infection and inflammatory diseases, *CELLS-BASEL.* 8 (2019) 123.
- [15] Y.T. Yen, H.R. Yang, H.C. Lo, Y.C. Hsieh, S.C. Tsai, C.W. Hong, et al., Enhancing autophagy with activated protein C and rapamycin protects against sepsis-induced

- acute lung injury, *SURGERY* 153 (2013) 689–698.
- [16] X. Yang, T. Jing, Y. Li, Y. He, W. Zhang, B. Wang, et al., Hydroxytyrosol attenuates LPS-induced acute lung injury in mice by regulating autophagy and sirtuin expression, *Curr. Mol. Med.* 17 (2017) 149–159.
- [17] F. Liu, C. Nie, N. Zhao, Y. Wang, Y. Liu, Y. Li, et al., MiR-155 alleviates septic lung injury by inducing autophagy via inhibition of transforming growth factor-beta-activated binding protein 2, *SHOCK* 48 (2017) 61–68.
- [18] I. de Laverre, A.D. Pavon, M.V. Paz, M. Oropesa-Avila, M. de la Mata, E. Alcocer-Gomez, et al., The connections among autophagy, inflammasome and mitochondria, *Curr. Drug Targets* 18 (2017) 1030.
- [19] 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 Dis.* 4 (2018) 52.
- [20] K. Li, Z. He, X. Wang, M. Pineda, R. Chen, H. Liu, et al., Apigenin C-glycosides of *Microcos paniculata* protects lipopolysaccharide induced apoptosis and inflammation in acute lung injury through TLR4 signaling pathway, *Free Radic. Biol. Med.* 124 (2018) 163–175.
- [21] K. Sinha, J. Das, P.B. Pal, P.C. Sil, Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis, *Arch. Toxicol.* 87 (2013) 1157–1180.
- [22] X. Zhang, R. Zhuang, H. Wu, J. Chen, F. Wang, G. Li, et al., A novel role of endocan in alleviating LPS-induced acute lung injury, *Life Sci.* 202 (2018) 89–97.
- [23] L. Lin, L. Zhang, L. Yu, L. Han, W. Ji, H. Shen, et al., Time-dependent changes of autophagy and apoptosis in lipopolysaccharide-induced rat acute lung injury, *Iran J. Basic Med. Sci.* 19 (2016) 632–637.
- [24] D.L.V.M. Rojo, M. Dodson, C. Gross, H.M. Mansour, R.C. Lantz, E. Chapman, et al., Role of Nrf2 and autophagy in acute lung injury, *Curr. Pharmacol. Rep.* 2 (2016) 91–101.
- [25] W. Dong, B. He, H. Qian, Q. Liu, D. Wang, J. Li, et al., RAB26-dependent autophagy protects adherens junctional integrity in acute lung injury, *AUTOPHAGY* 14 (2018) 1677–1692.
- [26] J. Li, J. Shi, P. Li, X. Guo, T. Wang, A. Liu, Genipin attenuates hyperoxia-induced lung injury and pulmonary hypertension via targeting glycogen synthase kinase-3 beta in neonatal rats, *NUTRITION* 57 (2019) 237–244.
- [27] Z. Li, T.B. Zhang, D.H. Jia, W.Q. Sun, C.L. Wang, A.Z. Gu, et al., Genipin inhibits the growth of human bladder cancer cells via inactivation of PI3K/Akt signaling, *Oncol. Lett.* 15 (2018) 2619–2624.
- [28] Z. Li, Y. Wu, X. Xu, J. Zhou, Y. Wang, H. Shen, et al., Autophagy as a double-edged sword in pulmonary epithelial injury: a review and perspective, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 313 (2017) L207–L217.
- [29] M.G. Kemp, Crosstalk between apoptosis and autophagy: environmental genotoxins, infection, and innate immunity, *Journal of Cell Death* 2017 (2017) 2114263837.
- [30] S. Song, J. Tan, Y. Miao, M. Li, Q. Zhang, Crosstalk of autophagy and apoptosis: involvement of the dual role of autophagy under ER stress, *J. Cell. Physiol.* 232 (2017) 2977–2984.
- [31] G. Qian, D. Liu, L. Hou, M. Hamid, X. Chen, F. Gan, et al., Ochrotoxin A induces cytoprotective autophagy via blocking AKT/mTOR signaling pathway in PK-15 cells, *Food Chem. Toxicol.* 122 (2018) 120–131.
- [32] S. Sifuentes-Franco, D.E. Padilla-Tejeda, S. Carrillo-Ibarra, A.G. Miranda-Díaz, Oxidative stress, apoptosis, and mitochondrial function in diabetic nephropathy, *Int. J. Endocrinol.* 2018 (2018) 1875813–1875870.
- [33] L. Zhang, W. Wang, B. Zhu, X. Wang, Epithelial mitochondrial dysfunction in lung disease, *Adv. Exp. Med. Biol.* 1038 (2017) 201.
- [34] B.B. Chen, T.A. Coon, J.R. Glasser, C. Zou, B. Ellis, T. Das, et al., E3 ligase subunit Fbxo15 and PINK1 kinase regulate cardiolipin synthase 1 stability and mitochondrial function in pneumonia, *Cell Rep.* 7 (2014) 476–487.
- [35] L.L. Zhu, M.Q. Li, F. He, S.B. Zhou, W. Jiang, Mitochondria targeted peptide attenuates mitochondrial dysfunction, controls inflammation and protects against spinal cord injury-induced lung injury, *Cell. Physiol. Biochem.* 44 (2017) 388–400.
- [36] K. Palikaras, E. Lionaki, N. Tavernarakis, Mechanisms of mitophagy in cellular homeostasis, physiology and pathology, *Nat. Cell Biol.* 20 (2018) 1013–1022.
- [37] M. Redmann, M. Dodson, M. Boyer-Guittaut, V. Darley-Usmar, J. Zhang, Mitophagy mechanisms and role in human diseases, *Int. J. Biochem. Cell Biol.* 53 (2014) 127–133.
- [38] Q. Zhang, S. Zhu, X. Cheng, C. Lu, W. Tao, Y. Zhang, et al., Euphorbia factor L2 alleviates lipopolysaccharide-induced acute lung injury and inflammation in mice through the suppression of NF-kappaB activation, *Biochem. Pharmacol.* 155 (2018) 444–454.
- [39] W. Li, R. Zhao, X. Wang, F. Liu, J. Zhao, Q. Yao, et al., Nobiletin ameliorated lipopolysaccharide-induced inflammation in acute lung injury by suppression of NF-kappaB pathway in vivo and vitro, *INFLAMMATION* 41 (2018) 996–1007.
- [40] P. Qin, G. Changpei, L. Rongpeng, L. Yi, T. Shirui, L. Xuefeng, et al., Atg7 deficiency intensifies inflammasome activation and pyroptosis in pseudomonas sepsis, *J. Immunol.* 198 (2017) 3205–3213.
- [41] L. Zhan, Y. Zhang, W. Su, Q. Zhang, R. Chen, B. Zhao, et al., The roles of autophagy in acute lung injury induced by myocardial ischemia reperfusion in diabetic rats, *J. Diabetes Res.* 2018 (2018) 5047526.