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Emodin reactivated autophagy and alleviated inflammatory lung injury in mice with lethal endotoxemia

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Abstract: An uncontrolled inflammation induced critical health problems with serious morbidity and death, which namely acute lung injury (ALI). Recently researchs have found the anti-inflammatory effects of emodin. Here, we investigated the potential effects of emodin on a mouse model with a lethal dose of the potential mechanisms and lipopolysaccharide (LPS)-induced inflammatory lung injury in mice. The pulmonary histological abnormalities, the Evans blue's leakage, the myeloperoxidase (MPO) activity, the grades of TNF-α, IL-6, nitric oxide (NO), lactic acid (LA) in lung tissues were determined 18 h post exposure of LPS. Based on the expression of LC3-II with BECN1 was determined using Western blotting. Besides, the LPS-exposed mice for survival rate was monitored. The results indicated that intervention with emodin was important for mitigating LPS-induced pulmonary histological change and LPS-induced leakage of Evans blue, which were associated with suppressed elevation of MPO activity and inhibited up-regulation of TNF-α, IL-6, NO with LA in lung tissues. Moreover, intervention with emodin enhanced the survival rate of LPS-exposed mice. Finally, therapy with emodin increased the LC3 and BECN1 in lungs of LPS-exposed mice. Treatment with 3-MA (the autophagy inhibitor) reversed the beneficial effects of emodin. In conclusion, emodin might provide pharmacological benefits in LPS-induced inflammatory lung injury, and the mechanisms might be related to the restoration of autophagy.

Key words: acute lung injury, anti-inflammatory, autophagy, emodin, lipopolysaccharide (LPS)

Introduction

Acute lung injury (ALI) with a severe situation, called Acute Respiratory Distress Syndrome (ARDS), is the universal critical disease with high death rate [24]. ALI could be induced by both direct harmful factors (pulmonary infection, inhalation injury, etc.) or indirect detrimental factors (sepsis, pancreatitis, blood transfusion,

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etc.) [2]. The pathological mechanisms underlying the development of ALI include an excessive activation of an inflammatory response, abnormal alterations of an alveolar capillary barrier, etc [5, 28, 33, 36]. Although great progress has been made in understanding the pathophysiology of ALI, ALI remains a life-threatening problem and exploration of novel reagents with beneficial effects in ALI attracts much research interests [39].

Emodin is an anthraquinone derivative that secluded from *Rheum palmatum* L., a herbal plant is majority used for traditional medicine of Chinese [1]. Recent studies have found that emodin might have multiple pharmacological activities and its anti-inflammatory effects contribute greatly to the therapeutic benefits of emodin under various pathological conditions [7, 29, 38]. In animal models with keratitis, nephritis and hepatitis, emodin suppressed the inflammatory response and alleviated tissue injury [4, 19, 48]. Furthermore, Studies have affirmed that the anti-inflammatory effects of emodin in microglia, macrophage and epithelial cells [16, 31, 45, 52]. Therefore, emodin has been suggested as a candidate for anti-inflammatory therapy [7].

Autophagy is essential cellular process involving evolutionarily conserved lysosomal degradation pathways and controlling of the removal of abnormally folded protein/aggregates and damaged organelles [26]. Lipopolysaccharide (LPS) could induce systemic inflammatory response very early (at 4 h) and reduce the expression of autophagy-related proteins (e.g. LC3-II, ATG2, RAB7) and this lasts up to 24 h. Recent studies reported that experimental induction of autophagy might exhibit a significant anti-inflammatory property [13, 46]. Therefore, a therapy targeting regulating autophagy might be a potential strategy for the treatment of acute lung injury induced by LPS. Numerous studies have demonstrated that emodin exerted a potential therapeutic effect by activating autophagy in colon cancer cells [41], cisplatin-induced nephrotoxicity of rat renal tubular cells [21], and in TNF-triggered cell death [12]. However, the role of emodin on autophagy in LPS-induced ALI remains undefined. Based on these studies, we hypothesized that emodin might play an active role in ALI and autophagy might be regulated by emodin to regulate its pulmonary protective effect.

LPS, the toxin from gram-negative bacteria, it is one of the major causes of systemic inflammation and tissue damage in almost all human organs [3]. Based on the unique histological and functional characters, the lung is the most susceptible organ in systemic inflammation [24]. In the current investigation, to research the protective actions and potential mechanisms of emodin in systemic inflammation-induced ALI, mice were uncovered to lethal dose of LPS with or without emodin administration. And then, the degree of inflammation, and the experimental animals' survival rate have been determined.

Materials and Methods

Drugs and reagents

LPS (*Escherichia coli*, 055: B5) and emodin (E7881, purity ≥90%) were bought from Sigma (St. Louis, MO, USA). The autophagy inhibitor 3-methyl-3H-purin-6-amine (3-MA) were the products from Cayman Chemical (Ann Arbor, MI, USA, 13242). The ELISA kits for judgement of mouse tumor necrosis factor alpha (TNF-α) and IL-6 were generated by NeoBioscience Technology Co. (Shenzhen, China). Myeloperoxidase (MPO) and lactic acid (LA) detection kits were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Nitric oxide (NO) detect kit was produced by Beyotime Institute of Biotechnology (Jiangsu, China). Evans Blue was the product from Molbase Institute of Biotechnology (Shanghai, China). The light Chain 3 (LC3) antibody was bought from Cell Signaling Technology (Danvers, MA, USA), with BECN1 antibody from Proteintech (Chicago, IL, USA), GAPDH antibody from Bioss (Bioss, Beijing, China). The BCA protein detect kit, HRP-combined goat anti-rabbit immune response or defense, and increased chemiluminescence (ECL) reagents were gained from Thermo Fisher Scientific (Rockford, IL, USA).

Animals

Male BALB/c mice (6–8 weeks, 20–22 g) were purchased at the Center of Experimental Animals of Medical University in Chongqing (Chongqing, China). The mice were acclimated for seven days before experimentation. Male mice were defended in a certain pathogen-free laboratory. Mice were associated with a criterion laboratory diet and water with a 12/12h light/dark cycle. The whole experimental programs relating creatures were approved by the Animal Care and Use Committee of Chongqing Medical University.

Experimental protocols

LPS (15 mg/kg, dissolved in neutral buffered saline, i.p.) was injected intraperitoneally into mice to induced lethal endotoxemia and lung injury. The mice were stochastically assigned to 4 groups (n=8 per group): 1) the control group, mice received vehicle injection; 2) the emodin control group, mice received emodin (20 mg/kg, dissolved in dimethylsulfoxide (DMSO) and finally dissolved in the appropriate medium, the final concentration of DMSO was 0.1%, i.p.) only; 3) the LPS group, mice with LPS-induced lethal endotoxemia and lung injury; 4) the LPS + emodin intervention group, LPS-expose mice received emodin 30 min before LPS challenge; 5) the LPS+ emodin+ 3MA inervention group, 3-MA (15 mg/kg, dissolved in dimethylsulfoxide (DMSO) and finally dissolved in the appropriate medium, the final concentration of DMSO was 0.1%, i.p.) was co-administered with the emodin 30 min before LPS challenge in LPS-expose mice. The plasma and lungs sample were collected 18 h post LPS exposure for further experiments.

Histopathological analysis

Lung samples were removed from the mice 18 h post LPS exposure. The left lungs were inserted in paraffin after putting in 10% neutral-buffered formalin solution. Microphotographs of hematoxylin&eosin-stained sections were caught with using a light microscope (Olympus, Tokyo, Japan) for conventional morphological evaluation.

Evans blue leakage

To evaluate the pulmonary barrier permeability, another set of animals were allocated (n=8 per group). In addition to the treatment described above, Evans blue (2%, 4 ml/kg) was injected via the caudal vein 0.5 h prior to the end of the experiment. And then, the animals were transcardially spread free of blood with sterile normal saline, and the lungs were rapidly removed. The sample was then weighed, homogenized in N-dimethylformamide (DMF), and hatched for 72 h at 60°C. In 30 min, centrifuge the specimens at 1,000 × g. There was assess the optical thickness of the supernatant at 632 nm and based on the standard absorbance curves of Evans blue to calculate the amount of Evans blue.

MPO activity determination

The right lung's lower lobes from each group were assessed for MPO levels, clean the frozen lung tissues with sterile PBS, weighed, and homogenized with phosphate buffer involving 0.5% hexadecyltrimethylammonium bromide. Use a BCA protein assay kit (Pierece Biotechnology, Rockford, IL, USA) to calculate the protein concentrations of the lysate and increase the protein lysate's equal measure to each well. Through the manufacturer's instructions, using the MPO invention kit to determine the enzyme activity spectrophotometrically (Nanjing Jiancheng, China). According to the absorbance to calculate the MPO activity at 450 nm, and standardized by the protein concentration of each sample.

Determination of TNF-α and IL-6

The right lung's lower lobes from each group were assessed for the standards of TNF-α and IL-6, based on the manufacturer's instructions, using the ELISA kits to determine the levels of TNF-α and IL-6 in lung tissue (NeoBioscience Technology Co., China). In brief, pipette the standards or samples into a microplate pre-coated with a monoclonal antibody particular against mouse TNF-α or IL-6. Increase an enzyme-linked antibody particular for mouse IL-6 or TNF-α or IL-6 to the wells after washing away any unbound substances. After washing step, increase a substrate solution to the wells. When adding the stop solution, the enzyme response yielded a blue product that turned yellow. The optical density in the wells was assessed at 450 nm and based on the standard absorbance curves to calculate the concentration of TNF-α or IL-6. And then, the value was standardized by the total protein's amount of each sample.

NO measurement

The lower lobes of the right lung from each group were assessed for NO levels, analyze the concentrations of NO in lung tissue with a NO colorimetric assay kit according to the Griess reaction (Beyotime Institute of Biotechnology, China).The concentration of NO was calculated based on the absorbance calculated at 540 nm and standardized by the total protein's amount of each sample.

LA measurement

The lower lobes of the right lung from each group were assessed for LA levels, the levels of LA in the lung tissue were analyzed with a LA assay kit by the manufacturer's instructions (Nanjing Jiancheng, China). The concentration of LA was calculated according to the

absorbance measured at 530 nm and normalized by the amount of total protein of each sample.

Analysis of Western blot

Each lung sample can extract proteins (40 μg), and determine the protein concentration with using the BCA method. Use 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to separate the protein draws and then transfers it onto nitrocellulose membranes. The membranes were first hatched with particular major antibodies after hatch with the blocking buffer (containing 5% no-fat dry milk, 10 Mm Tris–HCl, 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature: LC3 antibody, BECN1 antibody or GAPDH antibody overnight at 4°C, then hatch with the suitable HRP-conjugated secondary antibody for 2 h at room temperature. And finally, Protein bands were abstracted using the ChemiDoc Touch Imaging system (Bio-Rad Laboratories, Hercules, CA, USA) with the enhanced chemiluminescence system (Thermo Fisher Scientific).

Analysis of survival

The potential effects of emodin were determined on the mortality of LPS-challenged mice, another set of animals were allocated (n=20 per group). The experimental animal's survival was monitored in every 6 h for seven days and the survival rate calculated using the Kaplan-Meier method.

Analysis of statistical

Each experiment was repeated a minimum of 4 times. All results were normalized to untreated controls and were represented as mean ± SD. All data were represented as mean ± SD. Statistical Package was used to analyze data for Social Sciences (SPSS) software (version 19). The ANOVA was used to perform the comparisons of statistical with Turkey's post hoc test. The function of a log-rank test was for comparisons of the survival curve. $P<0.05$ was considered to express a statistically great disparity in values.

Results

Emodin alleviated lung injury, pulmonary vascular permeability and improved the LPS-exposed mice survival rate in LPS-exposed mice

In the present study, we first determined the effect of emodin on the pulmonary histological examination of mice with LPS-induced sepsis. The histological examination showed that LPS exposure induced significant abnormalities in lung tissue, including extensive leukocyte infiltration and pulmonary edema, these histopathological alterations were alleviated in emodin-intervention group. 3-MA, inhibition of autophagy, blocked the protective effects of emodin on pulmonary histological abnormalities in LPS-exposed mice (Fig. 1A). Next, we determined whether the pulmonary vascular permeability is modulated by emodin in LPS-exposed mice. Following LPS expose, the leakage of Evans blue was enhanced, suggests that LPS exposure might increase the pulmonary vascular permeability. However, intervention with emodin suppressed LPS-induced Evans blue leakage. In addition, 3-MA treatment significantly reversed these changes (Fig. 1B). Next, The emodin's potential effects were determined on the overall outcomes of LPS-exposed mice, the experimental animals subsist rate was monitored. The results rated that expose all of the mice to LPS died within 48 h, while after emodin intervention has 30% of the LPS-exposed mice subsisted (Fig. 1C). These data indicate that intervention with emodin might have beneficial effects and result in benefited outcomes in LPS-exposed mice. These data also suggest that autophagy might be involved in the protective effect of emodin on LPS-induced ALI.

Emodin suppressed pulmonary inflammation and metabolic disturbance in LPS-exposed mice

To further assess the neutrophils infiltration degree, the activity of MPO in lung tissues has been determined. As wished, the activity of MPO was up-regulated by LPS exposure, but this upregulation was significantly suppressed by emodin intervention (Fig. 2A). In histological examination, these data were in accordance with the reduced leukocyte infiltration observed. In addition, the pivotal pro-inflammatory cytokines TNF-α and IL-6 level in lung tissue have been confirmed. The results indicated that LPS challenge markedly elevated the TNF-α and IL-6 concentrations in pulmonary homogenates. When treating the LPS-exposed creatures with emodin, the TNF-α and IL-6 levels were significantly decreased (Figs. 2B and C). Consistently, similar the emodin effects for suppressive on LPS-induced production of pulmonary NO have been observed in the present study (Fig. 2D). Inhibition of autophagy by 3-MA blocked the suppressive effects of emodin on the activity of MPO, the induction IL-6, TNF-α, NO in LPS-

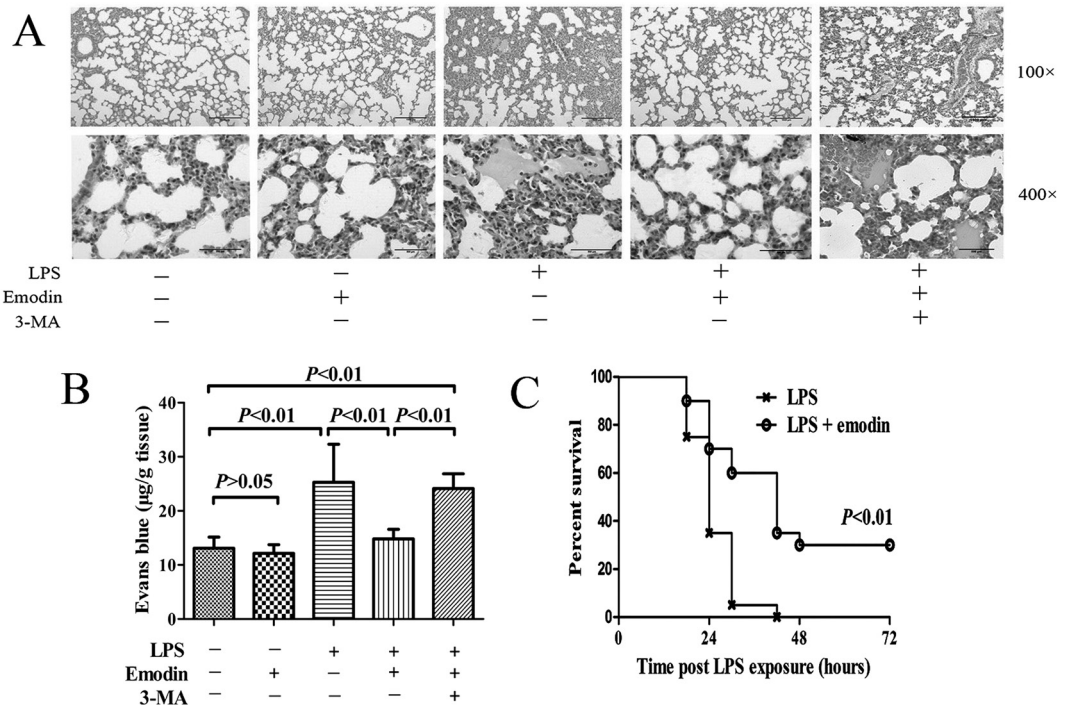

Fig. 1. Emodin alleviated pulmonary histological abnormalities, suppressed Evans blue leakage, and improved the survival rate of in lipopolysaccharide (LPS)-exposed mice. A. Emodin was administered in mice with intraperitoneal exposure to LPS. The histopathological alterations in lung tissue were observed 18 h post LPS exposure. Data are representative of four independent experiments and values were presented as mean ± SD (n=8). B. Emodin was administered in mice with intraperitoneal exposure to LPS. Evans blue was intravenously injected 0.5 h prior to the end of the experiment. And then, the animals were sacrificed and perfused free of blood. The lungs were removed, weighed, homogenized and the content of Evans blue in lung tissue was determined. Data are representative of four independent experiments and values were presented as mean ± SD (n=8). C. Emodin was administered in mice with intraperitoneal exposure to LPS. The survival of mice was recorded every 6 hours and the survival rate was described by the Kaplan-Meier curve (n=20).

exposed mice (Figs. 2A–D), suggesting that autophagy might contribute to the anti-inflammation effect of emodin on LPS-induced ALI.

In addition, the up-regulation of LA, a product of glycolysis pathway, is a reliable marker associated with the severity and mortality of severe inflammation [35]. The current research has found that LPS exposure obviously enhanced the pulmonary level of LA, which was suppressed by emodin intervention (Fig. 2E). About the intervention with emodin of data suggests that emodin may alleviate LPS-induced metabolic disturbance. Consistent with the results in the above study, 3-MA treatment also significantly reversed the suppressive effects of emodin on LA induction in LPS-exposed mice (Fig. 2E), these data further suggesting that autophagy might contribute to the protective effect of emodin on LPS-induced ALI.

Emodin activated autophagy in Lung in LPS-Induced ALI

The mechanism of emodin was visited on reducing LPS induced acute lung injury, we detected the autophagy-related protein levels in lung tissues. Following LPS expose, the BECN1 level and the LC3-II level expression markedly decreased (Figs. 3A and B). Nevertheless, emodin intervention notably upregulated the BECN1 level and LC3-II level (Figs. 3A and B). These data demonstrate that the mechanism of emodin protects against acute lung injury might associate with pulmonary autophagy activation.

Discussion

Natural products are considered an abundant source of new drug candidates for anti-inflammatory therapy [15, 18]. The traditional Chinese medicine has employed

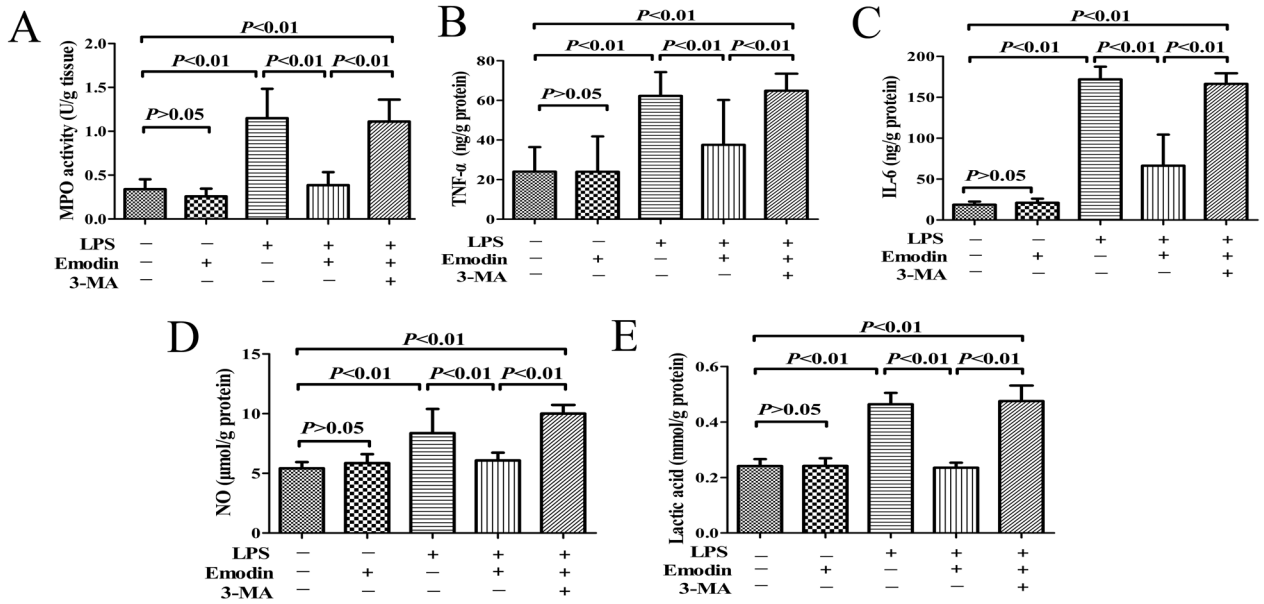

Fig. 2. Emodin reduced the activity of pulmonary myeloperoxidase (MPO), the levels of pulmonary tumor necrosis factor alpha (TNF-α) and IL-6, nitric oxide (NO), lactic acid (LA) in lipopolysaccharide (LPS)-exposed mice. A. The activities of MPO in lung tissue were determined by ELISA. B. The levels of TNF-α in lung tissue were determined by ELISA. C. The levels of IL-6 in lung tissue were determined by ELISA. D. The levels of NO in lung tissue were determined by ELISA. E. The levels of LA in lung tissue were determined by ELISA. Emodin was administered in mice with intraperitoneal exposure to LPS and the lung samples were harvested 18 h post LPS exposure. Data are representative of four independent experiments and values were presented as mean ± SD (n=8).

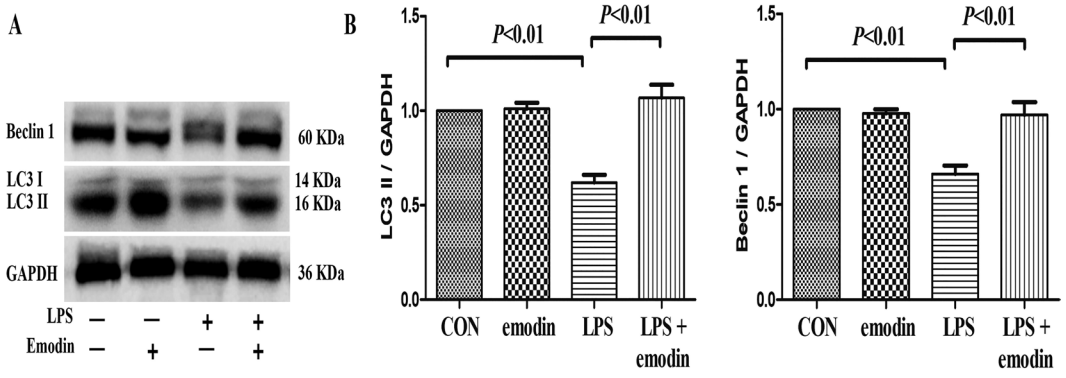

Fig. 3. Emodin enhanced autophagy activity in lipopolysaccharide (LPS)-exposed mice. Representative blots of independent experiments are show. A. Western blot analysis of pulmonary LC3-II and BECN1 protein expression were determined by immunoblot. B. The blots were semi-quantified and data were presented as mean ± SD (B) (n=4). Emodin was administered in mice with intraperitoneal exposure to LPS and the lung samples were harvested 18 h post LPS exposure.

the *Rheum palmatum* L. for thousands of years, and now emodin has been suggested as its most important active component [7]. The benefits of emodin anti-inflammatory were previously reported in LPS-induced keratitis already, nephritis and hepatitis [4, 19, 48]. The emodin also pressed LPS-induced pulmonary inflammation that found in current research, alleviated lung injury and improved the subsist rate in mice with lethal endotoxemia.

Increasing evidence indicates that activated macrophages mediate most cellular and molecular inflammation networks by producing cytokines such as ILs and tumor necrosis factor-α (TNF-α), NO, and prostaglandin E2 (PGE2) [43]. The pro-inflammatory mediators for

LPS-induced production, such as TNF-α, IL-6 and NO, the crucial roles were played in the inflammatory injury development and they are widely used as molecular markers of the inflammatory response [28, 44]. In the current research, the TNF-α and IL-6 for LPS-induced upregulation was significantly suppressed by emodin. Compared with the change in the inflammatory cytokines TNF-α, IL-6 of lung tissue in our study, previous studies have shown that the change of emodin on these inflammatory cytokines of serum in ALI is consistent with our results [47]. In agree with our findings, the suppressive effects of emodin on pro-inflammatory cytokines expression have been reported in LPS-exposed microglia, macrophage, epithelial cells, rheumatoid synoviocytes [25, 31, 45, 50, 52, 53]. In addition, the histological examination and the MPO assay indicated the LPS-induced neutrophil infiltration was attenuated in the emodin-treated group. The increased MPO activity is well considered as an indicator of the activation and accumulation of neutrophils in inflammation [51]. Therefore, emodin might affect both neutrophils and macrophages in this ALI model. The beneficial effects of emodin in the present study might attribute to, at least partially, its anti-inflammatory activities.

In addition to the pro-inflammatory mediators, the increased level of LA has been suggested to be associated with the degree of inflammatory injury. The elevation of LA might be an indicator of tissue hypoxia/hypoperfusion [8], it might also result from enhanced aerobic glycolysis under inflammatory situation [10]. Both tissue hypoxia and aerobic glycolysis are crucial pathological events contribute to the development of inflammation and tissue injury, and LA has been regarded as a reliable marker of severity and mortality in critically ill patients [17]. Emodin has been recently reported to regulate energy metabolism which was regarded as a novel mechanism for inflammation in the body [49]. The LPS-induced upregulation of LA was suppressed by emodin in the current research, which is consistent with increased subsist rate and alleviated pulmonary damage. These data further support that therapy with emodin could result in beneficial outcomes.

Pulmonary edema is a central pathological event that is closely associated with respiratory dysfunction, which might result from the impaired pulmonary endothelial barrier and increased vascular permeability [22]. In the present study, the evaluation of Evans blue leakage indicated that the increased pulmonary vascular permeability in LPS-exposed mice was suppressed by emodin. Consistently, the alleviated pulmonary edema in the emodin-treated group was also observed in the histological examination. In agree with our findings, the suppressive effect of emodin on pulmonary edema have been reported in previously [40]. The alleviated pulmonary edema would result in an improved respiratory function, which might also contribute to the beneficial outcomes in survival analysis. Because the inflammatory status changed dynamically after LPS exposure, which is coordinated with other pathological processes such as coagulation, oxidative stress, energy metabolism, lipid metabolites and apoptosis, endoplasmic reticulum (ER) stress and the presence of damaged mitochondria [13, 23, 37]. In addition, ALI can develop many destructive complications at later stages, including severe sepsis, severe trauma, and ischemia/reperfusion injury [34, 42]. The survival rate was not completely improved by emodin treatment. The increased vascular permeability might result from endothelial injury or/and dysregulated inter-endothelial junctions [39]. It has been reported that emodin could prevent endothelial cells from high glucose-induced cytotoxicity [9], which might be a potential mechanism responsible to the suppressed Evans blue leakage. In addition, pro-inflammatory mediators could disorganize the inter-endothelial junctions and lead to an increase in endothelial permeability [39]. The suppressive effects of emodin on pro-inflammatory mediators have been reported previously and were observed in the present study [16, 31, 45, 52], which might also contribute to the alleviated Evans blue leakage.

The conserved process of self-cannibalization, called autophagy, it liable for removing damaged organelles, anti-microbial responses, and long-lived proteins via a pathway of lysosomal degradation with considered to play an essential role in subsisting of cells permitted in the growth factors or nutrients absence [30]. Moreover, absence of autophagy spontaneously results in pulmonary injury formation, indicating that autophagy process is closely related to the pathogenesis and outcomes of acute lung injury [20]. Most importantly, activation of autophagy could protect against LPS-induced acute lung injury by maintaining endothelial cell (EC) permeability, and inhibiting inflammatory response [6, 14]. The upregulation of the conversion of LC3 II/I, means an autophagy activation specific biomarker. Combining measurement of the standards of BECN1 and LC3-II for protein indicates the induction of autophagic flux [27].

The current research disclosed that the levels of LC3-II and BECN1 were decreased in the lungs of LPS-induced ALI mice, indicating suppression of autophagy. By comparison, emodin intervention might rescue autophagy through promoted the LC3-II, BECN1 expressions as shown in our study. In addition, this finding was confirmed by the inhibition of autophagy with 3-MA treatment significantly reversed these beneficial effects of emodin on LPS-induced lung injury. Taken together, these data indicated that LPS inactivated the autophagic response, and emodin could reactivate autophagy in the LPS-induced ALI progression. So we surmised that emodin unleashed a protective effect against LPS-induced ALI in mice, at least partially, by restoring the impaired autophagy. However, the mechanisms by which emodin actives autophagy are unknown. Previous studies have demonstrated that Aloe-emodin induced autophagy via activating the ROS-JNK signaling pathway [32, 41]. Other studies have shown that aloe-emodin modulated autophagy via inhibiting of mTOR pathway and blocking nuclear localization of p53 protein [11]. A recent report indicated that emodin triggered autophagy by activating AMPK and inhibiting the mTOR signaling pathway [21]. Therefore, in future work, we will focus on studying atrophy-related signaling pathways and genes to further confirm the function of emodin in this process.

In conclusion, the current research has found that therapy with emodin suppressed lethal dose of LPS-induced inflammatory injury, decreased pulmonary hyperpermeability, alleviated pulmonary damage, these beneficial effects were associated with an improved survival rate, these alterations were accompanied with up-regulation of BECN1 and LC3-II. In addition, 3-MA (the autophagy inhibitor) reversed the protective effects of emodin on ALI mice. The mechanisms of the protection of emodin on inflammatory lung injury in mice with lethal endotoxemia appeared to be activating autophagy. Although the detailed mechanisms underlying of how the autophagy remains activated by emodin to be further investigated, the emodin may have potential value for the systemic inflammation-induced lung injury's pharmacological intervention in the current research.

Conflict of Interest

The authors confirm that there is no conflict of interest in this work.

Acknowledgments

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