Effects of piperlonguminine on lung injury in severe acute pancreatitis *via* the TLR4/NF-κB pathway

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ABSTRACT

Acute pancreatitis is an inflammatory response in the pancreas, involving activation of pancreatic enzymes. Severe acute pancreatitis (SAP) often causes systemic complications that affect distant organs, including the lungs. The aim of this study was to explore the therapeutic potential of piperlonguminine on SAP-induced lung injury in rat models. Acute pancreatitis was induced in rats by repetitive injections with 4% sodium taurocholate. Histological examination and biochemical assays were used to assess the severity of lung injury, including tissue damage, and levels of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4), reactive oxygen species (ROS), and inflammatory cytokines. We found that piperlonguminine significantly ameliorated pulmonary architectural distortion, hemorrhage, interstitial edema, and alveolar thickening in rats with SAP. In addition, NOX2, NOX4, ROS, and inflammatory cytokine levels in pulmonary tissues were notably decreased in piperlonguminine-treated rats. Piperlonguminine also attenuated the expression levels of toll-like receptor 4 (TLR4) and nuclear factor-kappa B (NF-κB). Together, our findings demonstrate for the first time that piperlonguminine can ameliorate acute pancreatitis-induced lung injury *via* inhibitory modulation of inflammatory responses by suppression of the TLR4/NF-κB signaling pathway.

Key words: Piperlonguminine; severe acute pancreatitis; lung injury; TLR4/NF-κB pathway.

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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome are potential complications of acute pancreatitis (AP) that can be classified as pancreatitis-associated acute lung injury (PALI).[1,2] Approximately 50% of mortality in patients with pancreatitis can be ascribed to PALI due to consequent pulmonary swelling, microvascular leakage, alveolar-capillary barrier disruption, and alveolar damage.[1-3] Since the early pathophysiological cellular events involved in PALI are currently elusive, effective and specific therapies for this disorder are largely lacking.

Piperlonguminine is a natural alkaloid derived from the long pepper, *Piper longum* L., and has numerous biological activities. The piperlonguminine compound is chemically characterized by the presence of two unsaturated imide functional groups, which exhibit anticancer activity and function to induce reactive oxygen species (ROS) production.[4] Piperlonguminine can exert anti-proliferative and anticancer effects by inducing cell cycle arrest, activation of caspases and mitogen-activated protein kinases (MAPKs), and down-regulation of anti-apoptotic proteins, such as Bcl-2 and NF-κB;[5-8] hence, piperlonguminine can trigger apoptosis and inflammatory responses.[9,10] For example, piperlongumine inhibits neuroinflammation *via* regulating NF-κB signaling in lipopolysaccharide (LPS)-stimulated BV2 microglia cells,[11] suggesting that piperlonguminine may play an important role in AP pathogenesis by regulating inflammatory responses and apoptosis.

Toll-like receptor 4 (TLR4) belongs to the pattern recognition receptor (PRR) protein family,[12] which is associated with inflammatory signaling.[13] PRRs are highly conserved receptors that recognize conserved pathogen-associated molecular patterns, thus representing the first line of defense against infections. TLR4 is associated with various inflammatory processes,[14,15] and the TLR4/NF-kB signaling pathway modulates LPS-induced immunity by regulating inflammatory responses and oxidative stress in ALI.[16,17] NF-κB is an inducible transcription factor that can induce gene expression and activate molecular signaling.[18] There is substantial evidence that TLR4 and NF-κB signaling are essential for inflammation,[19] but the roles of TLR4 and NF-κB in lung injury caused by AP are unknown.

The aims of this study were to examine whether piperlonguminine could ameliorate AP-induced lung injury *via* inhibition of pro-inflammatory cytokines and chemo-attractants, and to investigate the potential mediatory role of TLR4/NF-κB signaling in this process.

Materials and Methods

Animals

Male Sprague-Dawley rats (200-220 g, 6-8 weeks) were kept under standard conditions (temperature: 25±2°C; relative humidity: 55-60%; 12-h light/dark cycle) and allowed free access to water and food throughout the study. All animal experiments were carried out in line with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Committee.

The severe acute pancreatitis (SAP) rat model was constructed as described previously.[20] In brief, animals were anesthetized with isoflurane anesthesia (4% isoflurane for induction, 1.5% for maintenance of anesthesia) and a midline laparotomy was performed. First, a blunt needle was inserted transduodenally into the common bile duct. The hepatic duct was closed at the hilum with a bull's-tipped forceps to prevent reflux; 4% sodium taurocholate (1 mL/kg) was continuously infused for 1 min using a fine needle inserted into a 5 mm common bile duct. After the needle was left in place for 5 min, the needle and clamp were removed, and the laparotomy incision was closed. Pathology in the lung and pancreatic tissues observed in the rat model. Normal saline was injected into rats in the control group (NC). Rats involved in the dose study were treated by intraperitoneal injection with different doses of piperlonguminine (5, 10, and 15 mg/kg body weight) (ab142185; Abcam Biochemicals, Cambridge, MA, USA) dissolved in dimethyl sulfoxide (HY-Y0320; MedChemExpress, Monmouth Junction, NJ, USA) after SAP induction. Animals were sacrificed 24 h after piperlonguminine injection.

Cell counts in bronchoalveolar lavage fluid

The right lungs of model rats were ligated, and the left lungs were lavaged with PBS (P196987-500 mL; Aladdin, Seattle, WA, USA), and the resulting bronchoalveolar lavage fluid (BALF) immediately centrifuged (4°C, 300 g, 10 min). Cell counts in BALF cell pellets were recorded using a blood counting instrument.[21] A total of 200 cells per slide (magnification, 40×) were counted to predict differential cell counts. Numbers of each cell type were calculated based on the total number of cells in BALF and the ratio of each cell type.

ELISA

Cytokine release was analyzed in serum and BALF samples, following centrifugation at 4°C (3,000 g, 15 min) and 4°C (300 g, 10 min), respectively, according to the manufacturer's protocol. Samples or standards were pipetted into wells of microplates containing monoclonal antibodies, as follows: TNF-α (CSB-E11987r, CUSABIO), IL-1β (CSB-E08055r, CUSABIO), IL-6 (CSB-E04640r, CUSABIO), or IL-10 (CSB-E04595r, CUSABIO). After unbound antibody enzyme reagent was removed by washing, substrate solution was added to the wells. Enzyme reactions produced a blue product that turned yellow on addition of phosphoric acid stop solution. The intensity of the color was directly proportional to the amount of total target cytokine bound in the initial step, and the concentrations of factors of interest in the samples were calculated using standard curves.

Hematoxylin and eosin staining

Rat lung and bronchus tissue samples were fixed in 4% paraformaldehyde (orb90556; Biorbyt Ltd., Cambridge, UK), embedded in paraffin, and sectioned (4 μm thickness). Then, samples were stained with hematoxylin (H8070, Solarbio, Beijing, China) for 10 min and treated with eosin (E8080-10, Solarbio) for 5 min at room temperature. Histological images were acquired under a light microscope (Olympus, Tokyo, Japan) at 200× magnification.

RT-qPCR assay

Total RNA was extracted from lung tissues using TRIzol reagent (R0016, Beyotime Biotechnology, Shanghai, China), diluted to a final concentration of 1 μg/μL, and reverse transcribed to cDNA by TaqMan one-step reverse transcription (Applied Biosystems, Waltham, MA, USA). The following primer sequences were used: *NOX2* forward, 5'-GTATGCAGCCAAATGCCCTG-3' and reverse, 5'-AGTTACGCTGTTGTGCTGGT-3'; *NOX4* forward, 5'-GCTTCTCTTCACAACTGTTCC-3' and reverse, 5'-TAGGTAGAAGCTGTAACCATGAG-3'; *TLR4* forward, 5'-GCTGGATTTATCCAGGTGTG-3' and reverse, 5'-AGTACCAAGGTTGAGAGCTG-3'; and *NF-κB* forward, 5'-GGGAGATGTGAAGATGCTG-3' and reverse, 5'-AAGTGTAGGACACTGTCCC-3'. RT-qPCR experiments were carried out on an ABI Prism 7500 (Applied Biosystems), according to the manufacturer's instructions. Cycling conditions were 95°C for 3 min,

followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and *β-actin* served as an internal control.

Immunofluorescence assay

Lung tissues were fixed in methanol and washed with PBS, then permeabilized using 0.5% Triton X-100 (T9284, Sigma-Aldrich, St. Louis, MA, USA) at room temperature for 10 min. To guarantee the accuracy of immunofluorescence staining and exclude some nonspecific staining, we set up negative control (PBS) and positive control (known ROS- or TLR4-immunopositive samples). To analyze ROS accumulation, samples were incubated with 2,7-dichlorofluocein diacetate (10 μmol/L) for 60 min at 37ºC in the dark, then washed three times with PBS. To examine TLR4 expression and distribution, samples were incubated with primary antibody against TLR4 (ab22048, 1/100; Abcam) overnight at 4ºC, and then with secondary antibody (rabbit anti-rat IgG H&L, ab6734, 1/500; Abcam) at 37°C for 30 min. Nuclei were stained with DAPI. Expression levels were monitored using a laser scanning confocal microscope (Zeiss, Jena, Germany) at 200× magnification. The fluorescence intensities of ROS and TLR4 staining were analyzed using ImageJ software (ImageJ Software Inc., Bethesda, MD, USA).

Western blot assay

Total protein of lung tissue was extracted using RIPA buffer (Beyotime Biotechnology) containing protease and phosphatase inhibitors cocktails. A BCA protein assay kit (Beyotime Biotechnology) was used to determine protein concentration, according to the manufacturer's instructions. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Burlington, MA, USA), and then incubated with primary antibodies (Abcam) against: NOX2 (1:5000, ab129068), NOX4 (1:500, ab154244), TLR4 (1:500, ab95562), p-NF-κB p65 (1:1000, ab76302), NF-κB p65 (1:500, ab19870), p-IκB-α (1:10000, ab133462), IκB-α (1:1000, ab32518), or β-actin (1:500, ab8226) overnight at 4°C. Subsequently, membranes were incubated with goat anti-rabbit IgG H&L secondary antibody (1:5000, ab6702, Abcam) at 25ºC for 2 h. Proteins were visualized using ECL Western blotting detection reagents (Millipore). Immunoreactive bands were quantified using ImageJ (ImageJ Software Inc.,).

Lung and pancreas wet/dry weight ratio analysis

After induction of SAP, affected lungs were obtained from model rats under anesthesia. Blood stains on the surfaces of lungs were cleaned, and then the wet weight of each lung measured. Lung dry weights were obtained after heating at 70°C for 48 h. Water content in pancreatic tissue samples was analyzed by measuring the ratio of the initial wet weight of the pancreas to the final dry weight after incubation at 70°C for 48 h. Wet/dry (W/D) weight ratios were calculated as wet weight/dry weight.

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) and are expressed as mean ± SD. One-way analysis of variance followed by Tukey's *post-hoc* test was used to evaluate the significance of differences among multiple groups. A p-value <0.05 was considered statistically significant.

Results

The effect of piperlonguminine on SAP and lung injury in rats

To investigate the therapeutic effect of piperlonguminine on SAP and lung injury, SAP model rats were administered different doses of piperlonguminine (5, 10, and 15 mg/kg) or saline (NC). SAP induction increased inflammatory injury in the pancreas and lung in a time-dependent manner, while pancreatic and pulmonary tissue damage were remarkably reduced in the piperlonguminine-treated group, in a dose-dependent manner, as evidenced by reduced alveolar thickening and edema in tissue sections. In addition, pancreas and lung histopathological scores were lower in piperlonguminine-treated SAP-affected rats than in untreated SAP rats (Figure 1 A,B).

Effects of piperlonguminine on lung and pancreas edema in SAP rats

W/D weight ratios are widely used to detect and measure the degree of lung and pancreas edema. To determine whether SAP could cause lung injury, we compared the W/D weight ratios of lungs between the NC and SAP groups at 12 and 24 h. Lung W/D ratio in the SAP group was higher than that in the NC group ($p<0.05$), whereas after treatment with piperlonguminine, the W/D ratio of the lung was significantly reduced ($p<0.05$). Similarly, pancreas edema in SAP model rats was reduced following piperlonguminine treatment (Table 1).

Selection of the appropriate dose of piperlonguminine *in vivo*

To evaluate the most effective dose of piperlonguminine for treatment of SAP rats, serum of biochemical parameters, including amylase, lipase, SOD, plasma glutathione peroxidase (GSH-PX), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were examined. After treatment with piperlonguminine, serum levels of amylase and lipase were decreased at 12 and 24 h compared with the untreated SAP group (Figure 2 A,B). In contrast, the serum levels of SOD and GSH-PX increased in response to piperlonguminine treatment (Figure 2 C,D). In addition, ALT and AST activities were correspondingly decreased in piperlonguminine-treated SAP rats (Figure 2 E,F). Notably, at piperlongumi-

Table 1. The effect of piperlonguminine on the lung and pancreas edema in SAP rats.

Comparison of W/D in each group of rats ($\chi \pm s$, n=6))		12 h			24 h	
	Dose/mg·kg^{-1}	Pancreas W/D	Lung W/D	Dose/mg·kg^{-1}	Pancreas W/D	Lung W/D
Sham-NC		4.44±0.11	4.5±0.22		4.64±0.17	5.08±0.13
SAP		7.23±0.12*	6.86±0.12*		7.53±0.14*	7.08±0.04*
Piperlonguminine	5	6.35±0.12#	5.99±0.13#	5	6.72±0.14#	6.56±0.11#
	10	5.19±0.17#	5.21±0.16#	10	5.75±0.09#	5.82±0.19#
	15	5.1±0.15#	5.22±0.16#	15	5.48±0.05#	5.48±0.16#

SAP, severe acute pancreatitis; W/D, wet/dry; *p<0.05 *vs* NC; #p<0.05 *vs* SAP.

nine doses ranging from 5 to 10 mg/kg body weight, serum levels of the analyzed parameters exhibited dose-dependent responses, while at the 15 mg/kg body weight dose no further changes were observed relative to the effect of treatment with 10 mg/kg body weight piperlonguminine in either the pancreas or lung. Collectively, these data suggest that piperlonguminine can effectively ameliorate the pathological and serological changes in SAP rats at a dose of 10 mg/kg body weight. Hence, in subsequent experiments we focused on the influence on SAP rats of piperlonguminine at 10 mg/kg body weight.

Effects of piperlonguminine on serum inflammatory cytokines

To investigate the anti-inflammatory effect of piperlonguminine on SAP-associated lung injury, serum levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6), as well as the anti-inflammatory cytokine, IL-10, were analyzed by ELISA. As shown in Figure 3 A-D, the release of inflammatory cytokines (TNF-α, IL-1β, and IL-6) increased sharply in SAP rat serum after 12 and 24 h SAP induction. In contrast, piperlonguminine dramatically attenuated SAP-induced production of the inflammatory cytokines at 24 h, with TNF-α decreasing by 27.57%, IL-1β by 44.91%, and IL-6 by 51.29%. Further, the anti-inflammatory cytokine, IL-10, was consistently decreased by 50.41% in SAP rat serum, whereas it was restored to 63.51% on piperlonguminine treatment. These outcomes suggest that piperlonguminine can relieve the SAP-induced inflammatory response in rats.

Effects of piperlonguminine on NOX2, NOX4, and ROS levels in SAP rat lungs

To explore the impact of piperlonguminine on ROS production, NOX2 and NOX4 activities were examined in model rat serum and lung tissue. RT-qPCR and Western blot assays indicated that the mRNA and protein levels of NOX2 and NOX4 were increased in SAP rats, while they were decreased in the lungs of SAP rats after treatment with piperlonguminine (Figure 4 A-D). Functionally, we also detected ROS production by immunofluorescence assay and found that piperlonguminine decreased ROS levels in SAP model rats ($p<0.05$, Figure 4E).

Effects of piperlonguminine on lung inflammation

To assess levels of lung inflammation, we examined myeloperoxidase (MPO) and IL-1β expression levels, where MPO is a protein associated with inflammation in neutrophils.[22,23] As shown in Figure 5 A,B, MPO and IL-1β levels were increased in SAP model rats compared with the NC group, while piperlonguminine downregulated MPO and IL-1β expression levels. In addition, numbers of polymorphonuclear (PMN) cells in BALF were increased by SAP stimulation, whereas this change were partly reversed after piperlonguminine administration (Figure 5C).

Effects of piperlonguminine on the TLR4 and NF-κB signaling pathway in lung tissue

As shown in Figure 6 A,B, we observed that TLR4 and NF-κB expression levels were remarkably increased in lung tissues from SAP model rats. In contrast, piperlongumine decreased the expression levels of these two molecules. Consistent with these findings, immunofluorescence and Western blotting assays indicated that TLR4 was substantially decreased in response to piperlonguminine administration in SAP rat lungs (Figure 6 C,D). As IκB-α phosphorylation is required for initiation of NF-κB signaling activation, we next examined the phosphorylation levels of IκB-α (p-IκB-α) and p65 proteins. Levels of p-IκB-α and p-p65 proteins were significantly increased in SAP model rat lungs, whereas administration of piperlonguminine decreased the phosphorylation levels of IκB-α and p65 proteins in rat lung after 24 h of SAP induction

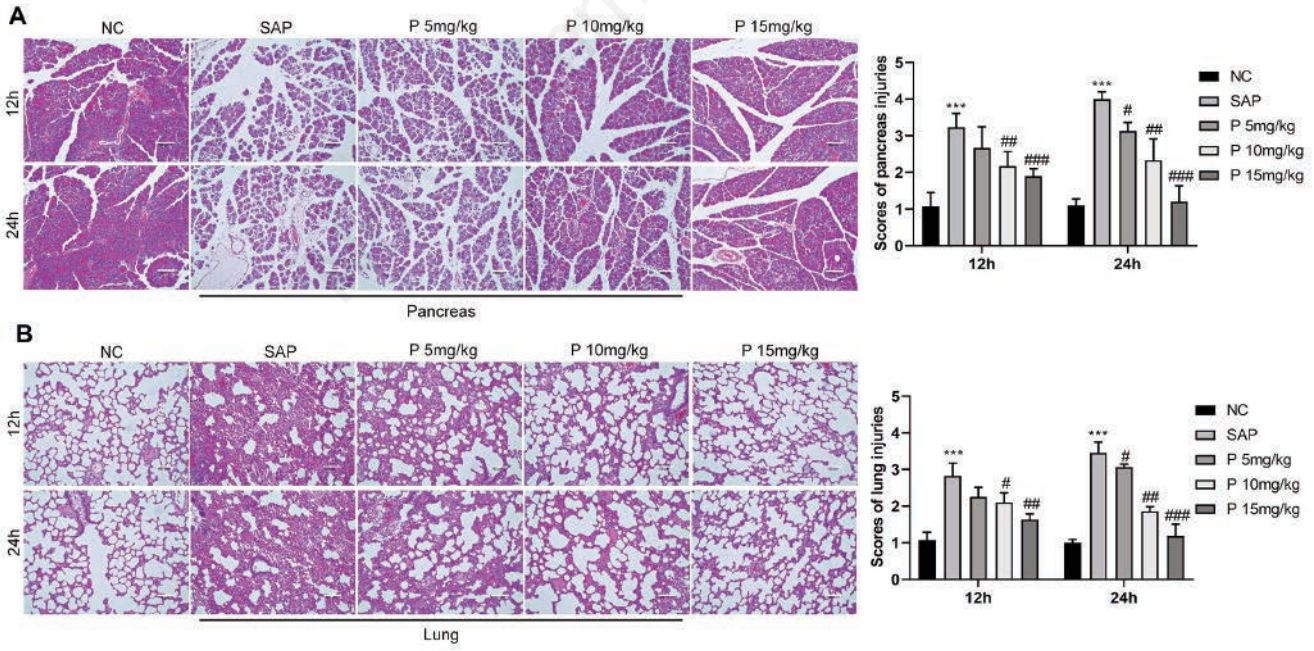

Figure 1. The effect of piperlonguminine on the SAP and lung injury in rats. Representative histopathological changes of pancreas and lung tissues were obtained from rats of different groups. A) Pancreas, edema, massive areas of acinar necrosis, inflammatory cell infiltration, and intrapancreatic hemorrhage in pancreas were observed in the SAP and piperlonguminine group; statistical analysis of pancreas score; scale bar: 100 μm, 200×. B) Lung, interstitial and intra-alveolar edema, inflammatory cell infiltration, and hemorrhage were detected; statistical analysis of lung score. All data were presented mean ± SD. ***p<0.001 *vs* NC group, #p<0.05, ##p<0.01, ###p<0.001 *vs* SAP group. P, piperlonguminine.

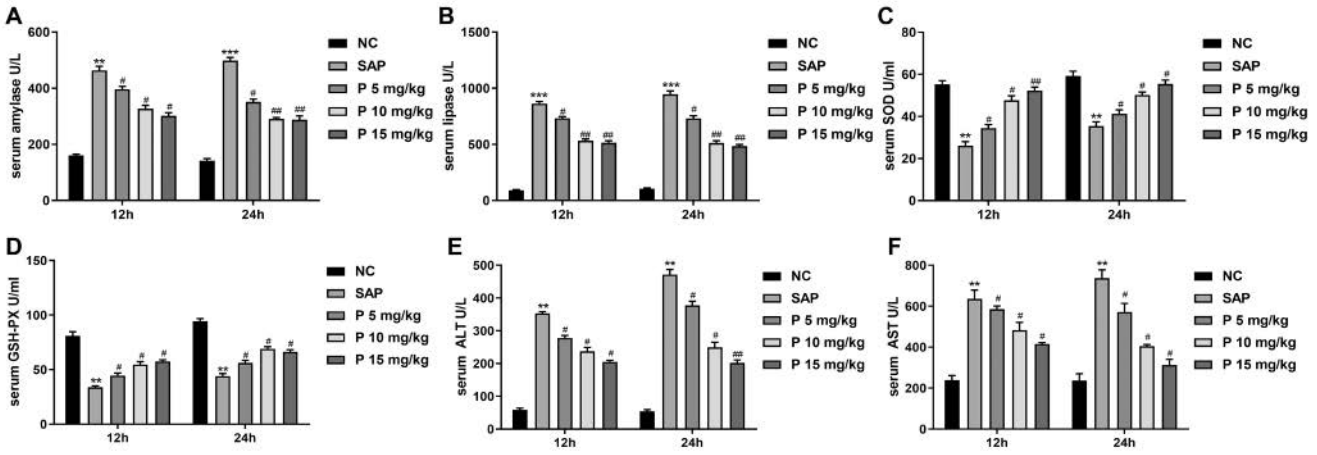

Figure 2. The appropriate dose selection of piperlonguminine *in vivo*. A) Serum amylase activity. B) Serum lipase activity. C) Serum SOD activity. D) Serum GDH-PX activity. E) Serum ALT activity. F) Serum AST activity; n=6. All data were presented mean ± SD. **p<0.01, ***p<0.001 *vs* NC group; #p<0.05, ##p<0.01 *vs* SAP group.

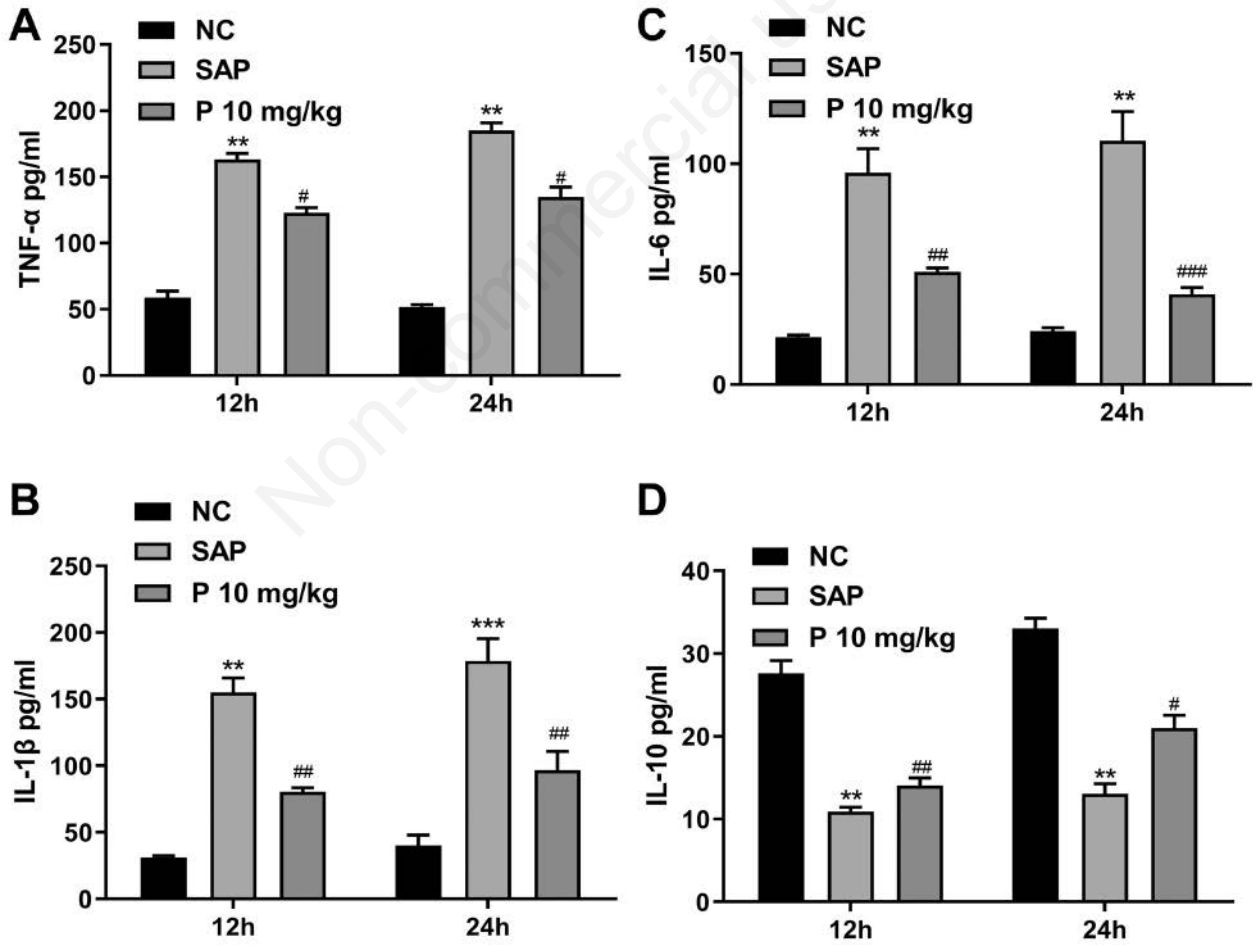

Figure 3. Effects of piperlonguminine on inflammatory cytokines in serum. Serum levels of TNF-α (A), IL-1β (B), IL-6 (C) and IL-10 (D) in rats suffering from 12 h and 24 h SAP; n=6. All data were presented mean ± SD. **p<0.01 *vs* NC group; #p<0.05, ##p<0.01, ###p 0.001 *vs* SAP group.

(Figure 6D). These results demonstrate that piperlonguminine can repress TLR4 expression, and inhibit NF-κB signaling activation by impeding IκB-α and p65 protein phosphorylation.

Discussion

ALI is considered the most frequent, and a potentially devastating, complication of SAP.[1,24] Although understanding of mechanisms underlying SAP-induced lung injury has improved, effective drugs are still lacking. In our study, we demonstrated the therapeutic effect of piperlonguminine on SAP-induced lung injury. Although many studies have confirmed therapeutic effects of *P. longum*, including piperlonguminine, on various cell and animal disease models,[4,9] the influence of piperlonguminine on SAP-associated lung injury and its related mechanisms have remained largely unexplored. Here, for the first time, we demonstrate that piperlonguminine can ameliorate lung injury caused by SAP.

SAP presents clinically as an inflammatory response; hence, the ratio between pro-inflammatory and anti-inflammatory cytokines can influence SAP progression.[24-26] As an inflammatory response initiating factor, IL-1β is highly expressed and secreted in the early phase of SAP, and increased IL-1β can trigger secretion of other inflammatory factors, further facilitating the SAP inflammatory response.[26] Moreover, TNF-α is a mainly pro-inflammatory cytokine secreted by monocytes and macrophages, and can activate and assist neutrophils in adhering and migrating to the injured area of the pancreas in the early stage of SAP. Subsequently, positive feedback from neutrophils induces accumulation of more inflammatory cells in the injured area of the pancreas, finally leading to aggravation of the SAP inflammatory response; therefore, TNF-α can serve as a biomarker that reflects the degree of pancreatic tissue inflammation.[27] It has been shown that piperlongumine reduces LPS-induced tissue damage and production of nitric oxide (NO) and TNF-α, IL-6, and IL-1β, showing anti-inflammatory activity in LPS-induced inflammation, injury of lungs, and sepsis.[28,29] Piperlongumine has therapeutic potential in the treatment of lung anti-inflammatory and antitumor diseases.[30-32] In our study, overall levels of TNF-α, IL-1β, and IL-6 were significantly increased in serum from SAP model rats compared with those in the control group. More importantly, after treatment with piperlonguminine (10 mg/kg body weight) these increases in cytokines

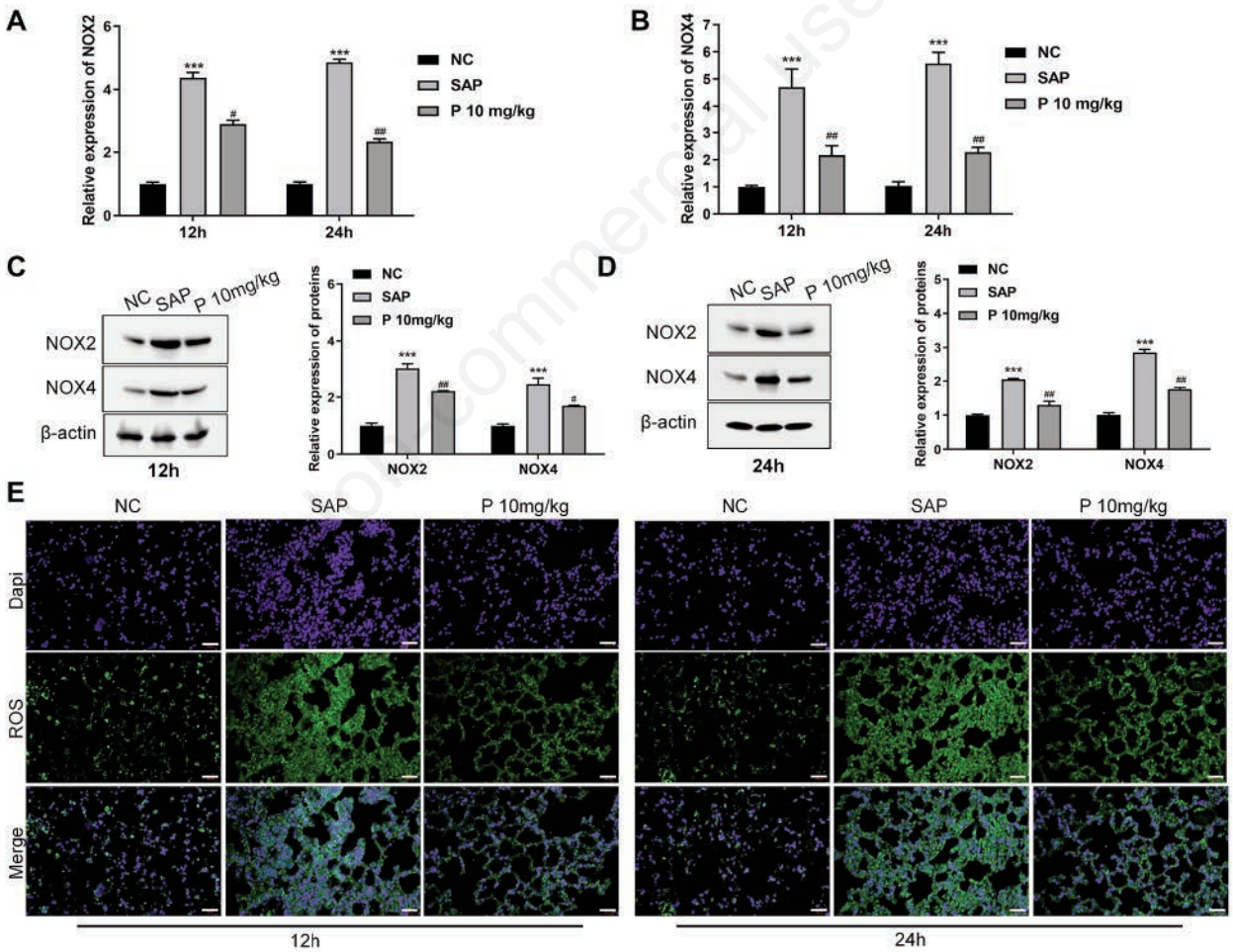

Figure 4. Effects of piperlonguminine on the expression of NOX2, NOX4 and ROS production in lung of SAP rats. A-D) The expression levels of NOX2 and NOX4 in the lung at 12 h and 24 h analyzed by RT-qPCR and western blotting. E) Immunofluorescence staining for ROS in the lung; scale bar: 20 μm, 200×. All data were presented mean ± SD. ***p<0.001 *vs* NC group; #p<0.05, ##p<0.01 *vs* SAP group

were partially attenuated in SAP rats. Further, the piperlonguminine dose used in our experiments was relatively low and did not result in any toxicity in the rats. These results suggest that piperlonguminine has strong anti-inflammatory properties and is non-toxic. NOX enzymes contribute to a wide range of pathological processes in SAP, including post-translational processing of proteins, regulating gene expression, and cell differentiation.[33] NOX2 and NOX4 are enzymes essential for ROS generation,[34] and doxorubicin-induced myocardial injury can be prevented and oxidative stress alleviated by down regulating NOX2 and NOX4 expression in rat myocardium.[35] Our results showed that NOX2 and NOX4 expression, as well as ROS production, were significantly

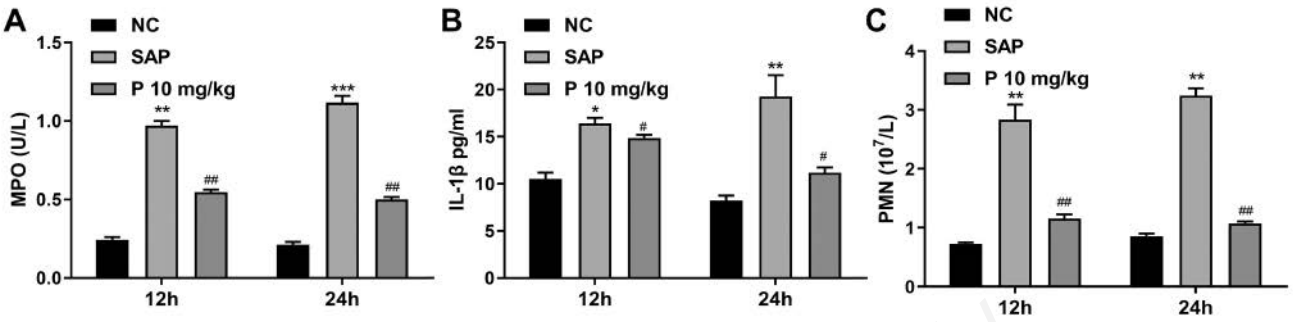

Figure 5. Effects of piperlonguminine on lung inflammation. By means of ELISA assay, pulmonary levels of MPO (A) and IL-1β (B) in rats were measured. C) Number of PMN in BALF. All data were presented mean ± SD. *p<0.05, **p<0.01, ***p<0.001 v. NC group; #p<0.05, ##p<0.01 *vs* SAP group.

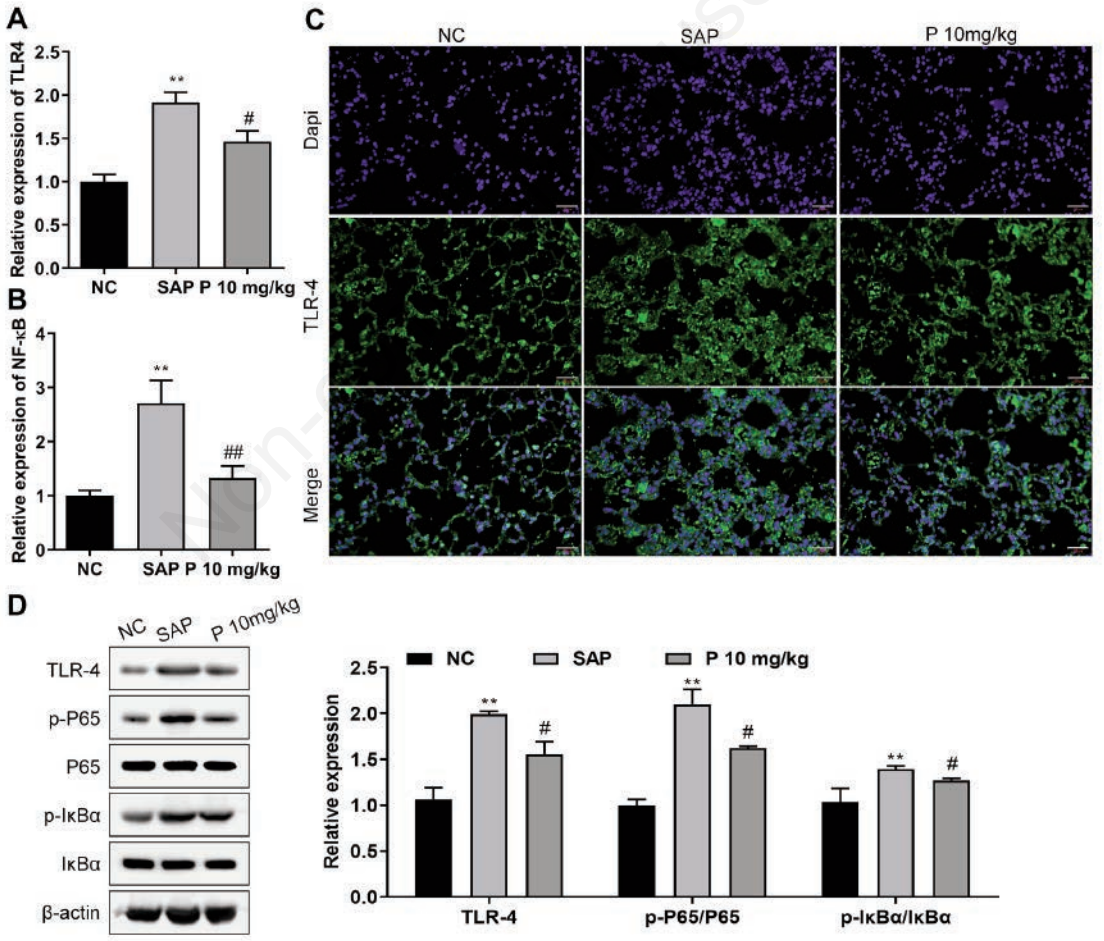

Figure 6. Effects of piperlonguminine on TLR4 and NF-κB signaling pathway in lung tissue. A,B) The expression of TLR4 and NF-κB in lung at 24 h analyzed by RT-qPCR. C) Immunofluorescence staining for TLR4 in lung; scale bar: 20 μm, 200×. D) Western blotting detection of TLR4 and NF-κB expression in lung tissue. All data were presented mean ± SD. **p<0.01 *vs* NC group; #p<0.05, ##p<0.01 *vs* SAP group.

increased in SAP rats, exacerbating the inflammatory response in lung tissues. In contrast, administration of piperlonguminine significantly relieved SAP-induced ROS generation and alleviated SAP-induced lung injury, indicating that the beneficial effects of piperlonguminine may be partially mediated through regulation of ROS. Further studies using N-acetyl-L-cysteine (a ROS scavenger) to identify the role of ROS in mediating the effects of piperlonguminine are warranted. As a type I transmembrane protein receptor, TLR4 is an important signaling receptor involved in various physiological and pathological processes, including cell proliferation, differentiation, inflammatory responses, and apoptosis. Further, extracellular signals can activate NF-κB to initiate inflammatory responses. In SAP, extracellular inflammatory factors, such as LPS, bind to TLR4 and then activate the NF-κB pathway, leading to an aggravation of inflammation. Recent studies have also demonstrated that activation of numerous inflammatory cells, such as neutrophils and macrophages, triggers excessive production of cytokines and inflammatory mediators, thus regulating the severity of AP and SAP-associated lung injury.[36-38] Piperlonguminine also has important roles in NF-κB regulation; for example, it can inhibit NF-κB activity and attenuate the aggressive growth characteristics of prostate cancer cells.[5] Piperlonguminine can also improve LPS-induced amyloidogenesis by suppressing the NF-κB pathway.[39] In our study, we found that piperlonguminine could inhibit TLR4 expression, and contribute to alleviation of lung injury by inhibiting activation of NF-κB signaling through down-regulation of IκB-α and p65 protein phosphorylation.

Despite these results, since piperlonguminine is known for its activation of the ERK and MAPK pathways, further studies should also include analysis of ERK and MAPK activation as positive controls for samples treated with piperlonguminine, as well as applying immunohistological or cell sorting techniques to determine which types of immune cell are enriched in the inflammatory tissues, which cell type expresses TLR4 and NF-κB in response to inflammation, and assess changes in CD14 expression.

In this study, we clearly demonstrate that piperlonguminine can significantly antagonize SAP-induced activation of the TLR4/NF-κB signaling pathway, thereby decreasing inflammatory cytokine production. Overall, piperlonguminine significantly ameliorates the SAP-associated systemic inflammatory response in rat lungs via regulation of the TLR4/NF-κB signaling pathway.

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