Introduction

Acute lung injury (ALI) and its sever conditions, acute respiratory distress syndrome (ARDS) is rapidly growing grave clinical disorder, generally resulting from sepsis, trauma, and severe pulmonary infections (1). The pathogenesis of ALI are often characterized by enlarged diffuse lung inflammation, alveolar-capillary obliteration, hypoxia, non-cardiogenic pulmonary edema and diminished lung compliance. First time, ARDS was defined by Ashbaugh and associates in 1967 (2), followed by the American-European Consensus Conference’s (AECC) in 1994 (3). About 190,000 cases of ARDS estimated in the United States as per the latest population data (4). Despite the availability of many therapeutic interventions (like ventilation and nutritional supports), ALI and ARDS are often coupled with low survival rates (35% to 50%) in the critically ill patients (5, 6, 7). The morbidity and mortality associated with this critical disease remain very high. At present, there is no any effective pharmacological treatment for the acute lung injury. Therefore, a safe and effective new drug or treatments are urgently needed for ALI and ARDS.

Lipopolysaccharide (LPS) is considered as a major constituent of the cell wall of Gram-negative bacteria and considered as a potent biological inducer of macrophages (8), thus, can act as suitable inducing agent of ALI in experimental rats. Moreover, it stimulates the NF-κB activation, which stimulates the over-production of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and IL-8 (9, 10). Consequently, excessive production of these inflammatory mediators induces the systemic inflammation and which further leads to the development of ALI/ARDS. It has been well-established that NF-κB signaling pathway is significantly involved in the inflammatory processes in LPS induced ALI/ARDS. While, Toll-like receptor 4 (TLR4) expressed on the cell surface, is another important receptor of LPS that can activate the NF-κB signaling pathway in and associated cytokines in ALI induced by LPS (11, 12).

Cinnamaldehyde, an essential oil obtained from the plant of *Cinnamomum cassia*, comprises cinnamic acid, cinnamol ether, cinnassiol, glycosides and some other constituents (13). From the ancient times, it has been used a source of medicine for numerous of ailments. Together with this, various studies have confirmed showed scientific evidences of cinnamaldehyde to act as anti-bacterial, anti-inflammatory, anti-oxidant, anti-ulcer, anti-diabetic, and anti-tumor effects accompanied with low cellular toxicity (13, 14). However, the anti-inflammatory and anti-oxidant properties of cinnamaldehyde have been recently demonstrated (15, 16), but no investigation has been carried out to elucidate its protective role in acute lung injury. Therefore, the present study was intended to investigate the anti-inflammatory effect of cinnamaldehyde in LPS-induced acute lung injury mice (ALI) model.

Materials and Methods

Animals

Male BALB/c mice weighing 20-24g of age of 7 to 10 weeks were used for the study. Mice were housed under a 12:12 h light-dark (LD) cycle under regular tempera-
nature (23 ± 2°C) in standard polypropylene laboratory cages. The animals were provided standard laboratory, food and water ad libitum prior to the experiment. All animal experiments were approved by Institutional animal ethical committee.

Reagents
Cinnamaldehyde (natural, ≥95%, catalogue#W228613) and LPS (Escherichia coli 0111:B4, catalogue#L2630) were procured from Sigma-Aldrich (St. Louis, MO). MIF ELISA kit (catalogue#DY1978) was procured from R&D systems (MN, USA). Mouse TNF-α, IL-6, IL-1β, IL-10, IL-13 were obtained from eBioscience (CA, USA). Whereas, the TLR4 (catalogue#14358), anti-NF-κBp65 (catalogue#6956), anti-p-NF-κBp65 (catalogue#3036), anti-IκBa (catalogue#7543), anti-p-IκBa (catalogue#9246) and β-actin (catalogue#3700) monoclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The myeloperoxidase determination kit was obtained from the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China).

LPS-induced ALI mouse model
Seventy-two Male BALB/c mice were randomly divided into six groups taking twelve mice in each group. The groups have been classified as follows, Control group, LPS group, LPS+CM (10, 20 and 40 mg/kg) group and LPS + VGX-1027 (0.5mg/mouse) group (standard). The LPS was instilled to BALB/c mice via the intranasal route (i.n.) (10 μg in 50 μL PBS per mouse) to induce lung injury. Whereas, the test drug, CM was given intraperitoneally (i.p.) 1 h after LPS treatment. The control mice receive PBS (i.n.) without LPS. After 6 h, a collection of bronchoalveolar lavage fluid (BALF) was carried for subsequent analysis.

Histopathological studies of lung
A light microscope was used to observe the pathological changes of the lung. In the current histological examination, the lungs were harvested, fixed in 10% formalin solution and embedded in paraffin. The 5 μm sections were cut and stained with hematoxylin and eosin (H&E) staining and were subsequently observed under a light microscope instrument at × 200 magnification (9).

Lung Wet/Dry weight ratio
The Lung Wet/Dry weight ratio was used as an index for estimation of edema in lungs. The left lungs were collected, and weighed to measure the ‘wet’ weight. The lungs were then dried in an oven at 80°C for 48 hours and re-weighed as dry weight. The Lung Wet/Dry weight ratio was calculated by dividing the wet by the dry weight (9).

Myeloperoxidase (MPO) activity
The lungs were harvested, homogenized, and centrifuged. The resulting supernatant were used for the determination of myeloperoxidase activity using the MPO activity assay kit (Nanjing Jiancheng Bioengineering Institute, China). All procedures were performed according to the manufacturer’s protocol (9).

Cells counts in bronchoalveolar lavage fluid (BALF)
The BAL samples were collected and centrifuged at 3000g for 7min. Then the sedimented cells were re-suspended in PBS solution and subjected to determine the total cell counts. Different cell counts were determined by using Wright-Giemsa stained kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

Enzyme-Linked Immunosorbent Assay
Inflammatory cytokines levels (TNF-α, IL-1β,IL-6, IL-10, IL-13) were measured in the BAL samples using an Enzyme-Linked Immunosorbent Assay (ELISA) kit. All procedures were performed according to the manufacturer’s instructions.

Western Blotting analysis for Total Protein Analysis
Total proteins obtained from left lung tissues were extracted by T-PER, Pierce. Bi-cinchoninic acid assay method was used to determine the protein concentration. Equal small fractions of protein (40μg) were separated on gel electrophoresis. After electrophoresis, the proteins were blotted to polyvinylidene fluoride membranes and were incubated with TLR4, NF-κB, and β-actin for 12 hours at 4°C. After the incubation for 2 h in 5% nonfat milk the secondary antibodies horseradish peroxidase-labeled (1:10000) were added and incubated for 1h at room temperature. ECL plus Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used for detection of the blots in this analysis.

Statistical analysis
All data were presented as mean ± standard deviation (SD). Statistically significant differences between groups were analyzed using one way ANOVA followed by Student-Newman-Keuls post-hoc test using the GraphPad Prism version 5.0. P value <0.05 was considered to indicate a statistically significant difference.

Results
Effects of CM on LPS induced histopathologic changes
In the present study we have used VGX-1027 as standard, which is an orally active isoxazoline compound ((S,R)-3-phenyl-4, 5-dihydro-5-isoxazoleacetic acid). It is a new class of immune modulators that inhibits the production of several proinflammatory cytokines responsible for the damaging effects in inflammatory diseases such as RA. We examined the effects of CM on histopathologic changes in LPS induced ALI mice. Hematoxylin and eosin (H&E) staining were used to determine the pathological changes in the present study. Here, we observed the normal intact lung structure with clear pulmonary alveoli in the control group. Whereas, the LPS-treated group clearly suggests the significant pathological changes containing the alveolar wall thickening inflammatory cell infiltration, fibrosis with collapse of air alveoli, interstitial edema, and some patchy hemorrhage. However, treatment with CM markedly attenuated the LPS induced pathological changes in the lung tissues as shown in Figure1.
Antiinflammatory activity of cinnamaldehyde.

CM Inhibited LPS-Induced inflammatory cell infiltration in the bronchoalveolar lavage fluid (BALF)

In the current study, anti-inflammatory action of CM was further evaluated on LPS induced neutrophils, macrophages and total cell number in bronchoalveolar lavage fluid (BALF). As shown in Figure 4, neutrophils, macrophages and numbers of total cells were increased in the LPS group compared to the control group. Moreover, CM and VGX-1027 significantly reduced the neutrophils (A), macrophages (B) and total cell number (C) in the BALF (Figure 4).

Effects of CM on inflammatory mediators in BALF

The anti-inflammatory effects of CM were measured on LPS-induced inflammatory cytokines. Here, the level of TNF-α, IL-1β, IL-6, IL-10, IL-13, and MIF concentrations were determined using ELISA kits. As shown in Figure 5, LPS-induced inflammatory mediators TNF-α (A), IL-1β (B), IL-6 (C), IL-13 (E), and MIF(F) production were significantly inhibited by CM and VGX-1027. However, CM also raised the IL-10 (D) in BALF (Figure 5).

Effects of CM on LPS-induced TLR4 expression and NF-κB activation

To study the anti-inflammatory mechanism of CM, TLR4 and NF-κB expressions were assessed in the lung tissues of LPS induced ALI mice. As shown in Figure 6, LPS significantly increased TLR4 (B) and NF-κBp65 (C) expression and IκBα degradation (D) (Figure 6).

Discussion

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are the critical illness syndrome.
of acute respiratory failure, that is resulted from sepsis, multiple transfusions, trauma, and severe pulmonary infections (1). It is mainly associated with bilateral alveolar infiltrates, disruption of the alveolar epithelium, protein-rich fluid edema and hypoxemia (17). Despite presence of many advance supportive treatments (like protective ventilation and nutritional supports), the incidence and mortality are still very high (30%-50%) (5, 6, 7). Due to absence of effective pharmacological treatment for ALI, more research is still required to discover a potential therapeutic agent to treat ALI/ARDS.

Cinnamaldehyde is an active constituent from volatile oil of cinnamon, isolated from the stem bark of *Cinnamomum cassia*, which exhibit many biological effects such as anti-bacterial, anti-inflammatory, anti-oxidant, anti-ulcer, anti-diabetic, and anti-tumor effects (13, 14). Cinnamaldehyde is associated with its inhibitory properties on cytokines products (such as TNFα, IL-6, IL-1β, and IL-8) induced inflammation. Many studies also support its antioxidant action via suppression of NF-κB activation (14, 15). It is confirmed that Cinnamaldehyde had many therapeutics effects with low toxicity. However, there are still no researches that Cinnamaldehyde may have therapeutic effects on inflammation after acute lung injury. Thus, in current study, we investigated an anti-inflammatory effect of cinnamaldehyde in LPS-induced LPS/ARDS.

LPS is one of the major potent bioactivator of macrophages that induce the acute lung injury (8). LPS stimulates the TLR4 which activates NF-κB signaling pathway and increase the release of pro-inflammatory factors.
mediators (9, 10). The release of tumor necrosis factor (TNFα), interleukin IL-6, IL-1β, and IL-8 are the key inflammatory mediators and play a critical role in inflammation-induced lung injury (9, 10). LPS-induced ALI mice model was used to evaluate the protective effects of CM on the inflammatory mediators; edema and histopathology changes in lungs.

During our histological evaluation, characteristic severe pathological changes were noted after 6 h of LPS challenge, including alveolar wall thickening, inflammatory cells infiltration, hyaline membrane formation, fibrosis, interstitial edema, and some patchy hemorrhage. We found that CM markedly attenuated inflammatory cells infiltration and thereby improved typical and severe pathological alterations in the lung tissues (Figure1). ALI and ARDS are generally manifested by pulmonary protein-rich fluid edema, and increased permeability of alveolar capillary barrier in the lung (18).

In present study, we measured the wet/dry weight ration as an index of pulmonary edema. We examined the lung edema by measuring the lung W/D weight ratio. The results revealed that CM significantly reduced LPS-induced pulmonary edema in lung of mice (Figure2). Myeloperoxidase (MPO) activity was measured to determine the activation and infiltration of neutrophils and macrophage in the lung tissues. After 6 h of LPS challenge, the MPO activity was significantly increased in comparison to the control group. However, this MPO activity was markedly reduced by CM (10mg, 20mg, and 40mg/kg) as shown Figure3.

Various studies confirmed that over-productions or infiltration of inflammatory cells (specifically neutrophils and macrophages) could cause direct or indirect alveolar epithelium and microvascular endothelium injuries in LPS induced ALI (19, 20, 21, 22). Our results showed that LPS significantly increased the neutrophils, macrophages and total cells in BALF, though these inflammatory cells count remarkably attenuated by CM (Figure4).

It has been reported that ALI is provoked by pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6 and Macrophage migration inhibitory factor (MIF) production, which consider to play key role in the pathogenesis of ALI/ARDS (23). Tumor Necrosis Factor (TNF-α) is generally produced by monocytes and macrophages, which triggers the pro-inflammatory signaling cascades, to lead damage of vascular endothelial cells (24, 25, 26). It is known to propagate the extension inflammatory process. Macrophage migration inhibitory factor (MIF) is rapidly expressed by monocytes and macrophages in response to bacterial toxins and pro-inflammatory mediators that promotes inflammatory responses. It is also widely accepted that MIF is implicated in the pathogenesis of ALI/ARDS (27, 28). The active cytokines IL-6 and IL-1β are crucial inflammatory mediator, associated with LPS-induced inflammation (29, 30). IL-10 is an immuno-modulatory, prevents the LPS-induced endotoxemia via suppressing the TNF-α production (29, 30, 31). It is considered to show a protective action against the LPS induced ALI mice.

Our current finding demonstrated the release of TNF-α, IL-1β, IL-6, IL-13 and MIF production markedly inhibited by CM in BALF of LPS-induced mice. Furthermore, the release of anti-inflammatory cytokines IL-10 also regulated by CM treatment (Figure5). Therefore, our data confirm the protective effects of CM by up-regulation of the pro-inflammatory cytokines in the lungs.

Nuclear transcription factor (NF-κB), is a nuclear protein, considered to be a regulator of inflammatory process. It is well confirmed that NF-κB stimulates the transcription of inflammatory cytokines and plays a crucial role in pathogenesis of lung inflammation (32). NF-κB(p50/p65) is normally located in the cytoplasm as inactive form, and considered to be associated with an inhibitor of IkB protein (32, 33). The LPS activated NF-κB, leads phosphorylation-mediated degradation of IkB. The phosphorylation induced degradation of IkB enables the translocation of NF-κB into nucleus and activate gene transcription of inflammatory cytokine (32, 33).

In present study, we found that increased TLR4 and NF-κB p65 expression and IkBα degradation, were significantly suppressed by CM in LPS induced ALI mice, and thereby CM showed preventive action on translocation of NF-κB into the nucleus of the lung (Figure6). TLR4 is important receptor of LPS, can activate NF-κB signaling pathway and subsequently regulates production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), IL-6 and IL-1β (11, 12). Various studies showed that TLR4 triggers the NF-κB signaling pathway to release of inflammatory mediators, associated with acute lung injury (ALI) induced by LPS (11, 12, 34, 35). It is widely accepted that TLR4–NF-κB signaling pathway is implicated in the pathogenesis of ALI/ARDS(9, 10). Therefore, we assessed the effects of CM on TLR4 expression in LPS induced ALI mice. The results revealed that CM inhibited TLR4 expression in LPS induced ALI mice (Figure6).

In conclusion, our finding demonstrated the anti-inflammatory and protective effect of Cinnamaldehyde on lipopolysaccharide induced acute lung injury in mice. The anti-inflammatory mechanism of Cinnamaldehyde was reported through suppression of TLR4-dependent NF-κB Activation. The results revealed that Cinnamaldehyde could be potential drug in acute lung injury.

Conflicts of Interest
The authors declare no conflict of interest.

References
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