Suppression of MAPK and NF-κB Pathways by Limonene Contributes to Attenuation of Lipopolysaccharide-Induced Inflammatory Responses in Acute Lung Injury

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Abstract—The present study aimed to investigate the protective role of limonene in lipopolysaccharide (LPS)-induced acute lung injury (ALI). ALI was induced in mice by intratracheal instillation of LPS (0.5 mg/kg), and limonene (25, 50, and 75 mg/kg) was injected intraperitoneally 1 h prior to LPS administration. After 12 h, bronchoalveolar lavage fluid (BALF) and lung tissue were collected. Limonene pretreatment at doses of 25, 50, and 75 mg/kg decreased LPS-induced evident lung histopathological changes, lung wet-to-dry weight ratio, and lung myeloperoxidase activity. In addition, pretreatment with limonene inhibited inflammatory cells and proinflammatory cytokines including tumor necrosis factor-α, interleukin-1β, and interleukin-6 in BALF. Furthermore, we demonstrated that limonene blocked the phosphorylation of IκBα, nuclear factor-κB (NF-κB) p65, p38 mitogen-activated protein kinase (MAPK), c-Jun NH2-terminal kinase, and extracellular signal-regulated kinase in LPS-induced ALI. The results presented here suggest that the protective mechanism of limonene may be attributed partly to decreased production of proinflammatory cytokines through the inhibition of NF-κB and MAPK activation.

KEY WORDS: limonene; lipopolysaccharide (LPS); nuclear factor-κB (NF-κB); mitogen-activated protein kinase (MAPK).

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common complications in the intensive care unit and take responsibility for significant morbidity and mortality. A recent epidemiological study revealed approximately 190,000 cases per year in the USA, with an associated 74,500 patients dying from ALI [1, 2]. ALI often showed as hypoxemia, alveolar-capillary barrier damage, and pulmonary inflammation and is associated with multiple organ failure at a later stage [3]. Although great advances in understanding the pathophysiology of ALI had been achieved, the available therapies have not reduced the mortality or increased the quality of life of survivors. Lipopolysaccharide (LPS) is a component of the Gram-negative bacterial cell wall, which can induce inflammatory responses and cause disturbance in the immune system function [4]. Intratracheal administration of LPS has gained wide acceptance as a clinically relevant model of severe lung injury [5]. Thus, we use this model to determine whether limonene could prevent ALI induced by LPS (intranasal—i.n.) administration in mice.

Nuclear factor-kappaB (NF-κB), a nuclear transcription factor, is a regulator of inflammatory processes. Chen et al. have reported that NF-κB plays an important role in the pathogenesis of lung diseases [6, 7]. NF-κB is required for maximal transcription of numerous cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [8].

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These cytokines are thought to be important in the generation of ALI. Therefore, it has been suggested that inhibitors of NF-κB function may be useful as anti-inflammatory agents [9]. Mitogen-activated protein kinases (MAPK) are also involved in inflammation [10]. Many lines of evidence have suggested that members of MAPKs, including p38 MAPK, extracellular regulated kinase (ERK), and c-Jun NH2-terminal kinase (JNK), are important players in signal transduction pathways activated by a range of stimuli and mediate a number of physiological and pathological changes in cell function [11]. It has been shown that inhibition of any of these three MAPK pathways (JNK, p38 MAPK, and ERK pathways) is sufficient to block induction of TNF-α by LPS [12]. Therefore, treatments aimed at inhibition of NF-κB and MAPKs may have potential therapeutic advantages in curing inflammatory diseases.

Limonene is a 10-carbon cyclohexanoid monoterpene derivative found in oils of lemon, orange, and grapefruit. Numerous pharmacological effects, such as anti-inflammation, antitumor, and anti-asthma activities, have been attributed to limonene [13–15]. It has been shown that limonene could inhibit LPS-induced production of nitric oxide, prostaglandin E2, and proinflammatory cytokines in RAW264.7 macrophages [13]. In addition, Hirota et al. have reported that limonene may have potential anti-inflammatory efficacy for the treatment of bronchial asthma by inhibiting cytokines, ROS production, and inactivating eosinophil migration [15]. However, no available study has evaluated the effects of limonene treatment on LPS-induced acute lung injury in a mouse model. Therefore, we sought to investigate whether an intraperitoneal (i.p.) injection of limonene could protect against nonspecific pulmonary inflammation in mice. Our results might provide a pharmacological basis on its folkloric use for the treatment of ALI.

MATERIALS AND METHODS

Chemicals and Reagents

Limonene and dexamethasone (DEX) were purchased from the National Institutes for Food and Drug Control (Beijing, China). LPS (Escherichia coli 055:B5) was purchased from Sigma Co. Mouse TNF-α, IL-1β, and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend, Inc. (San Diego, CA, USA). Rabbit mAb IκBα, p-NF-κB p65, SAPK/JNK, P-p38 MAPK, P-ERK, ERK, rabbit Ab P-SAPK/JNK, p38 MAPK, and mouse mAb p-IκBα were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). Horseradish peroxidase-conjugated goat antirabbit and goat mouse antibodies were purchased from GE Healthcare (Buckinghamshire, UK). Phosphatase inhibitor and PVDF membrane were purchased from Roche. All other chemicals were of reagent grade.

Animals

BALB/c mice (male, 8–10 weeks old, 18–20 g each) were purchased from the Center of Experimental Animals of Baiquen Medical College of Jilin University (Jilin, China). Mice were allowed to acclimatize to the laboratory for at least 7 days under climate-controlled conditions. All procedures were in accordance with the guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health.

Experimental Protocol for Acute Lung Injury Model

All mice were randomly divided into six groups: control, model, dexamethasone (0.5 mg/kg), and limonene (75, 50, and 25 mg/kg). Before inducing acute lung inflammation, limonene (25, 50, and 75 mg/kg) and dexamethasone (0.5 mg/kg) were given by i.p. injection. DEX was injected as a positive control. One hour later, 10 μg of LPS was instilled i.n. in 50 μl PBS to induce lung injury. Twelve hours after LPS administration, all animals were sacrificed by diethyl ether asphyxiation.

Bronchoalveolar Lavage

At 12 h after LPS challenge, mice were sacrificed by diethyl ether asphyxiation. The lungs were lavaged with 500 μl of sterile PBS (7.2) three times (total volume 1.5 ml). The recovery ratio of the fluid was about 87±2 %. Then, the bronchoalveolar lavage fluid (BALF) was immediately centrifuged at 3,000 rpm for 10 min at 4 °C, and the cell-free supernatants were stored at −80 °C for cytokine analysis. The sediment cells were resuspended in PBS for total cell and neutrophil counts using hemacytometer and cytosine by staining with the Wright–Giemsa staining method.

Cytokine Assay

Levels of TNF-α, IL-1β, and IL-6 in BALF were determined by ELISA kits according to the instructions recommended by the manufacturers. The optical density of each well was read at 450 nm.
Measurement of Wet-to-Dry Ratio of the Lungs

The mice were euthanized at 12 h after LPS challenge. The right lungs were excised and the “wet” weight was recorded. The lungs were then placed in an incubator at 80 °C for 48 h to obtain the “dry” weight. The ratio of the wet lung to the dry lung was an index of lung edema.

Myeloperoxidase Assay in Lung Tissues

Myeloperoxidase (MPO) is an important marker of polymorphonuclear leukocyte activation. The activity of MPO was determined in the lung tissue by MPO kit (Nanjing Jiancheng, China). Twelve hours after LPS treatment, mice under diethyl ether anesthesia were killed and the right lungs were excised. One hundred milligrams of lung tissue was homogenized and fluidized in extraction buffer to obtain 5 % of the homogenate. The sample including 0.9 ml homogenate and 0.1 ml of reaction buffer was heated to 37 °C in a water bath for 15 min, then the enzymatic activity was determined by measuring the changes in absorbance at 460 nm using a 96-well plate reader.

Pulmonary Histopathology

Mouse left lungs were excised at 12 h after LPS administration. The lungs were fixed in 4 % neutral buffered formalin for 24 h, embedded in paraffin, and cut into 3-μm sections. Hematoxylin–eosin stains were performed using standard protocol. After staining, pathological changes in the lung tissues were observed under a light microscope.

Western Blot Analysis

Lung tissues were harvested at 12 h after LPS administration and frozen in liquid nitrogen immediately until homogenization. Proteins were extracted with lysis buffer (RIPA with protease and phosphatase inhibitor) for 15 min on ice. Proteins, which were extracted from the lungs using Nuclear Protein Extraction Kit, were used to analyze phosphor-NF-κB p65. Protein concentrations were determined by BCA protein assay kit. Equal amounts of protein were loaded per well on a 10 % sodium dodecyl sulfate polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked with 5 % bovine serum albumin (BSA) (5 g BSA was dissolved in 100 ml TTBS) for 2 h at room temperature and incubated with primary antibody diluted 1:1,000 in 5 % BSA overnight at 4 °C. After that, with the use of peroxidase-conjugated secondary antimouse and antirabbit antibodies (1:5,000 dilution), the bound antibodies were detected by SuperSignal West Pico chemiluminescent substrate. β-Actin was detected as an internal control of protein loading.

Statistical Analysis

The data are expressed as mean values ± SEM. Differences between groups were analyzed by one-way ANOVA or Student’s t tests, with p<0.05 considered as significant.

RESULTS

Effect of Limonene on LPS-Induced Cytokine Production

To determine the effects of limonene on LPS-induced cytokine production, we measured the contents of TNF-α, IL-1β, and IL-6 in BALF by ELISA. As illustrated in Fig. 1, TNF-α (p<0.01), IL-1β (p<0.05), and IL-6 (p<0.01) levels were found to be significantly increased in the LPS group compared with the normal group. Limonene (25, 50, and 75 mg/kg) and DEX (0.5 mg/kg) pretreatment efficiently reduced the production of TNF-α (p<0.01 or p<0.05), IL-1β (p<0.01 or p<0.05), and IL-6 (p<0.01 or p<0.05) in a dose-dependent manner.

Effects of Limonene on Inflammatory Cell Count in BALF

Mice exposed to LPS showed an increase in the number of total cells (p<0.05) and neutrophils (p<0.01) as compared to the control group. Pretreatment with limonene (50 and 75 mg/kg) and DEX (0.5 mg/kg) significantly decreased the number of total cells (p<0.05) and neutrophils (p<0.05), compared to those in the LPS group (Fig. 2). However, limonene (25 mg/kg) did not decrease the number of total cells and neutrophils.

Effects of Limonene on LPS-Induced Lung Wet-to-Dry Ratio

The lung wet-to-dry (W/D) ratio was evaluated to indicate the pulmonary edema. Twelve hours after LPS challenge, the lung W/D ratios (p<0.01) were significantly higher than those in the control group. In the limonene groups (25, 50, and 75 mg/kg) and the DEX (0.5 mg/kg) group, there were significant reductions in the lung edema formation (p<0.05) (Fig. 3a).
Fig. 1. Effects of limonene on the production of inflammatory cytokines TNF-α, IL-1β, and IL-6 in the BALF of LPS-induced ALI mice. Limonene (25, 50, and 75 mg/kg) and DEX (0.5 mg/kg) were given by intraperitoneal injection 1 h prior to an instillation administration of LPS. 

(a) The concentrations of TNF-α in BALF after LPS i.n. administration and the effect of limonene at varied doses.

(b) The concentrations of IL-1β in BALF after LPS i.n. administration and the effect of limonene at varied doses.

(c) The concentrations of IL-6 in BALF after LPS i.n. administration and the effect of limonene at varied doses. The values presented are the means ± SEM (n=6 in each group). #p<0.05, ##p<0.01 vs the control group; *p<0.01, **p<0.001 vs the LPS group.
Effect of Limonene on LPS-Induced Polymorphonuclear Leukocyte Infiltration

MPO activity increase reflects polymorphonuclear leukocyte accumulation in the lung. Twelve hours after LPS administration, the MPO ($p<0.01$) activity in the lung tissues was significantly increased compared with the control group. Intraperitoneal injection of limonene (25, 50, and 75 mg/kg) and DEX (0.5 mg/kg) dramatically decreased MPO activity ($p<0.05$ or $p<0.01$) compared with the LPS group (Fig. 3b).

Effect of Limonene on LPS-Induced Pathological Changes of the Lung

The lungs, harvested at 12 h after LPS injection, were subjected to H&E staining. In the control group, no evident histological alteration was observed in lung specimens. In contrast, lung tissues from the experimental group administered LPS alone showed significant pathological changes, such as inflammatory cell infiltration and alveolar hemorrhage. However, LPS-induced pathological changes were significantly attenuated by limonene (25, 50, and 75 mg/kg) and DEX (0.5 mg/kg) (Fig. 4).
Effect of Limonene on LPS-Induced MAPK and NF-κB Activation

Activation of MAPKs and NF-κB signaling pathways plays key roles in the regulation of inflammatory mediator production. Therefore, we performed western blotting to investigate the activation of phosphor-ERK, phosphor-JNK, phosphor-p38, phosphor-IκB-α, and phosphor-NF-κB p65. LPS stimulation resulted in prominent phosphorylation of MAPKs (Fig. 5), phosphor-IκBα, and phosphor-NF-κB p65 (Fig. 6) in mouse lung tissues. Pretreatment with limonene (50 and 75 mg/kg) and DEX (0.5 mg/kg) prevented the phosphorylation of p38, ERK, JNK, IκBα, and NF-κB p65. However, limonene (25 mg/kg) prevented the phosphorylation of p38, JNK, IκBα, and NF-κB p65, but not the phosphorylation of ERK.

DISCUSSION

ALI and ARDS are the syndromes of acute respiratory failure that results from a disturbance of the alveolar-capillary barrier associated with several clinical disorders. ALI associated with inflammation is a severe disease that
presents high morbidity and mortality rates, and there are no effective drugs in the clinic [16]. Therefore, prevention of ALI is an important therapeutic goal. LPS is a principal component of the outer membrane of Gram-negative bacteria and can enter the blood stream and elicit inflammatory responses that may lead to shock and ultimately to death [17]. Intraperitoneal administration of LPS is a widely used model of ALI in mice. The symptoms of LPS-induced ALI expressed by the mouse model have close resemblance to the observed pathology in humans [18]. Thus, we used this model to study the prevention of limonene on LPS-induced ALI in mice. To the best of our knowledge, the current study is the first to demonstrate the effects of limonene on LPS-induced ALI.

In ALI, the predominant inflammatory cells are the neutrophils, which play an important role in the development of most cases of ALI [19]. Teder et al. reported that mice that failed to clear apoptotic neutrophils might lead to...
exacerbated inflammation and increased mortality in ALI [20]. Leukocyte activation produces reactive oxygen species and protease that lead to alveolar barrier disruption, permeability escalation, and direct tissue injury [21]. Widespread destruction of alveolar epithelium and flooding of the alveolar spaces with proteinaceous exudates containing large amounts of neutrophils represent the typical lesion in ALI [22]. MPO is an enzyme located mainly in the primary granules of neutrophils and its main function is to kill microorganisms, but under certain conditions, it produces excess oxidant leading to tissue damage [23]. In this study, we found that the recruitment of neutrophils in the airways and MPO activity in the lungs were dramatically increased after LPS administration. In contrast, pretreatment of limonene significantly decreased MPO activity and reduced neutrophil infiltration. Furthermore, limonene successfully abated lung tissue injury. This result was consistent with histological analysis of the lung and corroborated our findings in the airways. Lung W/D ratio was evaluated as an index of pulmonary edema, which is a typical symptom of inflammation not only in systemic inflammation, but also in local inflammation [24]. In the present study, it was found that limonene could decrease the LPS-induced lung W/D ratio. These results suggested that limonene has a protective effect on LPS-induced ALI.

The character of ALI is the acute inflammatory process in the airspaces and lung parenchyma injury.

Fig. 5. Effect of limonene on LPS-induced phosphorylation of ERK, JNK, and p38 MAPK in LPS-induced ALI mice. Mice were given different concentrations of limonene (25, 50, and 75 mg/kg) and DEX (0.5 mg/kg) with an intraperitoneal injection 1 h prior to an i.n. administration of LPS. Protein samples were analyzed by western blot with specific antibodies as described. Similar results were obtained in three independent experiments and one of three representative experiments is shown. The values presented are the mean ± SEM. #p<0.05, ##p<0.01 vs the control group; *p<0.05, **p<0.01 vs the LPS group.
involving the release of inflammatory mediators such as TNF-α, IL-1β, and IL-6. Several lines of evidence from several clinical studies indicated that proinflammatory cytokines, notably TNF-α, IL-1β, and IL-6, participate in the early development of inflammation; they have been shown to play a crucial role in ALI and ARDS [25]. Increased concentrations of TNF-α, IL-1β, and IL-6 in the BALF have been observed in patients with ALI and were related to poor outcome [26, 27]. The first endogenous mediator in the inflammatory process is TNF-α, which is produced from LPS-stimulated monocytes and macrophages that elicit the inflammatory cascade and contribute to the severity of lung injury [28]. The binding of TNF-α with receptors in the lung tissue leads to the leakage of enzymes out of the organelle, which causes damage to the lung parenchyma [29]. IL-6 is one of the most important mediators of fever, and in ARDS, high plasma and BALF levels are predictive of poor outcomes [30]. IL-1β can stimulate the production of a variety of chemotactic cytokines such as monocyte chemotactic peptide and macrophage inflammatory peptide-1α; they have earned a position of prominence at the head of the inflammatory cytokine cascade [31]. Both TNF-α and IL-6 induce adhesion molecule expression in vascular endothelial cells, resulting in recruitment of leukocytes into the inflammatory site [32]. Since cytokines have been considered as therapeutic targets for LPS-induced ALI, many treatments have had inhibitory effects on the gene expression of cytokines in the lung [33]. Therefore, TNF-α, IL-1β, and IL-6 served as predictive markers for ALI severity. In the present study, LPS caused a significant increase in TNF-α, IL-1β, and IL-6 expression in BALF compared with the control group; limonene was found to downregulate TNF-α, IL-1β, and IL-6 secretion at 12 h after LPS challenge. These results suggest that the protective effects of limonene on LPS-induced ALI were partly attributed to inhibition of TNF-α, IL-1β, and IL-6.

To further characterize the nature of the inhibitory effect of limonene on cytokine production, we examined the effects of limonene on the activation of the MAPKs and NF-κB signaling pathways. It is well known that MAPKs (ERK, JNK, and p38) and NF-κB are key signaling pathways accounting for the expressions of proinflammatory cytokines induced by LPS. Therefore, we investigated the possibility that limonene inhibits the production of TNF-α, IL-1β, and IL-6 by interfering with the activation of NF-κB and MAPK. The NF-κB pathway has been considered to play a pivotal role in the pathogenesis of ALI. The nuclear accumulation of NF-κB p65 was observed in alveolar macrophages from patients with acute lung injury caused by severe infection, in contrast to alveolar macrophages from control patients [34]. The heterodimers of NF-κB components, which are mostly p50/p65, are normally retained in the cytoplasm in an inactive form through
being associated with an inhibitor of κB (IkB) protein [35]. A wide variety of stimuli can cause the phosphorylation of IkB, a process that is followed by the protein’s ubiquitination and subsequent degradation. The phosphorylation-induced degradation of the IkB inhibitor enables the NF-κB dimers to enter the nucleus and activate specific target gene expression [36]. In the current study, we found that increased phosphor-IκB expression and NF-κB p65 activity in LPS-induced tissue were detected. However, pretreatment with limonene inhibited phosphor-IκB expression and prevented the translocation of NF-κB into the nucleus of the lung. In addition, recently, much attention has been given to the MAPK superfamily, especially p38 MAPK, due to its consistent activation by proinflammatory cytokines and to its role in nuclear signaling [37]. Previous lines of evidence have shown that inhibition of MAPKs pathway is involved in the decrease of sepsis-induced TNF-α, IL-1β, and IL-6 production [38]. LPS induces its inflammatory effects through the activation of the MAPK pathway. It has been shown that p38 MAPK induced by LPS is critical for LPS-induced cytokine release [39]. There is also evidence suggesting that inhibition of p38 MAPK could effectively attenuate LPS-induced lung injury [40]. Furthermore, some studies have found that activation of the p38 MAPK and JNK contributed to sepsis-induced organ injury, whereas inhibition of the p38 and JNK improved survival in septic mice [41, 42]. Schuh and Pahl reported that the ERK pathway plays an important role in LPS-mediated pulmonary inflammation [43]. Based on all of the above, to clarify the mechanisms of limonene in LPS-induced ALI, the expression of ERK, JNK, and p38 MAPK in lung tissue was examined by western blot analysis. Here we found that LPS stimulation led to evident phosphorylations of p38 MAPK, ERK, and JNK, which were consistent with the increase of TNF-α after challenge of LPS. Our results suggest that limonene attenuated the activation of ERK, p38 MAPK, and JNK in LPS-induced ALI. Therefore, we conclude that limonene may decrease LPS-induced proinflammatory cytokines in LPS-induced ALI by inhibiting the activation of NF-κB, ERK, p38 MAPK, and JNK signaling pathways.

In conclusion, the present study showed that limonene played a potent anti-inflammatory role in LPS-induced ALI in mice. Our results indicated that limonene attenuated LPS-induced inflammatory cell infiltration and reduced the activity of MPO. Administration of limonene reduced inflammatory cytokines (TNF-α, IL-1β, and IL-6) in BALF, decreased W/D ratio, and improved the pulmonary functions. Furthermore, we demonstrated that limonene inhibited ERK, JNK, p38 MAPK, and NF-κB activation in LPS-induced ALI. Therefore, limonene may be considered as a potential agent for preventing ALI in the future. Also, further and comprehensive studies are needed before clinical application.

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REFERENCES

Role of Limonene in LPS-Induced Acute Lung Injury


