Ginkgo biloba extracts attenuate lipopolysaccharide-induced inflammatory responses in acute lung injury by inhibiting the COX-2 and NF-κB pathways

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[ABSTRACT] In the present study, we analyzed the role of Ginkgo biloba extract in lipopolysaccharide (LPS)-induced acute lung injury (ALI). ALI was induced in mice by intratracheal instillation of LPS. G. biloba extract (12 and 24 mg·kg⁻¹) and dexamethasone (2 mg·kg⁻¹), as a positive control, were given by i.p. injection. The cells in the bronchoalveolar lavage fluid (BALF) were counted. The degree of animal lung edema was evaluated by measuring the wet/dry weight ratio. The super oxidase dismutase (SOD) and myeloperoxidase (MPO) activities were assayed by SOD and MPO kits, respectively. The levels of inflammatory mediators, tumor necrosis factor-α, interleukin-1β, and interleukin-6, were assayed by enzyme-linked immunosorbent assay. Pathological changes of lung tissues were observed by H&E staining. The levels of NF-κB p65 and COX-2 expression were detected by Western blotting. Compared to the LPS group, the treatment with the G. biloba extract at 12 and 24 mg·kg⁻¹ markedly attenuated the inflammatory cell numbers in the BALF, decreased NF-κB p65 and COX-2 expression, and improved SOD activity, and inhibited MPO activity. The histological changes of the lungs were also significantly improved. The results indicated that G. biloba extract has a protective effect on LPS-induced acute lung injury in mice. The protective mechanism of G. biloba extract may be partly attributed to the inhibition of NF-κB p65 and COX-2 activation.

[KEY WORDS] Ginkgo biloba extract; Lipopolysaccharide; Nuclear factor-κB; Cyclooxygenase

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Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS) are common complications in the intensive care unit and responsible for significant morbidity and mortality [1]. ALI often appears as hypoxemia, alveolar-capillary barrier damage, and pulmonary inflammation, and is associated with multiple organ failure at a later stage [2]. Currently, there is no specific treatment to control ALI or ARDS; searching for new therapies has become an important research effort. Animal models of ALI are important tools for studying mechanisms relevant to the ARDS in humans. Many methods can generate ALI in a variety of different animal species; each has its own advantages and limitations [1]. The most common cause of ALI is bacterial sepsis, and many investigators have used intraperitoneal or intratracheal administration of LPS as a method of provoking ALI [3].

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 [4-5]. NF-κB is required for maximal transcription of numerous cytokines, including...
Ginkgo biloba L. (Ginkgoaceae) is a popular herb used in traditional Chinese medicine (TCM) for thousands of years for ameliorating memory loss and improving blood circulation and respiratory function [12]. In current TCM practice, ginkgo is mainly used to calm wheezing, stop pain, and treat hypercholesterolemia, hypertension, coronary artery disease, angina pectoris, and cerebrovascular disease [13]. Recently, G. biloba extracts are amongst the top-selling phytomedicines in the world [14]. Several beneficial biological activities and pharmacological effects of G. biloba extracts have been proposed, which include free radical scavenging, anti-inflammatory, anti-tumor, anti-aging, and cardioprotective properties [12, 15]. Clinically, it has been prescribed to treat dementia, vaso-occlusion, and cochleovestibular disorders [16]. In murine RAW264.7 macrophages, G. biloba extract effectively inhibits LPS-induced production of nitric oxide and prostaglandin E2 [17]. Expression of toll-like receptor 4 is significantly reduced by G. biloba extract in LPS-stimulated THP-1 human monocytes [18]. However, the underlying mechanisms of G. biloba extract involved in LPS-induced ALI have not been elucidated in detail. The aim of the present study was to investigate the possible protective effect of G. biloba extract on the LPS-induced ALI in a mouse model.

Materials and Methods

Chemicals and reagents

Dexamethasone (DEX) was purchased from the National Institutes for Food and Drug Control (Beijing, China). G. biloba extract was obtained from Jiangsu Shenlong Pharmaceutical Co. (Yancheng, China). LPS (Escherichia coli 055:B5) was purchased from Sigma Co. (St. Louis, MO, USA). Mouse TNF-α, IL-1β, and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend, Inc. (San Diego, CA, USA). Rabbit monoclonal antibodies (mAb) against NF-κB and COX-2 were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit and goat mouse antibodies were purchased from GE Healthcare (London, UK). PVDF membranes were purchased from Roche (Basel, Switzerland). All other chemicals were of reagent grade.

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 lungs...
to the dry lungs was an index of lung edema.

**Measurement of MPO activity in lung homogenates and SOD levels in BALF**

The MPO activity in 10% lung homogenates and the SOD activities in the BALF were determined using test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer’s manual. The MPO activity was determined by using o-dianisidine as peroxidase substrate, and the data were presented as units per gram tissue. The total SOD activity included Cu-Zn and Mn SOD activity and was determined by hydroxylamine assay developed from xanthine oxidase, and the data were expressed as units per microgram protein. The absorbance at 460 nm was measured with Multiskan Spectrum Microplate Photometers (Thermo Fisher Scientific, Waltham, MA, USA).

**Pulmonary histopathology**

The mouse left lungs were excised at 12 h after LPS administration and fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, and cut into 3-μm sections. Hematoxylin-eosin stains were performed using the standard protocol. After staining, pathological changes in the lung tissues were observed under a light microscope.

**Western blot analysis**

The lung tissues were homogenized, washed with PBS, and incubated in lysis buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) to obtain extracts of lung proteins. The protein samples with equal amount of total proteins were fractionated by 10% SDS-PAGE gels and electrotransferred to nitrocellulose membranes. The blots were incubated with the appropriate concentration of specific antibody. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibody. The membranes were stripped and reblopped with anti-GAPDH antibody (Sigma) to verify the equal loading of protein in each lane.

**Statistical analysis**

All values were expressed as the mean ± SD. and analyzed by one-way analysis of variance (ANOVA), followed by Duncan’s Multiple Range Test using SPSS version 13.0 software; a P-value of less than 0.05 was considered statistically significant.

**Results**

**UPLC-PDA-TOF/MS fingerprint of G. biloba extract**

Representative UPLC-MS chromatograms of *G. biloba* extract were shown in Fig. 1. By comparing the mass/UV spectra and retention times with those of the reference compounds, seventeen compounds [(-)-epigallocatechin (1), (+)-catechin hydrate (2), (−)-epicatechin (3), quercetin 3-O-[6-(αL- rhamnosyl)]-β-D-glucoside (4), quercetin 3-Oβ-D-glucoside (5), bilobalide (6), quercetin 3-O-[4-(αL-rhamnosyl)]-β-D-glucoside (7), ginkgolide C (8), quercetin 3-O-α-L-rhamnoside (9), ginkgolide B (10), ginkgolide A (11), luteolin (12), apigenin (13), kaempferol (14), isorhamnetin (15), bilobetin (16), and genkwanin (17)] in the *G. biloba* extract were identified.

**Fig. 1** Representative UPLC-Q-TOFMS chromatograms of *G. biloba* extract in the negative ion mode

**Effects of *G. biloba* extract on LPS-induced cytokine production**

To determine the effects of *G. biloba* extract on LPS-induced cytokine production, the contents of TNF-α, IL-1β, and IL-6 in BALF were analyzed by ELISA. As illustrated in Fig. 2, the levels of TNF-α IL-1β and IL-6 were found to be significantly increased in the LPS group compared with the control group (all P < 0.01). The *G. biloba* extract (12 and 24 mg·kg⁻¹) and DEX (2 mg·kg⁻¹) pretreatment efficiently reduced the production of TNF-α (P < 0.01 and P < 0.05), IL-1β (P < 0.01), and IL-6 (P < 0.01) in a dose-dependent manner.

**Effects of *G. biloba* extract in BALF**

To confirm the efficacy of LPS exposure, the total cells, neutrophils, and leukocyte numbers in BALF were determined at the end of the intratracheal LPS protocol. Meanwhile, the SOD activity was determined. As illustrated in Fig. 3, the mice exposed to LPS showed an increase in the number of total cells (P < 0.01), neutrophils (P < 0.01), and leukocytes (P < 0.01), while the SOD activity decreased (P < 0.01), in comparison with the control group. *G. biloba* extract (12 and 24 mg·kg⁻¹)
Effects of *G. biloba* extract on the production of inflammatory cytokines TNF-α (a), IL-1β (b), and IL-6 (c) in the BALF of the LPS-induced ALI mouse model (mean ± SD, n = 6). *P* < 0.01 vs control group; *P* < 0.05, **P** < 0.01 vs LPS group.

could dose-dependently reduce the number of total cells (*P* < 0.01), neutrophils (*P* < 0.01), and leukocytes (*P* < 0.01), and increase the SOD activity in BALF (*P* < 0.01). After Dex treatment, the total cells, neutrophils, and leukocytes in BALF were also decreased, while SOD activity was increased in BALF.

**Effects of *G. biloba* extract on wet-to-dry ratio in LPS-induced lung injury mouse model**

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 *G. biloba* extract (12 and 24 mg·kg⁻¹) treated groups and the DEX (2 mg·kg⁻¹) treated group, there were significant reductions in the lung edema, compared with the model control group (*P* < 0.01) (Fig. 4a).

**Effect of *G. biloba* extract on LPS-induced polymorphonuclear leukocyte infiltration**

The MPO activity increase reflected polymorphonuclear leukocyte accumulation in the lung. Twelve hours after LPS administration, the MPO (*P* < 0.01) activity in the lung tissues significantly was increased, compared with the control group. Intraperitoneal injection of *G. biloba* extract (12 and 24 mg·kg⁻¹) and DEX (2 mg·kg⁻¹) dramatically decreased the MPO activity (*P* < 0.01), compared with the LPS group (Fig. 4b).

**Effect of *G. biloba* extract on LPS-induced pathological changes of the lung**

Histological evaluation of the lungs (Fig. 5) by light microscopy demonstrated a large amount of neutrophil sequestration and infiltration around the pulmonary vessel and airway, distributed in the alveolar and interstitial space after intratracheal LPS. The treatment groups showed a significantly reduced degree of inflammatory cell infiltration. The results indicated that *G. biloba* extract can reduce the degree of pathological inflammation in lung tissues in ALI.

**Effects of *G. biloba* extract on LPS-induced COX-2 and NF-κB activation**

Activation of COX-2 and NF-κB plays a key role in the regulation of inflammatory mediator production. In the present study, Western blotting was performed to investigate the activation of COX-2 and NF-κB p65. As shown in Fig. 6, compared with the control group, the levels of COX-2 and NF-κB p65 proteins in the model group were significantly increased. In the *G. biloba* extract-treated groups (12 and 24 mg·kg⁻¹), the expression of COX-2 and NF-κB p65 was significantly suppressed compared to the model group.

**Discussion**

ALI and ARDS are the syndromes of acute respiratory failure that result 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 for this disorder in the clinic [19]. 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 death [20]. Intraperitoneal administration of LPS is a widely used model of ALI in mice. The symptoms of LPS-induced ALI expressed in the mouse model have a close resemblance to the observed pathology in humans [21]. Thus, this model was used to study the preventive effects of *G. biloba* extract on LPS-induced ALI in mice. To our best knowledge, the current study was the first to investigate the possible protective effect of *G. biloba* extract in such an ALI model.

In ALI, the predominant inflammatory cells are the neutrophils, which play an important role in the development of ALI [22]. Teder et al [23] have reported that when mice failed to clear apoptotic neutrophils, it might lead to exacerbated inflammation and increased mortality in ALI. Leukocyte activation produces reactive oxygen species and protease that leads to alveolar barrier disruption, permeability escalation, and direct tissue injury [24]. Widespread destruction of the alveolar epithelium and flooding of the alveolar spaces...
with proteinaceous exudates containing large amounts of neutrophils represent the typical lesion in ALI [25]. The 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 [26]. In the present study, we found that the recruitment of neutrophils in the airways and the MPO activity in the lungs dramatically increased after LPS administration. The pretreatment with *G. biloba* extract significantly decreased the MPO activity and reduced neutrophil infiltration. Furthermore, the *G. biloba* extract successfully prevented lung tissue injury. The evaluated Lung wet/dry weight (W/D) ratio is as an index of pulmonary edema, which is a typical symptom of inflammation in systemic and local inflammation. In the present study, we found that the *G. biloba* extract could decrease the LPS-induced lung W/D ratio. These results suggested that *G. biloba* extract had a protective effect on LPS-induced ALI. The characteristics of ALI include the acute inflammatory process in the airspaces and lung parenchyma injury involving the release of inflammatory mediators such as TNF-α, IL-1β, and IL-6. Several lines of evidence from various clinical studies have indicated that pro-inflammatory cytokines, notably TNF-α, IL-1β, and IL-6, participate in the early development of inflammation and play a crucial role in ALI and ARDS [27]. In the present study, LPS caused a significant increase in the levels of TNF-α, IL-1β, and IL-6 in BALF, compared with the control group. The *G. biloba* extract down-regulated TNF-α, IL-1β, and IL-6 secretion. These results suggested that the

Fig. 5 Effects of *G. biloba* extract on LPS-mediated lung histopathologic changes. Lungs (*n* = 3) from each experimental group were processed for histological evaluation at 12 h after LPS challenge: *a* the lung section from the control mouse; *b* the lung section from LPS-induced ALI model mouse; *c* the lung section from the mice exposed to LPS and administered with 2 mg·kg$^{-1}$ DEX; *d* the lung section from the mice exposed to LPS and administered with 12 mg·kg$^{-1}$ *G. biloba* extract and *e* the lung section from the mice exposed to LPS and administered with 24 mg·kg$^{-1}$ *G. biloba* extract; Representative histological section of the lungs was stained by hematoxylin and eosin (H&E staining, magnification × 200)

Fig. 6 Effects of *G. biloba* extract on the expressions of COX-2 and NF-κB p65 in five groups

protective effects of *G. biloba* extract on LPS-induced ALI were partly attributed to inhibition of pro-inflammatory cytokine production.

To further characterize the nature of the inhibitory effects of *G. biloba* extract on cytokine production, the effects of *G. biloba* extract on the activation of the COX-2 and NF-κB signaling pathways were examined. It is well known that COX-2 and NF-κB are key signaling pathways accounting for the expression of proinflammatory cytokines induced by LPS [9, 28]. The NF-κB pathway has been considered to play a pivotal role in the pathogenesis of ALI [28]. Therefore, the possibility that *G. biloba* extract inhibited the production of TNF-α, IL-1β, and IL-6 by inhibiting the activation of COX-2 and NF-κB was investigated. We found that increased NF-κB p65 activity was detected in LPS-treated group. However, the pretreatment with *G. biloba* extract inhibited NF-κB p65 expression. The COX enzymes can convert arachidonic acid to prostanoids, which plays an important role in the development of both the early and late phases of endotoxemia [29]. COX-2, the inducible form of cyclooxygenases, plays an important role in lung injury, and is up-regulated by several proinflammatory stimuli, such as cytokines and growth factors. In the present study, *G. biloba*
extract prevented the LPS-induced increase in COX-2 activity in the mouse ALI model.

Conclusions

G. biloba extract has protective effects on LPS-induced acute lung injury in mice, and the protective mechanism may be attributed at least in part to the inhibition of NF-κB and COX-2 activation.

References
