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The effects of germacrone on lipopolysaccharide-induced acute lung injury in neonatal rats

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Abstract

Germacrone is one of the main bioactive components in the traditional Chinese medicine *Rhizoma curcuma* and has been shown to possess an anti-inflammatory activity. Our present study aimed to investigate the protective effects of germacrone on lipopolysaccharide (LPS)-induced acute lung injury in neonatal rats. Results showed that germacrone treatment significantly decreased the expression of pro-inflammatory cytokines IL-6 and TNF-α. Meanwhile, the expression of anti-inflammatory mediators TGF-β1 and IL-10 was obviously increased following germacrone administration. The LPS-induced pathological changes in neonatal rats were also attenuated by germacrone treatment. *In vitro*, MTT and EdU incorporation assay indicated that germacrone administration significantly increased the A549 cell viabilities in a dose-dependent manner. Besides, flow cytometry and TUNEL analysis showed that the cell apoptosis rate was significantly reduced in a concentration-dependent manner after germacrone injection. At the molecular level, we found that germacrone treatment promoted the expression of claudin-4 both *in vivo* and *in vitro* as shown by real time PCR and western blot. Collectively, our study demonstrated that germacrone protected neonatal rats against LPS-induced ALI partially by modulation of claudin-4.

Key words: Germacrone, acute lung injury, lipopolysaccharide, claudin-4.

Introduction

Acute respiratory distress syndrome (ARDS), a manifestation of acute lung injury (ALI), remains one of the leading causes of death in pediatric and adult intensive care units, which occurs primarily in preterm infants as a consequence of severe lung injury (1, 2). Premature infants are likely to be exposed to infection *in utero* or during postnatal life, accelerating the development of ARDS/ALI. The pathophysiological mechanism of ARDS/ALI is believed to be associated with the uncontrolled inflammatory response in lungs (3). Accumulating evidences suggest that many pro- and ani-inflammatory cytokines, including interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), including interleukin 10 (IL-10) and transforming growth factor beta 1 (TGF-β1) play critical roles for initiating and amplifying the lung injury (4-6).

The formation of pulmonary edema in ALI results from increased endothelial and epithelial permeability and reduced clearance of edema fluid by the alveolar epithelium (7). It is suggested that the expression of claudin proteins account for differences in permeability of paracellular pores among epithelia (8,9). Previous studies demonstrated that increased claudin-4 expression in ALI represents a mechanism to limit pulmonary edema and that the regulation of alveolar epithelial claudin expression may be a novel target for ALI therapy (10).

Many natural products possess immunomodulatory and anti-inflammotary activities, which have long been used for treating human diseases. *Rhizoma curcuma* is widely applied as traditional herbal medicine in China, Japan and other Asian countries (11). Germacrone, a major bioactive component of *Rhizoma curcuma*, has been shown to possess anti-inflammatory, immunomodulatory and neuroprotective properties (12, 13). Several studies reported that germacrone had potent protective effects on acute liver injury in mice induced by tumor necrosis factor-alpha and lipopolysaccharide (14, 15). Investigation of germacrone also found that it produced an obvious anti-inflammatory activity against carrageenan-induced hind paw edema in rats (16). However, there are no reports related to the protective effects of germacrone on LPS-induced lung injury in neonatal rats. Therefore, our present study assessed the therapeutic effects of germacrone and the relevance of claudin-4 on the LPS-induced lung injury in neonatal rats.

Materials and methods

Cell Culture

Human type II-like alveolar epithelial cells A549 was cultured in RPMI1640 supplemented with 10% FBS, 2 mmol/L glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were maintained at 37°C in a humidified 5% CO_2 incubator. Then, cells were incubated with LPS (10 μg/mL) and indicated concentrations of germacrone for 24 h.

MTT assay

Cells viability was evaluated by MTT assay (Promega, USA) in accordance with the manufacturer's instructions. Cell density of $3x10^3$ (cells/well) was seeded into 96-well plates and left to adhere overnight. 10 ml of 5 mg/ml MTT was added and incubated in dark at 37°C for 2 h. The absorbance was determined on MRX II microplate reader (Dynex, Chantilly, VA, USA) with the wavelength of 490nm.

EdU incorporation assay

A549 cells were exposed to EdU (5-ethynyl-2'-deoxyuridine) (Invitrogen, Carlsbad, CA, USA) for 2h at 37°C. Cells were fixed with formaldehyde and treated with 0.5% Triton X-100. After washing with phosphate buffered saline (PBS) for three times, the cells were reacted with 100 μL Apollo reaction cocktail and visualized under a fluorescent microscope (Leica Microsystems, Wetzlar, Germany).

Real time PCR

Total RNA was extracted with a TRIzol kit (Invitrogen Carlsbad, CA, USA) and converted to cDNA with a first strand cDNA synthesis kit (Fermentas, Burlington, Canada). Real time PCR was performed using SYBR Green SuperMix-UDG (Invitrogen Carlsbad, CA, USA). The primer sequences used for PCR are as follows: claudin-4 (forward 5'-CCTTTCCCATACGGTCTTGCT-3', reverse 5'-CCCGTACCTTCCACAGACTG-3'), GAPDH (forward 5'-CAGTGCCAGCCTCGTCTCATA-3', reverse 5'-TGCCGTGGGTAGAGTCATA-3'). Amplification was performed at 50°C for 2 min, at 95°C for 2 min, followed by 40 cycles of denaturing at 95°C for 15 s and annealing at 60°C for 30 s. All reactions were performed in triplicate. GAPDH was used as a reference gene.

Western blot

Total protein from lung homogenates and cells was separated by electrophoresis on a 10% SDS-polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes. Membranes were incubated overnight with primary antibodies including anti-claudin-4 and anti-GAPDH (Cell Signaling, Beverly, MA, USA). Anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase was used (Pierce Chromatography Cartridges, USA). Immunoreactive bands were detected by enhanced chemiluminescence (ECL) kit (SYNGENE, USA).

Flow cytometry analysis

Cells were exposed to different concentrations of germacrone for 24 h. The cells were washed with PBS, detached with trypsin and harvested. Apoptosis cells were detected with annexin V-FITC/PI according to the protocol of Annexin V-FITC cell Apoptosis Detection Kit (BD, USA).

TUNEL assay

Paraffin-embedded lung tissues were labeled using a TUNEL assay kit (Roche Diagnostics, Basel, Switzerland). The number of TUNEL-positive (apoptotic) cells on three sections per rat was counted under a fluorescence microscope at x 400 (Carl Zeiss Microsystems, Thornwood, NY, USA).

Animals

This study was conducted under a protocol approved by the institutional animal care and research committee. Wistar rats with dated pregnancies were maintained at the same center and were bred in house. They were provided free access to water and standard laboratory chow. The animals' lighting was provided from 6 a.m. to 6 p.m. Thirty Wistar rats were randomly divided into 3 groups (n=10): control group, ALI group and germacrone group. According to the previous report, ALI was induced by LPS (Sigma, St. Louis, MO, USA) via intratracheal injection (13). Control animals were injected with an equal volume of sterile saline. Additional neonatal rats were treated intraperitoneally with 10 mg/kg of germacrone 1 hour before LPS injection.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the contents of IL-6, TNF-α, TGF-β1 and IL-10 in the serum according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Absorbance was measured at 450 nm by microplate assay.

Histology

Animals were killed using sodium pentobarbital and the lungs were immediately harvested, post-fixed in 4% paraformaldehyde for 24 hours. Paraffin-embedded sections with the thickness of 5 mm were stained with hematoxylin and eosin (HE) for visualization under a light microscope (Leica Microsystems, Wetzlar, Germany) at 200 x magnification.

Measurement of lung wet/dry weight ratio

The severity of pulmonary edema was assessed by the wet to dry weight ratio (W/D ratio). The left lower lungs weighed and then dehydrated at 60°C for 72 h in an oven.

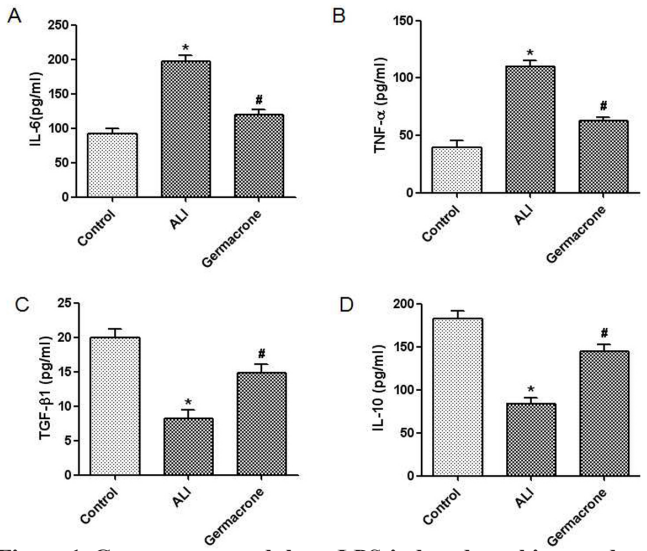

Figure 1. Germacrone modulates LPS-induced cytokine production *in vivo*

Neonatal rats were treated intraperitoneally with 10 mg/kg of germacrone 1 hour before LPS injection. ELISA was performed to determine the expression of IL-6 (A), TNF-α (B), TGF-β1 (C) and IL-10 (D) in serum in the rats. * $P<0.05$ vs. Control. # $P<0.05$ vs. ALI.

Statistical analysis

For statistical analysis, all the data were presented as means ± SD of at least triplicate determinations and treated for statistics analysis by SPSS program. Comparison between groups was made using ANOVA and statistically significant difference was defined as $P<0.05$.

Results

Germacrone modulates LPS-induced cytokine production in vivo

TNF-α and IL-6 are proximal proinflammatory cytokines upregulated after LPS administration. To determine whether germacrone attenuated cytokine production, the TNF-α and IL-6 levels were evaluated by ELISA after treatment with germacrone. We found that the expression of IL-6 and TNF-α in ALI group were significantly increased in the serum as determined by ELISA. However, the levels of IL-6 and TNF-α were obviously decreased after pre-treatment with germacrone at the concentration of 10 mg/kg (Fig. 1A and B). Meanwhile, the anti-inflammatory mediator TGF-β1 and IL-10 were decreased after LPS injection, which were reversed by germacrone treatment (Fig. 1C and D). These data suggested that germacrone modulated LPS-induced cytokine production *in vivo*.

Germacrone decreases LPS-induced lung injury and apoptosis in neonatal rats

HE staining was used to evaluate the pathological changes in the lungs of rats after germacrone treatment. Histologic examination revealed evidence of notable inflammatory cells infiltration, interstitial edema, and intra-alveolar hemorrhage. However, such pathological changes in the lung tissues were remarkably relieved after germacrone administration (Fig. 2A). Then, lung wet/dry weight ratio (W/D) was employed to assess the pulmonary edema in the lungs. Higher W/D was observed in the neonatal rats received LPS injection compare

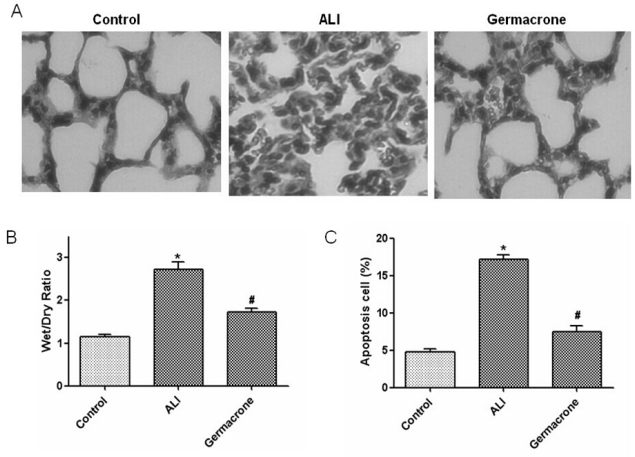

Figure 2. Germacrone decreases LPS-induced lung injury

(A) Lung tissue sections were stained with hematoxylin and eosin (H&E) to determine the pathological changes with or without germacrone treatment. The figures demonstrated representative views (x 200) from each group. (B) After LPS injection with or without germacrone treatments, neonatal rats were sacrificed and the lungs were obtained for wet/dry weight ratio determination. * P<0.05 vs. Control. # P<0.05 vs. ALI. (C) After LPS injection with or without germacrone treatments, TUNEL assay was performed to measure the apoptosis of lung tissues. * P<0.05 vs. Control. # P<0.05 vs. ALI.

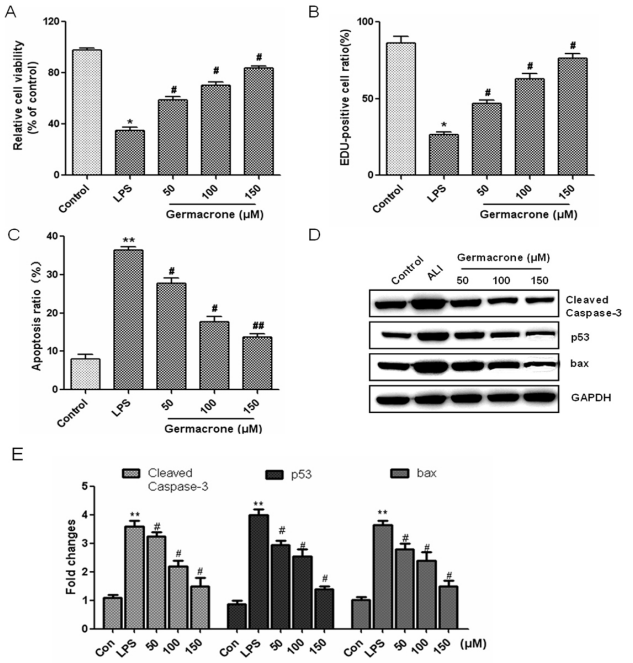

Figure 3. Effect of germacrone administration on A549 cell viability and apoptosis

Cells were treated with LPS (10 μg/mL) or different concentrations of germacrone (50 μM, 100 μM and 150 μM) for 24 h. MTT (A) and EdU incorporation assay (B) were performed to measure the A549 viability. (C) Flow cytometry was used to determine the apoptosis rate of A549 cells. (D and E) Western blot was performed to detect the protein expression of Caspase 3, p53 and bax. * P<0.05, ** P<0.01 vs. Control. # P<0.05 vs. LPS.

with the control group. However, germacrone administrations significantly reduced the W/D (Fig. 2B). In addition, We found that the apoptosis rate in ALI group was significantly increased compared with the control group. However, treatment with germacrone obviously reversed the apoptotic death in the lung tissues (Fig. 2C).

Effect of germacrone treatment on A549 cell viability and apoptosis

The lung epithelial cell line A549 was used to explore the roles of germacrone on cell viability and apoptosis. Data from MTT assay showed that cell viabilities were significantly reduced after LPS treatment. However, germacrone administration significantly improved the cell viabilities in a dose-dependent fashion (Fig. 3A). Moreover, germacrone concentration-dependently promoted cell proliferation as measured by EdU incorporation assay (Fig. 3B). Flow cytometric analysis revealed that germacrone administration significantly reduced the LPS-induced cells apoptosis (Fig. 3C). In addition, the expression levels of key apoptosis-related molecules (Caspase 3, p53 and bax) were remarkably reduced after germacrone treatment (Fig. 3D and E). These findings suggested that germacrone administration protected against LPS-induced lung injury *in vitro*.

Germacrone promoted the expression of claudin-4 in vivo and in vitro

Claudin-4, one member of the claudins family, plays an important role in acute lung injury. In the present study, we found that the mRNA and protein levels of claudin-4 were significantly reduced in rat lung tissues

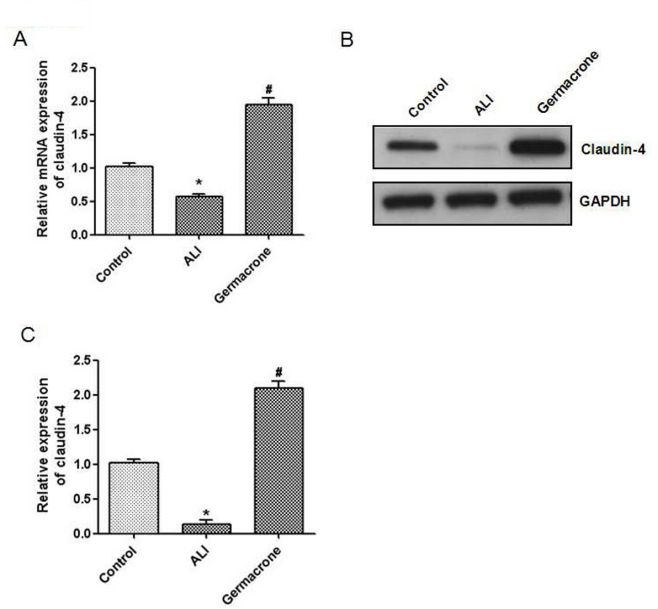

Figure 4. Germacrone treatment increased the claudin-4 expression *in vitro*

Neonatal rats were treated with 10 mg/kg of germacrone 1 hour before LPS injection. Real time PCR and western blot were performed to measure the mRNA (A) and protein (B and C) expression of claudin-4. The expression of each protein was measured by relative band intensities. * P<0.05 vs. Control. # P<0.05 vs. LPS.

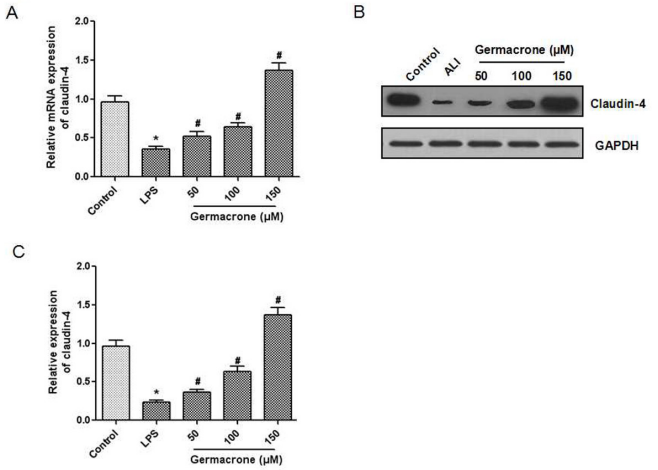

Figure 5. Germacrone treatment increased the claudin-4 expression *in vitro*

Cells were administrated with LPS (10 μg/mL) or different concentrations of germacrone (50 μM, 100 μM and 150 μM) for 24 h. Then the claudin-4 expression was determined by real time PCR (A) and western blot (B and C). The expression of each protein was measured by relative band intensities. * P<0.05 vs. Control. # P<0.05 vs. LPS.

after LPS injection. Nevertheless, germacrone administration significantly increased the claudin-4 expression as shown by real time PCR (Fig. 4A) and western blot (Fig. 4B and C). Moreover, we examined the claudin-4 expression in A549 cells after germacrone administration. Real time PCR showed that germacrone significantly increased the mRNA expression of claudin-4 in a dose dependent manner (Fig. 5A). Besides, the protein expression of claudin-4 was also concentration-dependently elevated following germacrone treatment (Fig. 5B and C). Collectively, these data demonstrated that germacrone administration resulted in the significant up-regulation of claudin-4 both *in vivo* and *in vitro*.

Discussion

Germacrone is a biologically active component of *Rhizoma curcuma*, and has been shown to possess antimicrobial, antioxidant and immunoregulatory activities (12, 13). In the present study, we explored the protective effects of germacrone on lung injury induced by LPS injection in neonatal rats.

Increasing evidences have suggested that inflammatory mediators play an important role in the pathogenesis of lung injury. Among them, TNF-α and IL-6 were considered as the most important pro-inflammatory mediators in innate immune response (17, 18). Transforming growth factor β (TGF-β) and IL-10 have shown to play an anti-inflammatory role in immunity and autoimmunity (19, 20). In the current study, germacrone administration significantly decreased the LPS-induced upregulation of TNF-α and IL-6 in serum. Moreover, the anti-inflammatory mediator TGF-β1 and IL-10 were reduced after LPS injection, which was reversed by germacrone treatment. Pulmonary edema was typical pathological changes in ALI, leading to deterioration of pulmonary gas exchange and reduction of lung compliance (21, 22). Our study demonstrated that the LPS treatment led to typically pathological features of lung injury. But such pathological changes were reduced by germacrone administration. Furthermore, we found that germacrone treatment remarkably attenuated LPS-induced pulmonary edema as shown by lung wet/dry weight ratio. Taken together, these findings demonstrated that germacrone administration protected neonatal rats against LPS-induced lung injury through attenuation of inflammation and pulmonary edema *in vivo*.

Next the lung epithelial cell line A549 was used to investigate the i*n vitro* effect of germacrone. According to our data, germacrone administration significantly improved the A549 cell viability in a dose dependent manner as shown by CCK-8 and EdU incorporation assays. Apoptosis is the process of programmed cell death which plays a critical role in developmental biology and in remodeling of tissues during repair (23, 24). In our study, flow cytometry and TUNEL analysis showed that germacrone treatment significantly reduced the apoptosis rate of A549 cells. Taken together, our results demonstrated that germacrone could promote cell viability and reduce cell apoptosis, thus protecting against LPS-induced lung injury.

We further investigated the possible molecular mechanism underlying the protective effects of germacrone on LPS-induced ALI in neonatal rats. Claudins are a family of proteins that are the most important components of the tight junctions, where they establish the paracellular barrier controlling the molecules flow between the cells of an epithelium (25, 26). Claudin-4, one member of the claudins family, is required for maximal epithelial barrier function and its expression affects the paracellular transport by decreasing ion conductance and conferring relative chloride selectivity (10). It also plays an important role in determining alveolar fluid clearance rates in human lungs (27). The expression of claudin-4 was significantly enhanced after germacrone treatment both *in vivo* and *in vitro*, suggesting that the protective effects of germacrone was partly associated with claudin-4.

In summary, the present study for the first time demonstrated that germacrone protected against LPS-induced ALI in neonatal rats partially by modulation of claudin-4. These results suggested that germacrone may be considered as an effective candidate drug for the potential treatment of ALI.

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