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Zerumbone reduced the inflammatory response of acute lung injury in endotoxin-treated mice via Akt-NFκB pathway

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ABSTRACT

Zerumbone, a cyclic eleven-membered sesquiterpene, is the major component of the essential oil isolated from the wild ginger, *Zingiber zerumbet*. There are several beneficial pharmacological activities of zerumbone including anti-inflammatory, antioxidant, and anticancer activities. Acute lung injury (ALI) is an acute pulmonary inflammatory disorder with high morbidity and mortality rate. In present study, we aimed to investigate the protective effects and mechanisms of zerumbone on endotoxin, lipopolysaccharide (LPS)-induced ALI. Mice were pretreated with zerumbone at various concentrations for 30 min followed by intratracheal administration of LPS for 6 h. Pretreatment with zerumbone not only reduced leukocytes infiltration into the alveolar space but also inhibited lung edema in LPS-induced ALI. Decreased secretion of proinflammatory cytokines such as TNFα and IL-6 caused by LPS were reversed by zerumbone. LPS-induced expressions of proinflammatory mediators, iNOS and COX-2, were inhibited by zerumbone. In addition, NFκB activation and Akt phosphorylation were inhibited by zerumbone in LPS-induced ALI. All these results suggested that the protective mechanisms of zerumbone on endotoxin-induced ALI were via inhibition of Akt-NFκB activation.

1. Introduction

Acute lung injury (ALI) is an acute pulmonary inflammatory disorder and life-threatening disease. There are several histopathological features in ALI including alveolar leukocytes infiltration, increased alveolar wall thickness, lung edema, haemorrhage, and hyaline membrane formation [1]. Lipopolysaccharide (LPS), also called endotoxin, is the major component of Gram-negative bacterial outer membranes and the most important risk factor for ALI via multitude of pulmonary and extrapulmonary insults [2]. Instillation of LPS into the lung induces activation of alveolar macrophages and epithelial cells which are predominant cell types in the alveolar-capillary barrier. The proinflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 from activated alveolar macrophages and epithelial cells alter the integrity of alveolar-capillary barrier and activation of peripheral neutrophils [3]. Disruption of alveolar-capillary barrier can be induced by LPS, and results in leakage of neutrophils and plasma proteins into the alveolar space and parenchyma [4]. Nuclear factor (NF)-κB, a proinflammatory transcription factor, is one of the most important factor which participating in the regulation of proinflammatory mediators generation [5]. Phosphoinositide 3-kinase and its downstream effector, the protein kinase Akt, have been demonstrated to modulate NF-κB activation in LPS-induced ALI [6].

Up to date, the incidence and mortality rate for ALI are still high. In the United States, the incidence of ALI is around 200,000 patients per year with a mortality rate of 40% [7]. However, an effective clinical treatment is deficient and still in development. Zerumbone,

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2,6,9,9-tetramethyl-*(2E,6E,10E)*-cycloundeca-2,6,10-trien-1-one, is a cyclic eleven-membered sesquiterpene and the main component of the essential oil isolated from the wild ginger, *Zingiber zerumbet* [8,9]. In mice, carrageenan-induced paw edema is inhibited by zerumbone [10]. Ultraviolet (UV) B radiation-induced inflammatory photokeratitis which results in corneal damage is protected by zerumbone [11]. After over-dosage of paracetamol administration, the inflammation and necrosis in the rat liver tissues are reduced by zerumbone [12]. In murine RAW264.7 macrophages, zerumbone effectively inhibits LPS-induced production of nitrite via expression of COX-2 mRNA and protein [13]. At present study, we aimed to determine the anti-inflammatory activity of zerumbone in ALI after LPS administration in animal model and to explore the mechanism involved.

2. Materials and methods

2.1. Materials

Antibodies against iNOS, COX-2, β-actin, and phosphorylated and non-phosphorylated forms of Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were obtained from Jackson Immuno Research Laboratories (Baltimore, MD, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and TNF-α were obtained from Cayman (Ann Arbor, MI, USA). Lipopolysaccharide (LPS; *Escherichia coli*, serotype 0111:B4), dimethyl sulfoxide (DMSO), and other reagents, unless specifically stated elsewhere, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The final volume of DMSO in all reaction mixtures was <0.5%.

2.2. Mice and experimental design

Male ICR mice (8–10 weeks old) weighing 25–30 g were purchased from the BioLASCO (Taipei, Taiwan). Mice were housed and maintained at a constant temperature (21 ± 1 °C) and humidity under a 12 h light dark cycle. Mice were fed with commercial diet and water ad libitum. All procedures performed on the animals were approved by the Institutional Animal Ethics Committee of Chung Shan Medical University. The animals (n = 60) were randomly divided into six groups, including a control and five treatment groups. The mice of the control group first received vehicle intraperitoneally (IP) for 30 min followed by intratracheal (IT) administration of 50 μl of saline; while the mice of the five treatment groups were injected with zerumbone at concentration of 0, 0.1, 1, or 10 μmol/kg or 1 mg/kg dexamethasone IP for 30 min followed by IT instillation of 100 μg/50 μl of LPS. After 6 h, the mice were sacrificed by pentobarbital (40 mg/kg) IP and tissue samples were collected. In addition, bronchoalveolar lavage fluid (BALF) was also collected and pooled together [14].

2.3. Measurement of lung edema

LPS-induced lung edema was assessed by the wet to dry weight (W/D) ratio. After excision of lungs, the wet weights were recorded. Then the lungs were placed in a dry oven at 80 °C for 24 h to obtain the dry weight. The W/D ratio was calculated to evaluate the extent of lung edema [14].

2.4. Bronchoalveolar lavage and cell counting

After the mice were sacrificed, the lungs were lavaged with sterile saline and cell numbers were counted as described previously [15]. Briefly, the lungs were lavaged with 1 ml of ice-cold sterile saline three times via tracheal cannula. BALF was recovered and centrifuged at 800 *g* for 10 min at 4 °C, the supernatant was stored at −20 °C for measurement of cytokines expression. In addition, the pellets were resuspended and stained with Geimsa solution for cell counting under the microscope.

2.5. Measurement of cytokines expression

The expression of cytokines, including TNF-α and IL-6, in BALF supernatant were measured by ELISA assay kits. The concentrations were interpolated from the standard curves for recombinant TNF-α and IL-6. All expression of cyokines measurements were performed in duplicate [16].

2.6. Western blot analysis of lung tissue

After the mice were sacrificed, the lungs were harvested and frozen in liquid nitrogen immediately. The lungs were then homogenized in tissue protein extraction solution and proteins were extracted (T-PER; Pierce, Rockford, IL), separated by SDS–PAGE, and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dried milk for 1 h, washed with PBS containing 0.1% Tween-20 (PBST), then probed with antibodies including iNOS, COX-2, β-actin, and phosphorylated and non-phosphorylated forms of Akt. After washing again, the horseradish peroxidase-labeled IgG was added for 1 h, and the blots were developed using enhanced chemiluminescence [5].

2.7. NFκB activation

As previously described [4], NFκB activation in nuclear extracts was measured by NFκB (p65) Transcription Factor Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

Statistical analyses were performed using ANOVA followed by the Bonferroni *t*-test for multi-group comparisons. $P < 0.05$ was considered significant for all tests. Data were expressed as mean ± standard deviation (S.D.) of at least three separate experiments.

3. Results

3.1. Effects of zerumbone on lung edema and leukocyte infiltration in LPS-induced ALI

Increased permeability of alveolar-capillary membrane is one of the important pathological characteristic in ALI which leads to lung edema and leukocytes, including neutrophil, infiltration [15]. Lung edema was determined by the W/D ratio. Data had shown that lung edema was significantly increased after administration of LPS when compared to untreated control group ($p < 0.05$), while for mice pretreated with zerumbone at concentration of 10 μmol/kg, the lung edema was significantly reduced ($p < 0.05$) (Fig. 1A). In addition, leukocytes infiltration was quantified by Giemsa stain. After LPS administration, extensive leukocytes infiltration into BALF was significantly increased, when compared to untreated control group ($p < 0.05$). LPS induced leukocytes infiltration was inhibited after pretreating with zerumbone in a concentration-dependent manner, significant inhibitory effect began at 1 μmol/kg ($p < 0.05$) (Fig. 1B). These results indicated that the protective effect of zerumbone in LPS-induced ALI mice.