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Calcitriol inhibits tumor necrosis factor alpha and macrophage inflammatory protein-2 during lipopolysaccharide-induced acute lung injury in mice

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ABSTRACT

Acute lung injury is a common complication of sepsis in intensive care unit patients with an extremely high mortality. The present study investigated the effects of calcitriol, the active form of vitamin D, on tumor necrosis factor alpha (TNF-α) and macrophage inflammatory protein-2 (MIP-2) in sepsis-induced acute lung injury. Mice were intraperitoneally (i.p.) injected with lipopolysaccharide (LPS, 1.0 mg/kg) to establish the animal model of sepsis-induced acute lung injury. Some mice were i.p. injected with calcitriol (1.0 μg/kg) before LPS injection. An obvious infiltration of inflammatory cells in the lungs was observed beginning at 1 h after LPS injection. Correspondingly, TNF-α and MIP-2 in sera and lung homogenates were markedly elevated in LPS-treated mice. Interestingly, calcitriol obviously alleviated LPS-induced infiltration of inflammatory cells in the lungs. Moreover, calcitriol markedly attenuated LPS-induced elevation of TNF-α and MIP-2 in sera and lung homogenates. Further analysis showed that calcitriol repressed LPS-induced p38 mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) phosphorylation. In addition, calcitriol blocked LPS-induced nuclear translocation of nuclear factor kappa B (NF-κB) p65 and p50 subunit in the lungs. Taken together, these results suggest that calcitriol inhibits inflammatory cytokines production in LPS-induced acute lung injury.

1. Introduction

Severe sepsis is the leading cause of death for patients in intensive care units [1,2]. Generally, patients with severe sepsis develop multiple organ failure including acute lung injury (ALI) and its severe form, the acute respiratory distress syndrome (ARDS), with a deregulated inflammatory response [3,4]. It is increasingly recognized that bacterial endotoxin, also known as lipopolysaccharide (LPS), a component of the outer membrane in Gram-negative bacteria, was involved in the pathogenesis of sepsis-induced acute lung injury [5,6]. Thus, LPS has been widely used to establish animal models of sepsis-induced acute lung injury [7]. As no effective treatment for sepsis-induced acute lung injury is established, novel therapeutic strategy is urgently needed to tackle sepsis-induced acute lung injury.

Numerous studies demonstrate that toll-like receptor (TLR)4-mediated inflammatory responses plays an important role in the development of sepsis-induced acute lung injury [8,9]. Indeed, LPS can activate inflammatory signaling pathways in airway neutrophils, such as mitogen-activated protein kinase (MAPK), PI3K/protein kinase B (Akt) and nuclear factor kappa B (NF-κB) signaling pathways, which are responsible for sepsis-induced acute lung injury [10–13]. The blockade of either PI3K/Akt or MAPK or NF-κB signaling pathway could effectively inhibit LPS-evoked inflammatory responses and protect against sepsis-induced acute lung injury [14–17].

Vitamin D, a secosteroid hormone, is known for its classical functions in calcium uptake and bone metabolism [18]. Recently, vitamin D is recognized for its non-classical actions including the modulation of innate immune, antioxidant effect and the anti-

Abbreviations: ARDS, acute respiratory distress syndrome; CYP, cytochrome P450; ELISA, enzyme linked immunosorbent assay; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MIP-2, macrophage inflammatory protein-2; NF-κB, nuclear factor kappa B; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha; VDR, vitamin D receptor; VitD3, vitamin D3.

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inflammatory activity [19–21]. Vitamin D itself is devoid of biological activity. The active form of vitamin D, calcitriol [1,25(OH)2D3], is produced by cytochrome p450 (CYP)27B1 and inactivated by CYP24A1 [22]. The actions of vitamin D are mediated by vitamin D receptor (VDR) that binds calcitriol to induce both transcriptional and non-genomic responses [23]. Indeed, VDR is highly expressed in the lungs [24]. Nevertheless, it remains unclear whether vitamin D protects against sepsis-induced acute lung injury.

The aim of the present study was to investigate the effects of calcitriol, the active form of vitamin D, on tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine, and macrophage inflammatory protein-2 (MIP-2), a chemokine, during LPS-induced acute lung injury in mice. The present study demonstrates that calcitriol inhibits LPS-induced TNF-α and MIP-2 through the blockade of several inflammatory signaling pathways.

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and calcitriol [1,25(OH)2D3] were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphor-MAPK p38 (pp38), p38, NF-κB p65, β-actin, α-tubulin and Lamin A/C antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). VDR antibodies were from Abcam (Cambridge, MA). Phosphor-Akt (pAkt) and Akt antibodies were from Cell Signaling Technology (Beverley, MA). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). All the other reagents were from Sigma or as indicated in the specified methods.

2.2. Animals and treatments

Adult male CD-1 mice (8 week-old, 28–32 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment. All mice were divided into four groups randomly. All mice except controls were intraperitoneally (i.p.) injected with LPS (1.0 mg/kg). In calcitriol + LPS group, mice were pretreated with three doses of calcitriol (1.0 μg/kg, i.p.) at 48, 24 and 1 h before LPS. The doses of calcitriol used in this study referred to others [25]. One hour after LPS injection, half of mice were euthanized with carbon dioxide and cervical dislocation. Six hours after LPS injection, the other half mice were euthanized with carbon dioxide and cervical dislocation. Left lungs were collected for measurements of inflammatory cytokines. After the lung vasculature was flushed, the superior lobe of right lung was excised for histopathologic examination. The middle and lower lobes of right lung were excised for immunoblots. This study was approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (Permit Number: 14-0016). All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

2.3. Lung histology

Lung tissues were fixed in 4% formalin and embedded in paraffin according to the standard procedure. Paraffin-embedded lung tissues were serially sectioned. At least five consecutive longitudinal sections were stained with hematoxylin and eosin (H&E) and scored for the extent of pathology on a scale of 0–5, where 0 was defined as no lung abnormality, and 1, 2, 3, 4, and 5 were defined as the presence of inflammation involving 10%, 10–30%, 30–50%, 50–80%, or >80% of the lungs, respectively.

2.4. Enzyme linked immunosorbent assay

The levels of TNF-α and MIP-2 in sera and lung homogenates were measured by using enzyme linked immunosorbent assay (ELISA) kits. All procedures were done in accordance with the manufacturer's instructions.

2.5. Immunoblots

Pulmonary lysate was prepared by homogenizing 50 mg lung tissue in 300 μl lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulphate, 1 mM phenylmethylsulfonyl fluoride) supplemented with a cocktail of protease inhibitors (Roche). For nuclear protein extraction, pulmonary lysate was suspended in hypotonic buffer and then kept on ice for 15 min. The suspension was then mixed with detergent and centrifuged for 30 s at 14,000×*g*. The nuclear pellet obtained was resuspended in complete lysis buffer in the presence of the protease inhibitor cocktail, incubated for 30 min on ice, and centrifuged for 10 min at 14,000×*g*. Protein concentrations were determined with the bicinchoninic acid

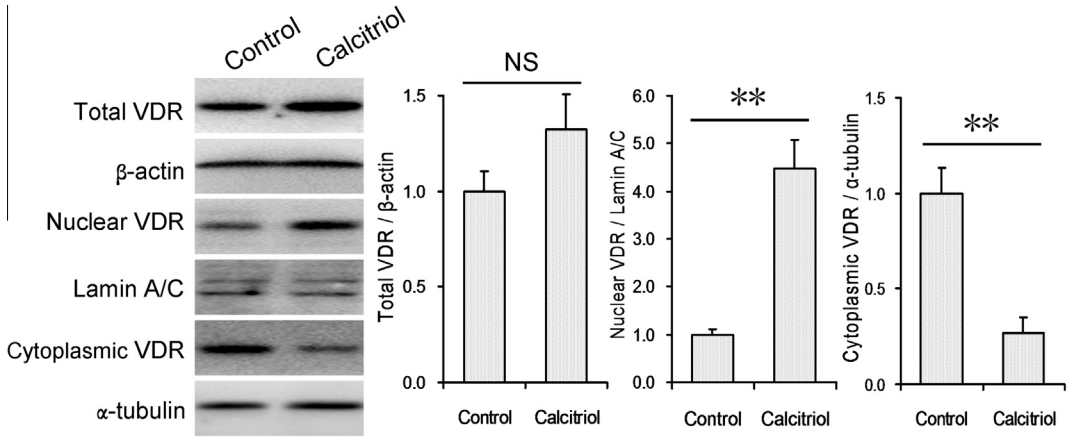

Fig. 1. Calcitriol-induced VDR activation in the lungs. In control group, mice were i.p. injected with normal saline. In calcitriol group, mice were pretreated with three doses of calcitriol (1.0 μg/kg) at 48, 24 and 1 h before normal saline injection. Lungs were collected 1 after normal saline injection. Total, nuclear and cytoplasmic VDR were measured using immunoblots. All experiments were duplicated for four times. All data were expressed as means ± S.E.M. (n = 4). $^{**}P < 0.01$, NS: NO significance.