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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

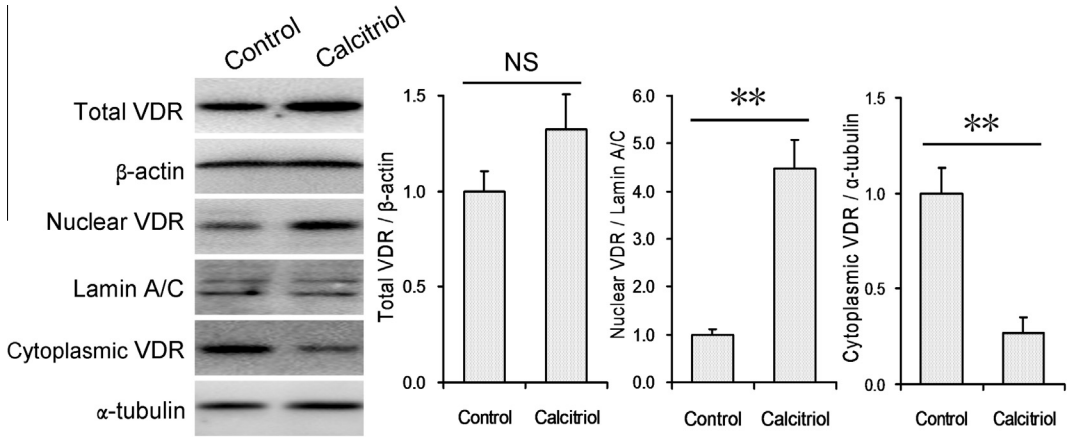

Fig. 1. Calcitriol-induced VDR activation in the lungs. In control group, mice were i.p. injected with normal saline. In calcitriol group, mice were pretreated with three doses of calcitriol (1.0 μg/kg) at 48, 24 and 1 h before normal saline injection. Lungs were collected 1 after normal saline injection. 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.

(BCA) protein assay reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. For immunoblots, same amount of protein (40 ~ 80 μg) was separated electrophoretically by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were incubated for 2 h with the following antibodies: pAkt, Akt, pp38, NF-κB p65 and VDR. For total proteins, β-actin or α-tubulin was used as a loading control. For nuclear protein, lamin A/C was used as a loading control. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse antibody for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an ECL detection kit.

2.6. Statistical analysis

Normally distributed data were expressed as means ± SEM. ANOVA and the Student-Newmann-Keuls post hoc test were used to determine differences among different groups. Data that were not normally distributed were assessed for significance using non-parametric tests techniques (Kruskal-Wallis test and Mann–Whitney *U* test). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Calcitriol-induced VDR activation in the lungs

The effect of calcitriol on pulmonary VDR was analyzed. We showed that total VDR level was slightly increased in calcitriol-pretreated mice ($P > 0.05$). As expected, the level of pulmonary nuclear VDR subunit was markedly elevated in calcitriol group (Fig. 1). Correspondingly, pulmonary cytoplasmic VDR level was significantly decreased in calcitriol-pretreated mice (Fig. 1).

3.2. Calcitriol alleviates infiltration of inflammatory cells in LPS-induced acute lung injury

The effects of calcitriol on the infiltration of inflammatory cells in LPS-induced acute lung injury were analyzed. A mild pulmonary edema was observed in LPS-treated mice (data not shown). Histological examination showed an infiltration of numerous inflammatory cells in the lungs (Fig. 2A). As expected, pretreatment with calcitriol significantly attenuated LPS-induced pulmonary edema (data not shown). Moreover, pretreatment with calcitriol significantly alleviated LPS-induced infiltration of inflammatory cells in the lungs (Fig 2A and B).

3.3. Calcitriol down-regulates LPS-induced TNF-α and MIP-2 in sera and lung homogenates

The effects of calcitriol on LPS-induced serum TNF-α and MIP-2 are presented in Fig 3A and B. As expected, serum TNF-α and MIP-2 levels were markedly elevated 1 h after LPS injection and remained increased 6 h after LPS injection. Interestingly, LPS-induced elevation of serum TNF-α level was significantly attenuated by calcitriol (Fig. 3A). In calcitriol + LPS group, LPS-induced elevation of serum MIP-2 level was significantly attenuated 6 h after LPS injection (Fig. 3B). The effects of calcitriol on LPS-induced TNF-α and MIP-2 in lung homogenates were then analyzed. As expected, the levels of TNF-α and MIP-2 in lung homogenates were markedly elevated 1 h after LPS injection and remained increased 6 h after LPS injection (Fig 3C and D). At 1 h after LPS injection, LPS-induced elevation of TNF-α in lung homogenates was attenuated by calcitriol (Fig. 3C). Although calcitriol had little effect on MIP-2 level in lung homogenates 1 h after LPS injection, LPS-induced elevation of MIP-2 in lung homogenates was attenuated by calcitriol at 6 h after LPS injection (Fig. 3D).

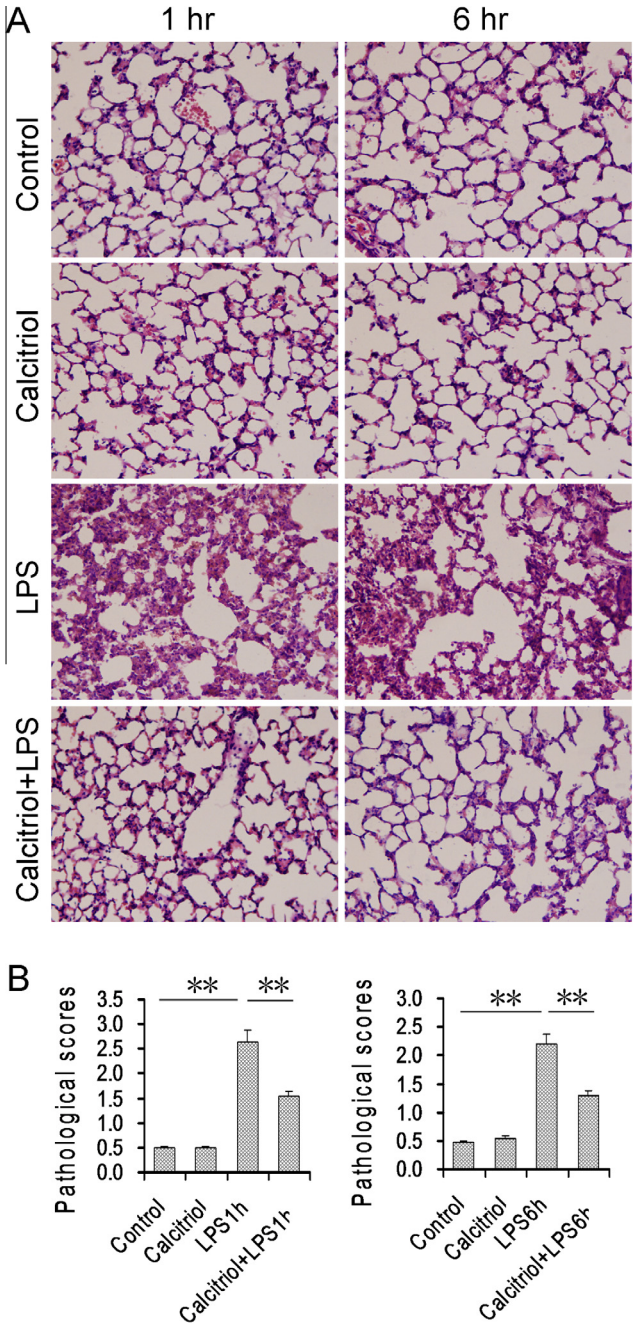

Fig. 2. Calcitriol alleviates on LPS-induced acute lung injury. In LPS group, mice were i.p. injected with a single dose of LPS (1.0 mg/kg). In calcitriol + LPS group, mice were pretreated with three doses of calcitriol (1.0 μg/kg) at 48, 24 and 1 h before LPS injection. Lungs were collected 1 and 6 h after LPS injection. (A) Representative H&E staining of lung sections. Original magnification: ×200. (B) Pathological scores of pulmonary damage among different groups. All data were expressed as means ± S.E.M (n = 10). $^{**}P < 0.01$.

3.4. Calcitriol inhibits LPS-activated PI3K/Akt signaling in the lungs

The effects of calcitriol on LPS-activated pulmonary PI3K/Akt signaling are presented in Fig. 4. Although LPS and calcitriol had no effect on the expression of Akt protein in the lungs (Fig. 4A),

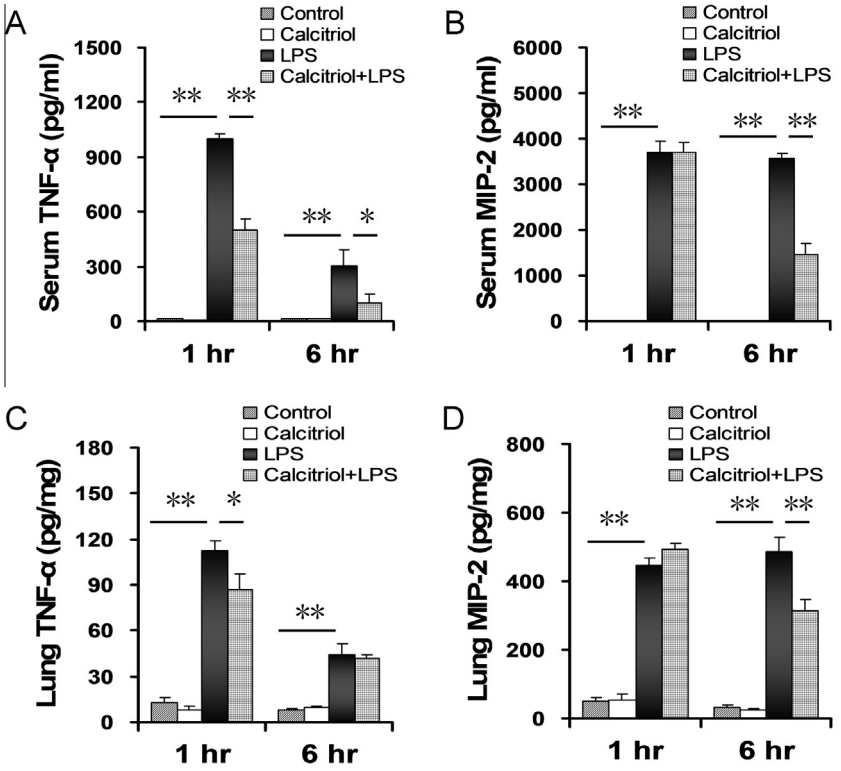

Fig. 3. Calcitriol down-regulates LPS-induced TNF-α and MIP-2 in serum and lung homogenates. In LPS group, mice were i.p. injected with a single dose of LPS (1.0 mg/kg). In calcitriol + LPS group, mice were pretreated with three doses of calcitriol (1.0 μg/kg) at 48, 24 and 1 h before LPS injection. Lungs were collected 1 and 6 h after LPS injection. Serum and lung tissue were collected 1 and 6 h after LPS injection. TNF-α and MIP-2 were measured using ELISA. (A) Serum TNF-α. (B) Serum MIP-2. (C) TNF-α in lung homogenates. (D) MIP-2 in lung homogenates. All data were expressed as means ± S.E.M (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$.

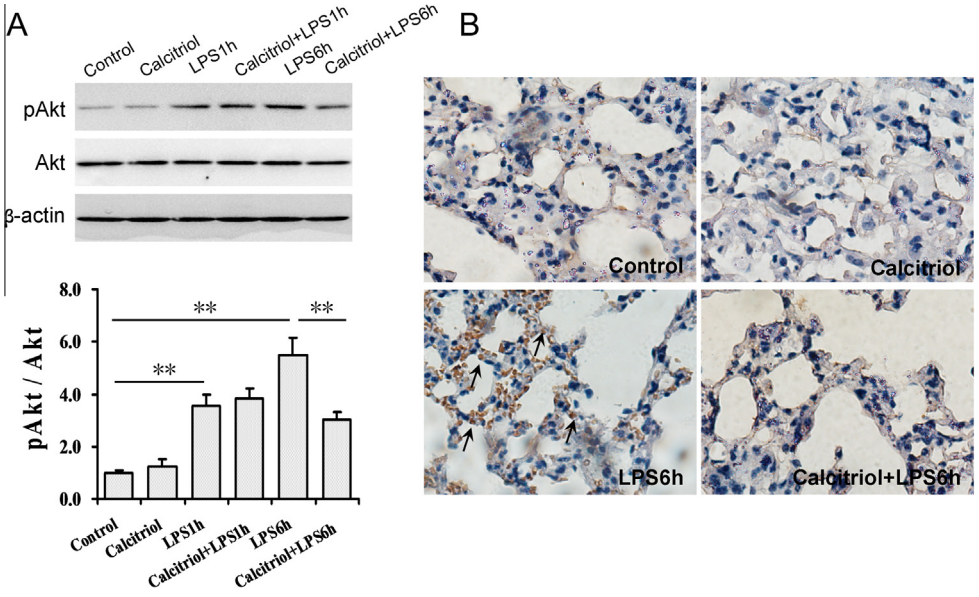

Fig. 4. Calcitriol inhibits LPS-activated PI3K/Akt signaling in the lungs. In LPS group, mice were i.p. injected with a single dose of LPS (1.0 mg/kg). In calcitriol + LPS group, mice were pretreated with three doses of calcitriol (1.0 μg/kg) at 48, 24 and 1 h before LPS injection. (A) Lungs were collected 1 and 6 h after LPS injection. Pulmonary pAkt and Akt were measured using immunoblots. (B) Lungs were collected 6 h after LPS injection. Pulmonary pAkt were measured using immunohistochemistry. Representative photomicrographs of pulmonary histology are shown. Original magnification: 400×. All experiments were duplicated for four times. All data were expressed as means ± S.E.M. (n = 4). $^{**}P < 0.01$.

the level of phosphorylated Akt in the lungs was obviously elevated 1 h after LPS injection and remained increased 6 h after LPS injection (Fig. 4A). LPS-evoked pulmonary Akt phosphorylation was significantly attenuated when mice were pretreated with calcitriol before LPS injection (Fig. 4A). Moreover, further analyzed using immunohistochemistry showed that LPS-evoked pulmonary Akt phosphorylation was mainly distributed in alveolar epithelial cell and pulmonary interstitium. Calcitriol pretreatment almost completely blocked LPS-induced pulmonary Akt phosphorylation in mice (Fig. 4B).

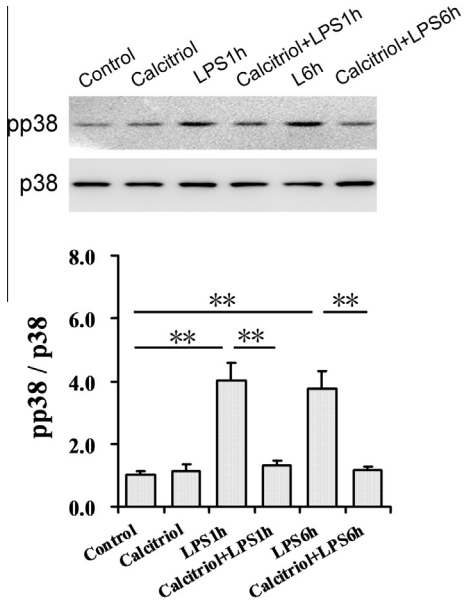

Fig. 5. Calcitriol inhibits LPS-activated p38 MAPK signaling in the lungs. In LPS group, mice were i.p. injected with a single dose of LPS (1.0 mg/kg). In calcitriol + LPS group, mice were pretreated with three doses of calcitriol (1.0 μg/kg) at 48, 24 and 1 h before LPS injection. Lungs were collected 1 and 6 h after LPS injection. Lungs were collected 1 and 6 h after LPS injection. Pulmonary pp38 was measured using immunoblots. All experiments were duplicated for four times. All data were expressed as means ± S.E.M. (n = 4). $^{**}P < 0.01$.

3.5. Calcitriol inhibits LPS-activated p38 MAPK signaling in the lungs

The effects of calcitriol on LPS-activated pulmonary p38 MAPK signaling are presented in Fig. 5. As expected, the level of phosphorylated p38 in the lungs was obviously elevated 1 h after LPS injection and remained increased 6 h after LPS injection. Interestingly, pretreatment with calcitriol significantly attenuated LPS-induced pulmonary p38 MAPK phosphorylation.

3.6. Calcitriol inhibits LPS-activated NF-κB signaling in the lungs

The effects of calcitriol on LPS-induced nuclear translocation of NF-κB p65 in the lungs were analyzed. As shown in Fig. 6, no significant difference on pulmonary total NF-kB p65 and p50 subunit levels were observed among different groups. As expected, the levels of nuclear NF-κB p65 in the lungs were significantly elevated 1 h after LPS injection and remained increased 6 h after LPS injection. Correspondingly, pulmonary cytoplasmic NF-κB p65 level was significantly decreased in LPS-treated mice (Fig. 6A). In addition, pulmonary nuclear NF-κB p50 levels were significantly elevated 1 h after LPS injection and remained increased 6 h after LPS injection. Correspondingly, pulmonary cytoplasmic NF-κB p50 levels were significantly decreased in LPS-treated mice (Fig. 6B). Calcitriol pretreatment almost completely blocked LPS-induced pulmonary translocation of NF-κB p65 and p50 subunit from cytoplasm to nuclei (Fig. 6).

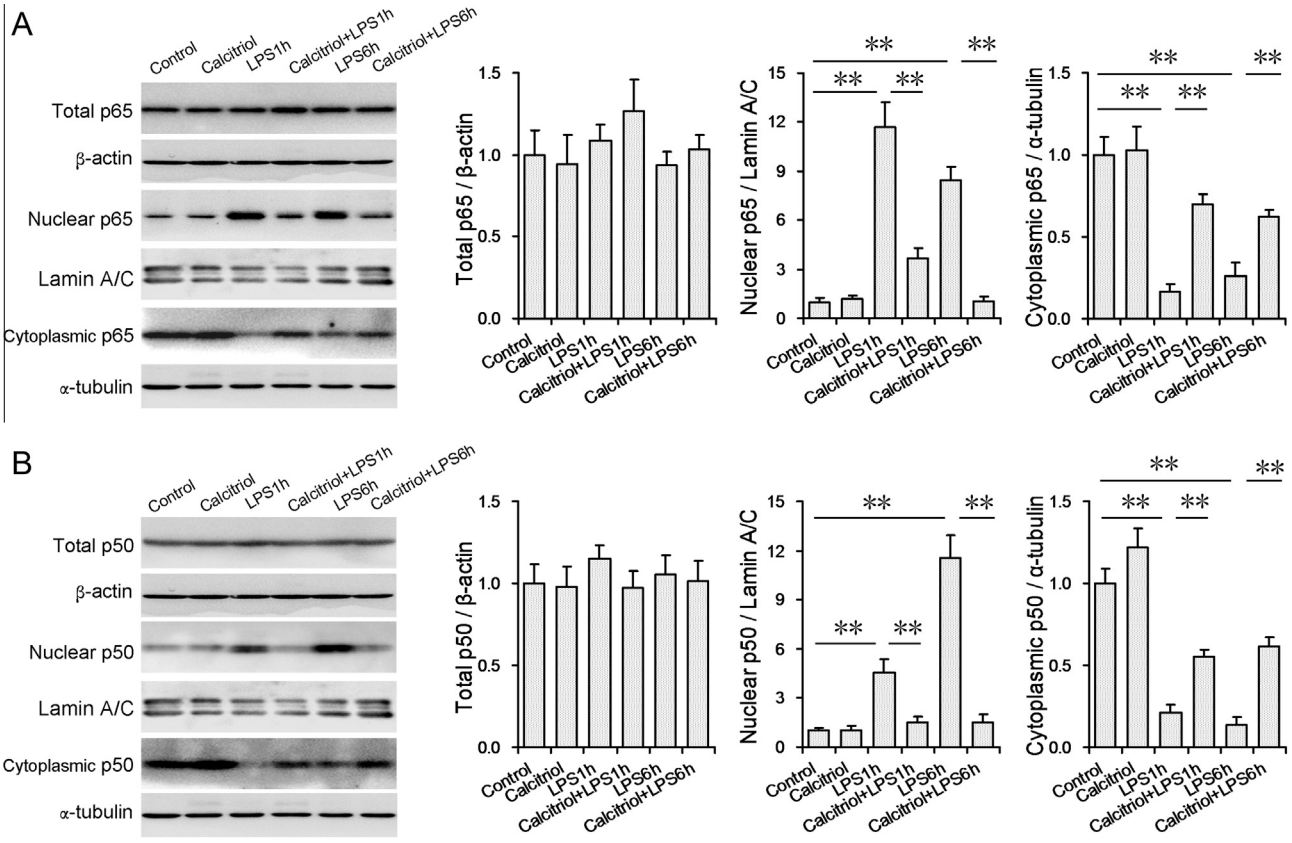

Fig. 6. Calcitriol inhibits LPS-activated NF-κB signaling in the lungs. In LPS group, mice were i.p. injected with a single dose of LPS (1.0 mg/kg). In calcitriol + LPS group, mice were pretreated with three doses of calcitriol (1.0 μg/kg) at 48, 24 and 1 h before LPS injection. Lungs were collected 1 and 6 h after LPS injection. Lungs were collected 1 and 6 h after LPS injection. (A) Total, nuclear and cytoplasmic NF-κB p65 were measured using immunoblots. (B) Total, nuclear and cytoplasmic NF-κB p50 were measured using immunoblots. All experiments were duplicated for four times. All data were expressed as means ± S.E.M. (n = 4). $^{**}P < 0.01$.

4. Discussion

According to a recent report, pretreatment with calcitriol, the active form of vitamin D, ameliorated seawater aspiration-induced acute lung injury in rats [26]. The present study investigated the effects of calcitriol on early inflammatory responses during sepsis-induced acute lung injury in mice. As expected, nuclear VDR level in the lung was markedly elevated, indicating pulmonary VDR signaling was activated in calcitriol-pretreated mice. Interestingly, pretreatment with calcitriol obviously alleviated LPS-induced pulmonary edema and infiltration of inflammatory cells in the lungs. These results are in agreement with a recent report [27], in which calcitriol significantly repressed neutrophil recruitment in the lung in a hamster model of LPS-induced acute lung injury. These results demonstrate that calcitriol can inhibit early inflammatory responses in sepsis-induced acute lung injury.

It is increasingly recognized that inflammatory cytokines play important roles in early inflammatory responses during sepsis-induced acute lung injury [9,10]. Indeed, pro-inflammatory cytokines, such as TNF-α, are the major mediator of early inflammatory responses during sepsis-induced acute lung injury [28,29]. CXC chemokines, such as MIP-2, play key roles in the recruitment of inflammatory cells into the lungs in sepsis-induced acute lung injury [30,31]. Indeed, calcitriol has an anti-inflammatory activity. Several reports demonstrated that calcitriol repressed the release of inflammatory cytokines in dendritic cells and macrophages [32–34]. According to an in vitro study, calcitriol inhibited not only Th1 cytokine IFN-γ but also Th2 cytokine IL-4 in naive CD62 ligand + CD4 + T cells [35]. The present study investigated the effects of calcitriol on systemic and local inflammatory cytokines in LPS-induced acute lung injury. We found that LPS-induced releases of TNF-α and MIP-2 in sera were obviously repressed when mice were pretreated with calcitriol before LPS. Correspondingly, LPS-induced elevation of TNF-α and MIP-2 in lung homogenates was markedly attenuated in calcitriol-pretreated mice. These results suggest that calcitriol inhibits not only systemic but also local inflammatory cytokines in sepsis-induced acute lung injury.

The MAPK signaling is one of the most important signaling cascades that regulate LPS-induced inflammatory genes [36,37]. An earlier study observed that calcitriol repressed the release of inflammatory cytokines in LPS-stimulated human umbilical vein cord endothelial cells through the blockade of p38 MAPK signaling [38]. According to a recent report, calcitriol dose-dependently repressed LPS-induced p38 MAPK phosphorylation and the releases of IL-6 and TNF-α in human monocytes [39]. The PI3K/Akt signaling is another important signaling pathway that regulates LPS-induced inflammatory cytokines [40]. A recent study found that calcitriol suppressed Akt phosphorylation in LPS-stimulated murine macrophages [41]. The present study investigated the effects of calcitriol on LPS-activated p38 MAPK and PI3K/Akt signaling during sepsis-induced acute lung injury. As expected, LPS-induced pulmonary Akt and p38 MAPK phosphorylation was attenuated when mice were pretreated with calcitriol before LPS. These results suggest that calcitriol inhibits LPS-induced pulmonary inflammatory cytokines partially through blockade of p38 MAPK and PI3K/Akt signaling pathways.

NF-κB signaling plays a central role in the regulation of inflammatory genes [42]. Under unstimulated conditions, NF-κB subunits bind with the inhibitor of kappa B (I-κB) in the cytoplasm. I-κB phosphorylation promotes translocation of NF-κB subunits to the nucleus [43]. Several studies demonstrated that calcitriol blocked LPS-induced activation of NF-κB signaling in mouse embryonic fibroblasts, human adipocytes and macrophages [44–46]. The present study investigated the effects of calcitriol on pulmonary NF-κB signaling during LPS-induced acute lung injury. As expected, the levels of nuclear NF-κB subunit p65 in the lungs were markedly elevated in LPS-treated mice. Interestingly, LPS-induced pulmonary NF-κB p65 translocation from cytoplasm to nuclei was almost completely blocked in calcitriol-pretreated mice. These results suggest that calcitriol inhibits LPS-induced pulmonary inflammatory cytokines partially through blockade of NF-κB signaling pathways. The mechanism through which calcitriol inhibits LPS-induced pulmonary NF-κB activation remains obscure. A recent study found that VDR blocked nuclear translocation of NF-κB p65/p50 subunits through by interacting with I-κB kinase β protein [47]. According to a recent report from our laboratory, VitD3 inhibits LPS-induced renal NF-κB activation through reinforcing the physical interaction between VDR and NF-κB p65 subunit [48]. Indeed, the present study showed that pulmonary VDR was activated in calcitriol-treated mice. Therefore, we guess that calcitriol blocks nuclear localization of NF-kB subunits through activating VDR in the lungs.

A small case-control study observed that vitamin D status was negatively correlated with disease severity in children with severe, therapy-resistant asthma [49]. According to a large prospective cohort study, there was an association between lower plasma 25 (OH)D levels and a higher risk of chronic obstructive pulmonary disease [50]. Indeed, vitamin D deficiency is common in bronchiectasis and correlates with disease severity [51]. Vitamin D supplementation helps regulate inflammation in patients with cystic fibrosis [52]. Moreover, vitamin D supplementation as an adjunctive therapy accelerates resolution of inflammatory responses during tuberculosis treatment. The present study demonstrates that pretreatment with calcitriol inhibits early inflammatory responses in sepsis-induced acute lung injury.

The present study laid emphasis on whether pretreatment with calcitriol inhibits TNF-α and MIP-2 during LPS-induced acute lung injury in mice. However, the present study has several limitations. First, only a single dose of calcitriol was used in the present study. A dose-dependent effect of calcitriol needs to be explored in another study. Second, the present study does not exclude the role of immune cells. Third, a recent study showed that treatment with calcitriol for seven day can increase the serum calcium level in mice [53]. Thus, although calcitriol may be therapeutic for sepsis-induced acute lung injury, the associated elevation in serum calcium may have adverse consequences that would prevent its clinical application.

In summary, the present study investigates the effects of calcitriol on TNF-α and MIP-2 during LPS-induced acute lung injury in mice. Our results indicate that calcitriol inhibits TNF-α and MIP-2 in LPS-induced acute lung injury. Moreover, calcitriol down-regulates LPS-induced pulmonary inflammatory cytokines partially through blockade of p38 MAPK, PI3K/Akt and NF-κB signaling pathways. Overall, calcitriol may have potential preventive and therapeutic utilities as an adjunctive agent for protecting against early inflammatory responses in sepsis-induced acute lung injury.

Disclosure

The authors have declared that no competing interests exist.

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