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Calcitriol inhibits bleomycin-induced early pulmonary inflammatory response and epithelial–mesenchymal transition in mice

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HIGHLIGHTS

- Calcitriol attenuates BLM-induced inflammatory cytokines in the lungs.
- Calcitriol inhibits BLM-activated NF-κB signaling in the lungs.
- Calcitriol inhibits BLM-activated PI3K/Akt and p38 MAPK in the lungs.
- Calcitriol inhibits BLM-induced EMT in the lungs.
- Calcitriol inhibits BLM-activated TGF-β-Smad signaling in the lungs.

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ABSTRACT

Early pulmonary inflammation and epithelial–mesenchymal transition (EMT) play important roles during lung fibrosis. Increasing evidence demonstrates that calcitriol, the active form of vitamin D3, has anti-inflammatory activities. The aim of this study was to investigate the effects of calcitriol on bleomycin (BLM)-induced early pulmonary inflammation and subsequent EMT. Mice were intratracheally injected with BLM (3.0 mg/kg). In three calcitriol + BLM groups, mice were intraperitoneal (i.p.) injected with different doses of calcitriol (0.2, 1.0 or 5.0 μg/kg) daily, beginning at 48 h before BLM injection. Twenty-four hours, seven and fourteen days after BLM injection, pulmonary inflammation and EMT were evaluated. As expected, BLM-induced infiltration of inflammatory cells in the lungs was attenuated by calcitriol. BLM-induced pulmonary inflammatory cytokines were repressed by calcitriol. Moreover, BLM-induced nuclear translocation of nuclear factor kappa B (NF-κB) p65 was blocked by calcitriol. In addition, BLM-induced phosphorylation of pulmonary p38 MAPK and protein kinase B (Akt) was inhibited by calcitriol. Further analysis showed that BLM-induced α-smooth muscle actin (α-SMA), a marker for EMT in the lungs, was significantly attenuated by calcitriol. BLM-induced transforming growth factor-beta 1 (TGF-β1) up-regulation and Smad phosphorylation were attenuated by calcitriol. In conclusion, calcitriol inhibits BLM-induced early pulmonary inflammation and subsequent EMT.

1. Introduction

Idiopathic pulmonary fibrosis, characterized by fibroblast proliferation and extracellular matrix remodeling, is a chronic pulmonary disease of unknown origin ultimately leading to death (Borchers et al., 2011; King et al., 2011). Bleomycin (BLM), a widely used anti-neoplastic drug, causes a dose-dependent interstitial pulmonary fibrosis in humans and experimental animals (Adamson and Bowden, 1974; Lazo et al., 1990; Chen and Stubbe, 2005). BLM-induced pulmonary fibrosis, resembling human interstitial pulmonary fibrosis (Moore and Hogaboam, 2008), has been the most commonly used model for idiopathic pulmonary fibrosis (Moeller et al., 2008). Although the mechanisms of BLM-induced pulmonary fibrosis are not completely understood, alveolar cell

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damage followed by an infiltration of numerous inflammatory cells and a diffuse inflammatory response, epithelial–mesenchymal transition (EMT) and subsequent extracellular matrix deposition are involved in the pathogenesis of BLM-induced pulmonary fibrosis (Tanjore et al., 2009; Hashimoto et al., 2010).

Vitamin D, a secosteroid hormone, is known for its classical functions in calcium uptake and bone metabolism (Holick, 2006). Recently, vitamin D is recognized for its non-classical actions including the modulation of innate immune, antioxygenation and anti-inflammatory activity (Hewison, 2010; Xu et al., 2015; Chen et al., 2015a,b). 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 (Schuster, 2011). The actions of vitamin D are mediated by vitamin D receptor (VDR) that binds calcitriol to induce both transcriptional and non-genomic responses (Dimitrov et al., 2014). Although VDR is highly expressed in the lungs (Menezes et al., 2008), its function remains unclear.

Vitamin D deficiency is common and increasingly recognized as a global public health problem (Chen et al., 2015c). Increasing evidence demonstrates that there is a cause association between vitamin D deficiency and childhood asthma (Paul et al., 2012; Litonjua, 2012; Poon et al., 2013). Moreover, vitamin D deficiency is linked with an increased risk of respiratory infections (Lowery et al., 2012; Chalmers et al., 2013; Jeon et al., 2013; Hong et al., 2014). According to a double-blind and randomized controlled trial, vitamin D3 supplementation alleviates eosinophilic airway inflammatory response in patients with nonatopic asthma with severe eosinophilic airway inflammation (de Groot et al., 2015). In addition, vitamin D3 supplementation protects against moderate or severe exacerbation in patients with chronic obstructive pulmonary disease (Martineau et al., 2015).

The aim of the present study was to investigate the effects of calcitriol on early pulmonary inflammatory response and subsequent EMT in the process of BLM-induced pulmonary injury. Our results showed that pretreatment with calcitriol alleviated early pulmonary inflammatory response through blocking activation of several inflammatory signaling in the lungs. We demonstrate that calcitriol attenuates BLM-induced EMT through suppressing pulmonary transforming growth factor-beta 1 (TGF-β1)-Smad signaling.

2. Materials and methods

2.1. Chemicals and reagents

BLM and calcitriol were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphor-MAPK p38 (pp38), NF-κB p65, α-SMA, β-actin and Lamin A/C antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Phosphor-Akt (pAkt) and Akt antibodies were from Cell Signaling Technology (Beverley, MA, USA). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). TRI reagent was from Molecular Research Center, Inc. (Cincinnati, Ohio). RNase-free DNase was from Promega Corporation (Madison, WI). All the other reagents were from Sigma or as indicated in the specified methods.

2.2. Animals and treatments

Adult male C57BL/6J mice (8 week-old, 24–26 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. The present study consisted of two independent experiments.

Experiment 1, to investigate the effects of pretreatment with different doses of calcitriol on BLM-induced early pulmonary pathological damage and inflammatory cytokines, thirty mice were divided into five groups. In BLM alone group, mice were intratracheally injected with 3.0 mg/kg of BLM (dissolved in 50 μL phosphate buffered saline (PBS). The doses of BLM used in the present study referred to others (Pilling et al., 2014). In three BLM + calcitriol groups, mice were intraperitoneally (i.p.) injected with different doses of calcitriol (0.2, 1.0 or 5.0 μg/kg) daily, beginning at 48 h before BLM injection, the second at 24 h before BLM injection, and the third at 1 h before BLM injection. The doses of calcitriol used in the present study referred to others with minor modification (Ito et al., 2013). Control mice were i.p. injected with normal saline (NS) daily, beginning at 48 h before PBS injection, the second at 24 h before PBS injection, and the third at 1 h before PBS injection. All mice were euthanized by exsanguination during pentobarbital anesthesia (75 mg/kg, i.p.) 24 h after an intratracheal BLM injection. Whole lung was weighed and left lungs were collected for measurements of inflammatory cytokines. After the lung vasculature was flushed, right lung were excised for histopathologic examination.

Experiment 2, to investigate the effects of calcitriol pretreatment on BLM-induced pulmonary pathological damage and inflammatory signaling at different time points, four–two mice were divided into three groups. In BLM alone group, eighteen mice were intratracheally injected with a single dose of BLM (3.0 mg/kg). In BLM + calcitriol group, eighteen mice were i.p. injected with calcitriol (1.0 μg/kg) daily, beginning 48 h before an intratracheal BLM injection. In Control group, six mice were i.p. injected with NS daily, beginning 48 h before an intratracheal PBS injection. In BLM alone and BLM + calcitriol groups, six mice each group were euthanized by exsanguination during pentobarbital anesthesia either 24 h or 7 d or 14 d after an intratracheal BLM injection. Whole lung was weighed and 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: 13-0016). 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.

Table 1
Oligonucleotide sequences and size of primers.

Genes	Sequences	Sizes (bp)
18S	Forward: 5′-GTAACCCGTTGAACCCCATT-3′ Reverse: 5′-CCATCCAATCGGTAGTAGCG-3′	151
tnf-α	Forward: 5′-CCCTCCTGGCCAACGGCATG-3′ Reverse: 5′-TCGGGGCAGCCTTGTCCCTT-3′	109
il-1β	Forward: 5′-GCCTCGTGCTGTCGGACCCATAT-3′ Reverse: 5′-TCCTTTGAGGCCCAAGGCCACA-3′	143
il-6	Forward: 5′-AGACAAAGCCAGAGTCCTTCAGAGA-3′ Reverse: 5′-GCCACTCCTTCTGTGACTCCAGC-3′	146
mcp-1	Forward: 5′-GGCTGGAGAGCTACAAGAGG-3′ Reverse: 5′-GGTCAGCACAGACCTCTCTC-3′	93
mip-1α	Forward: 5′-GCAACCAAGTCTTCTCAGCG-3′ Reverse: 5′-TGGAATCTTCCGGCTGTAGG-3′	77
mip-2	Forward: 5′-TTGCCTTGACCCTGAAGCCCCC-3′ Reverse: 5′-GGCACATCAGGTACGATCCAGGC-3′	175

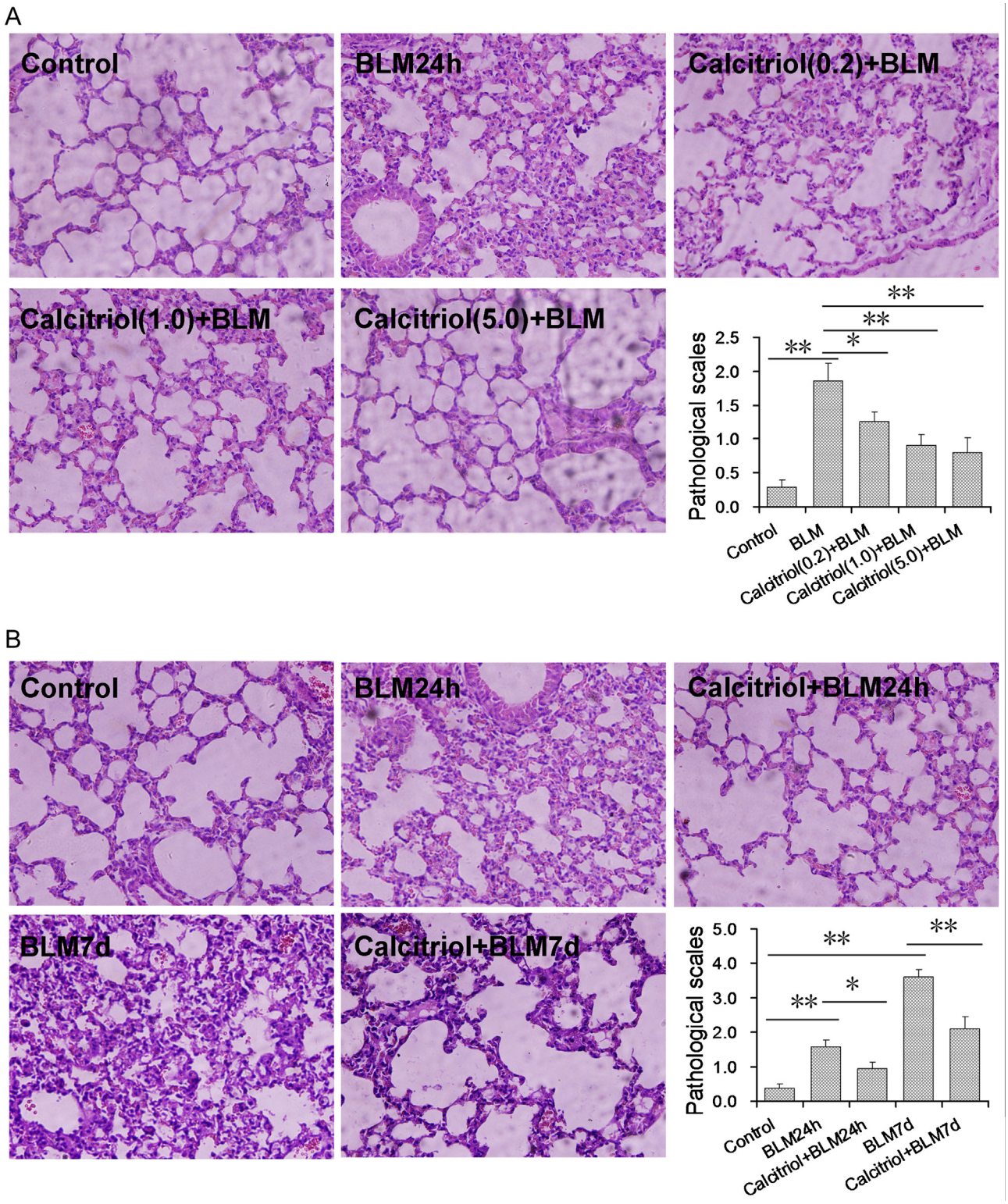

Fig. 1. Calcitriol alleviates BLM-induced pulmonary pathological damage. (A) A dose–effect relationship. In BLM alone group, mice were intratracheally injected with a single dose of BLM (3.0 mg/kg, dissolved in 50 μL PBS). In three BLM + calcitriol groups, mice were i.p. injected with different doses of calcitriol (0.2, 1.0 or 5.0 μg/kg) daily, beginning at 48 h before BLM, the second at 24 h before BLM injection, and the third at 1 h before BLM injection. Lungs were collected 24 h after BLM. Lung cross sections were stained with H&E. Original magnification: 200×. Infiltration of inflammatory cells in the lungs was evaluated. All data were expressed as means ± SEM ($n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$. (B) A time-course analysis. All mice except controls were intratracheally injected with a single dose of BLM (3.0 mg/kg, dissolved in 50 μL PBS). In BLM + calcitriol group, mice were i.p. injected with calcitriol (1.0 μg/kg) daily, beginning at 48 h before BLM. Lungs were collected 24 h and 7 d after BLM. Lung cross sections were stained with H&E. Original magnification: 200×. Infiltration of inflammatory cells in the lungs was evaluated. All data were expressed as means ± SEM ($n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$.

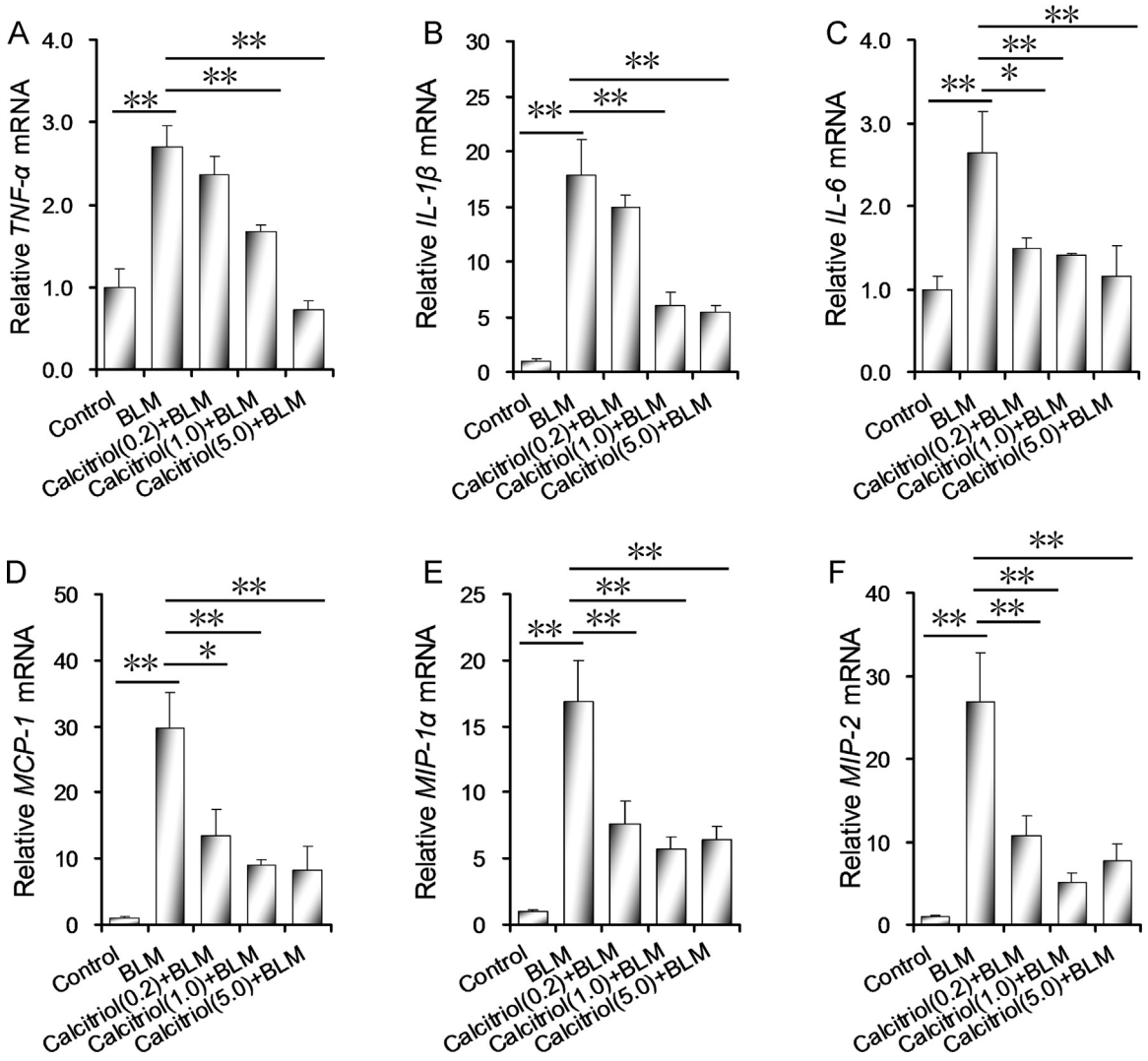

Fig. 2. Effects of different doses of calcitriol on BLM-induced pulmonary proinflammatory cytokines and chemokines. In BLM alone group, mice were intratracheally injected with a single dose of BLM (3.0 mg/kg, dissolved in 50 μL PBS). In three BLM + calcitriol groups, mice were i.p. injected with different doses of calcitriol (0.2, 1.0 or 5.0 μg/kg) daily, beginning at 48 h before BLM, the second at 24 h before BLM injection, and the third at 1 h before BLM injection. Lungs were collected 24 h after BLM. Pulmonary inflammatory cytokines and chemokines were measured using real-time RT-PCR. (A) TNF-α; (B) IL-1β; (C) IL-6; (D) MCP-1; (E) MIP-1α; (F) MIP-2. All data were expressed as means ± SEM ($n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$.

2.3. Pulmonary 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 1–5 were defined as the presence of inflammation involving 10%, 10–30%, 30–50%, 50–80%, or >80% of the lungs, respectively.

2.4. 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 dodecylsylphate, 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 (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, p38, NF-κB p65 and α-SMA. For total proteins, β-actin 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.

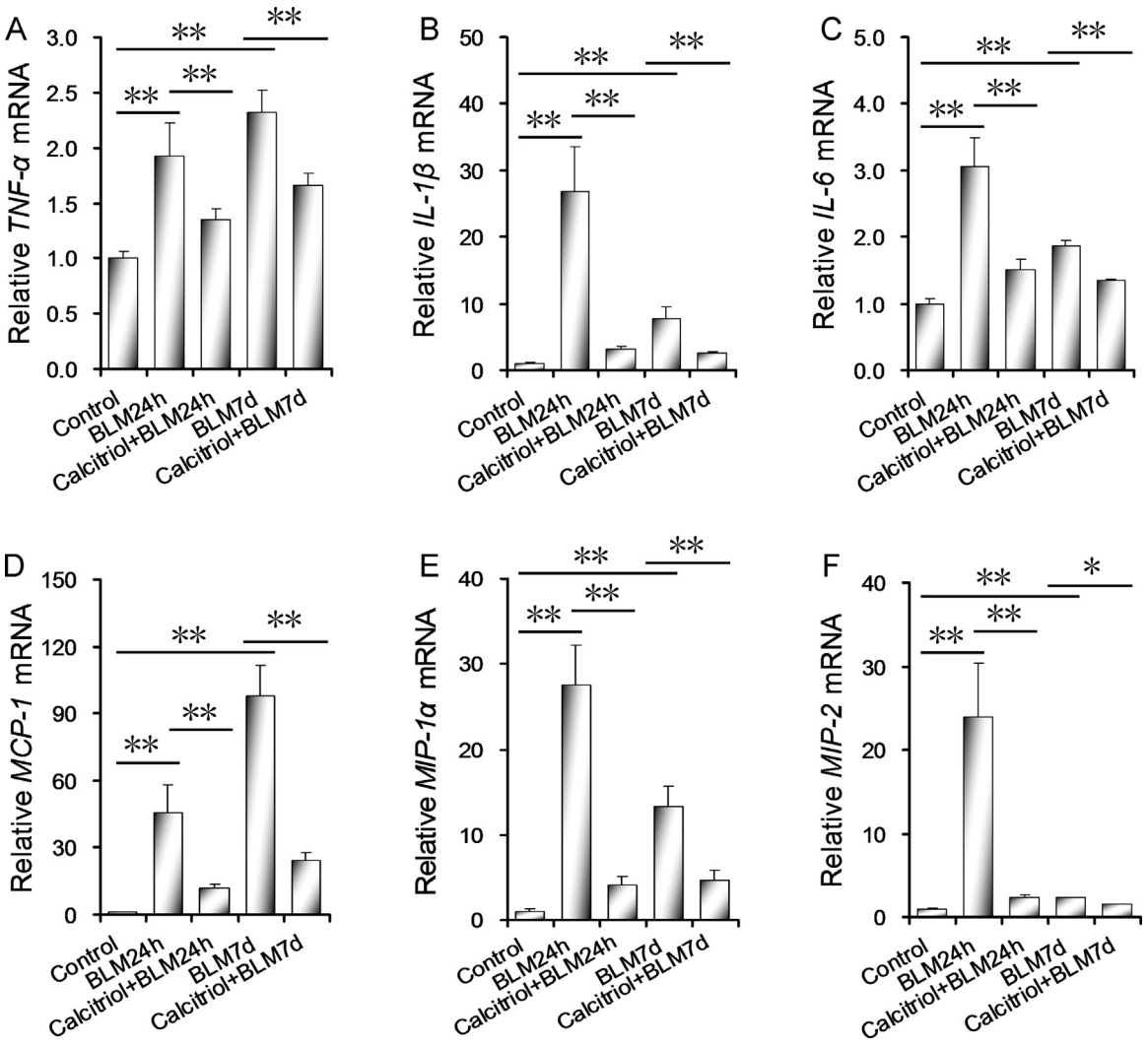

Fig. 3. Calcitriol attenuates BLM-induced pulmonary inflammatory cytokines and chemokines—a time-course analysis. In BLM group, mice were intratracheally injected with a single dose of BLM (3.0 mg/kg, dissolved in 50 μL PBS). In BLM + calcitriol group, mice were i.p. injected with calcitriol (1.0 μg/kg) daily, beginning at 48 h before BLM. Lungs were collected 24 h and 7 d after BLM. Pulmonary inflammatory cytokines and chemokines were measured using real-time RT-PCR. (A) TNF-α; (B) IL-1β; (C) IL-6; (D) MCP-1; (E) MIP-1α; (F) MIP-2. All data were expressed as means ± SEM ($n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$.

2.5. Isolation of total RNA and real-time RT-PCR

Total RNA was extracted using TRI reagent. RNase-free DNase-treated total RNA (1.0 μg) was reverse-transcribed with AMV. Real-time RT-PCR was performed with a GoTaq qPCR master mix using gene-specific primers as listed in Table 1. The amplification reactions were carried out on a LightCycler 480 Instrument (Roche Diagnostics GmbH, Mannheim, Germany) with an initial hold step (95 °C for 5 min) and 50 cycles of a three-step PCR (95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s). The comparative CT-method was used to determine the amount of target, normalized to an endogenous reference (18 s) and relative to a calibrator ($2^{-\Delta\Delta Ct}$) using the Lightcycler 480 software (Roche, version 1.5.0). All RT-PCR experiments were performed in triplicate.

2.6. Immunohistochemistry

For immunohistochemistry, paraffin-embedded lung sections were deparaffinized and rehydrated in a graded ethanol series. After antigen retrieval and quenching of endogenous peroxidase, sections were incubated with either α-SMA or NF-κB p65 monoclonal antibody (1:200 dilution) at 4 °C overnight. The color reaction was developed with HRP-linked polymer detection system and counterstaining with hematoxylin.

2.7. 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 attenuates BLM-induced infiltration of inflammatory cells

First, the effects of different doses of calcitriol on BLM-induced pulmonary pathological damage were analyzed. A mild pulmonary edema, as determined by a mild elevation of lung weight, was observed in BLM-treated mice (data not shown). Consistent with pulmonary edema, alveolar structure was damaged and an

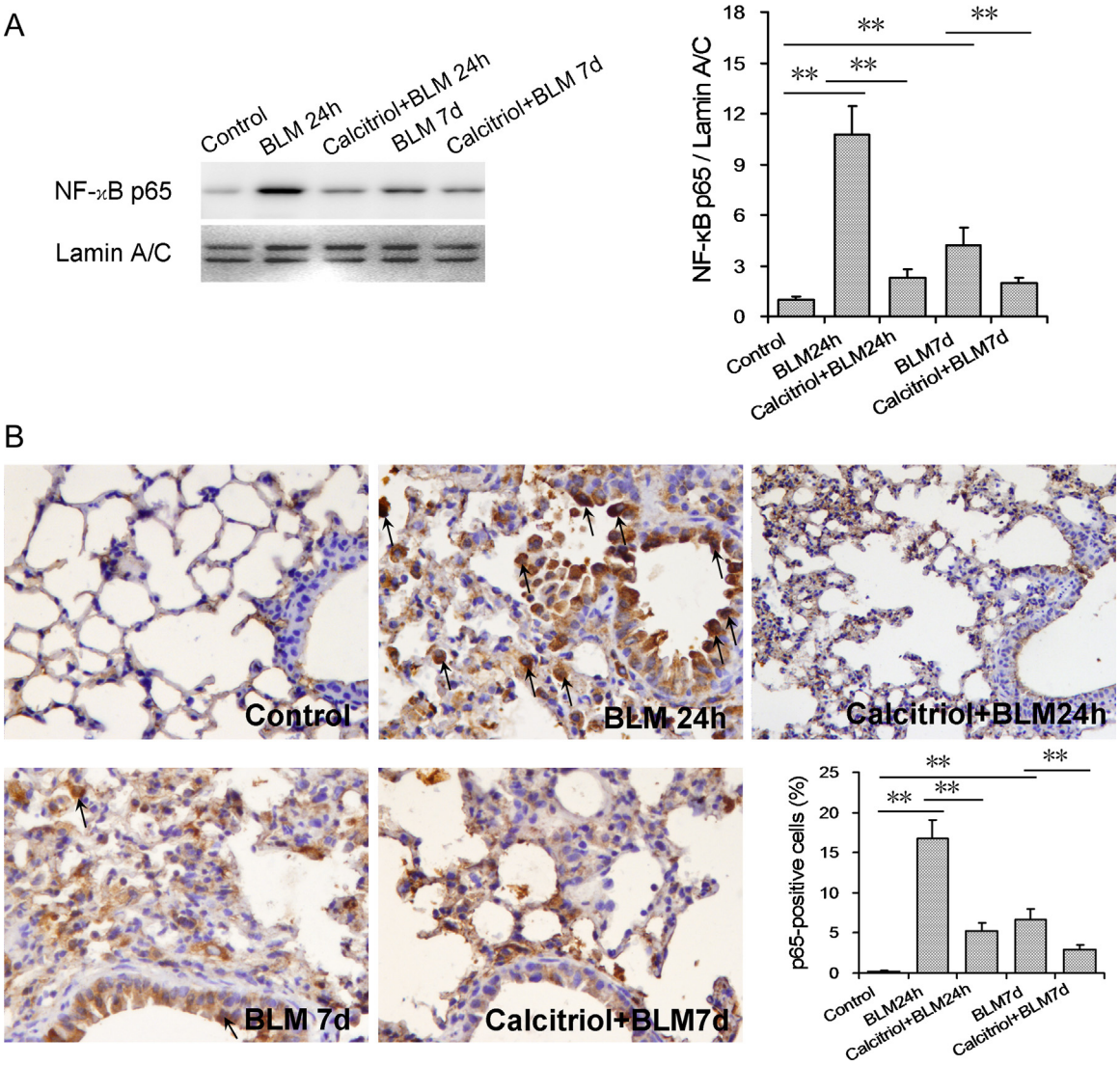

Fig. 4. Calcitriol inhibits BLM-activated NF-κB signaling in the lungs. In BLM group, mice were intratracheally injected with a single dose of BLM (3.0 mg/kg, dissolved in 50 μL PBS). In BLM + calcitriol group, mice were i.p. injected with calcitriol (1.0 μg/kg) daily, beginning at 48 h before BLM. Lungs were collected 24 h and 7 d after BLM. (A) Nuclear NF-kB p65 was measured using Immunoblots. All experiments were replicated six times. (B) Pulmonary nuclear translocation of NF-kB p65 was analyzed using immunohistochemistry. Representative photomicrographs of pulmonary histological specimens from mice treated with saline, BLM alone (24 h), calcitriol plus BLM (24 h), BLM alone (7 d), calcitriol plus BLM (7 d). Original magnification: 400×. Arrowheads indicate p65-positive cells. Pulmonary p65-positive cells were counted. All data were expressed as means ± SEM ($n = 6$). $^{**}P < 0.01$.

infiltration of a small amount of inflammatory cells was observed in the lungs of BLM-treated mice (Fig. 1A). All doses of calcitriol attenuated BLM-induced pulmonary edema (data not shown). In addition, all doses of calcitriol alleviated BLM-induced infiltration of inflammatory cells in the lungs (Fig. 1A). The effects of calcitriol on BLM-induced pulmonary pathological damage at different time points were then analyzed. An obvious pulmonary edema, as determined by a mild elevation of lung weight, was observed in BLM-treated mice (data not shown). Moreover, an infiltration of numerous inflammatory cells was observed in the lungs of BLM-treated mice (Fig. 1B). Calcitriol attenuated BLM-induced pulmonary edema (data not shown). In addition, calcitriol alleviated BLM-induced infiltration of inflammatory cells in the lungs (Fig. 1B).

3.2. Calcitriol inhibits BLM-induced inflammatory cytokines and chemokines in the lungs

The effects of different doses of calcitriol on BLM-induced early inflammatory cytokines and chemokines in the lungs were analyzed. As shown in Fig. 2, mRNA levels of pulmonary TNF-α, IL-1β and IL-6, three pro-inflammatory cytokines, were elevated 24 h after BLM injection (Fig. 2A–C). Moreover, mRNA levels of pulmonary MCP-1, MIP-1α and MIP-2, three chemokines, were up-regulated 24 h after BLM injection (Fig. 2D–F). As shown in Fig. 2A–F, calcitriol pretreatment alleviated BLM-induced pulmonary pro-inflammatory cytokines and chemokines in a dose-dependent manner. The effects of calcitriol on BLM-induced pulmonary pro-inflammatory cytokines and chemokines at different time points were then analyzed. As shown in Fig. 3A–C, mRNA levels of pulmonary TNF-α, IL-1β and IL-6, three pro-inflammatory cytokines, were elevated 24 h after BLM injection and remained increased 7 d after BLM injection. Moreover, mRNA levels of pulmonary MCP-1, MIP-1α and MIP-2, three chemokines, were up-regulated 24 h after BLM injection and remained up-regulated 7 d after BLM injection (Fig. 3D–F). Calcitriol pretreatment attenuated BLM-induced elevation of pulmonary pro-inflammatory cytokines and chemokines at all time points (Fig. 3A–F).

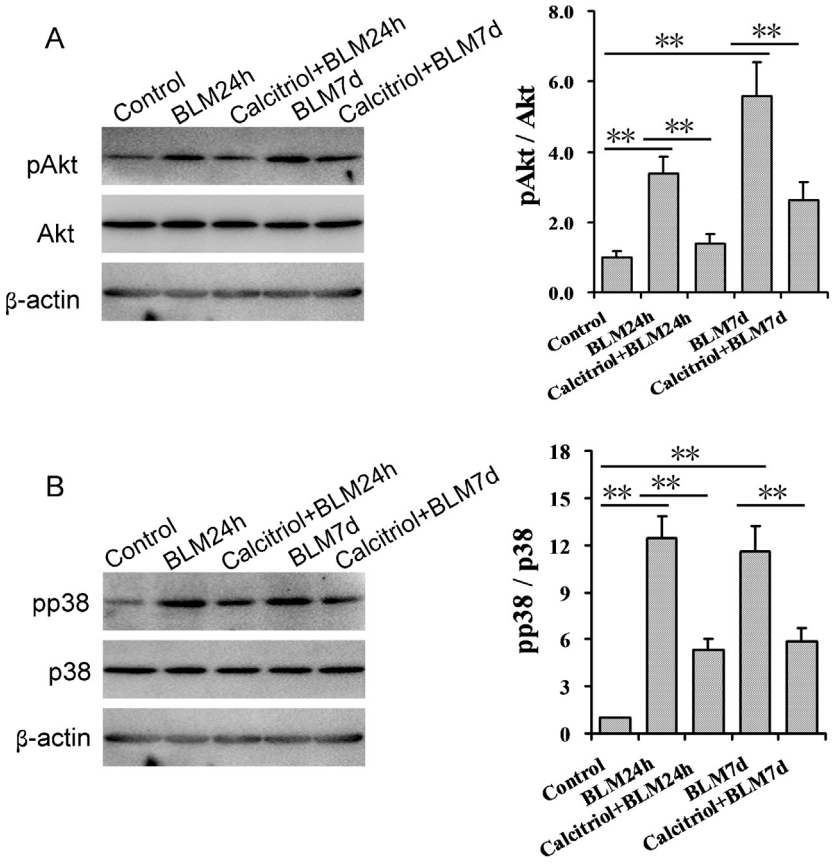

Fig. 5. Calcitriol attenuates BLM-induced p38 MAPK and Akt phosphorylation in the lungs. In BLM group, mice were intratracheally injected with a single dose of BLM (3.0 mg/kg, dissolved in 50 μL PBS). In BLM + calcitriol group, mice were i.p. injected with calcitriol (1.0 μg/kg) daily, beginning at 48 h before BLM. Lungs were collected 24 h and 7 d after BLM. (A) Pulmonary p-Akt and Akt were measured using immunoblots. (B) Pulmonary pp38 and p38 were measured using immunoblots. All experiments were replicated six times. All data were expressed as means ± S.E.M. ($n = 6$). $^{**}P < 0.01$.

3.3. Calcitriol inhibits BLM-activated NF-κB signaling in the lungs

The effects of calcitriol on BLM-activated NF-κB signaling in the lung were analyzed. As shown in Fig. 4A, the level of nuclear NF-κB p65 in the lungs was elevated 24 h after BLM injection and remained increased 7 d after BLM injection. Calcitriol attenuated BLM-induced elevation of nuclear NF-κB p65 in the lungs. Immunohistochemistry showed that numerous NF-κB p65-positive cells were observed in the lungs of BLM-treated mice (Fig. 4B). Further analysis showed that the number of NF-κB p65-positive cells was elevated in the lungs of BLM-treated mice (Fig. 4B). Calcitriol inhibited BLM-induced NF-κB activation in the lungs (Fig. 4B).

3.4. Calcitriol inhibits BLM-activated PI3K/Akt and p38 MAPK in the lungs

The effects of calcitriol on BLM-activated PI3K/Akt signaling in the lungs were analyzed. Although BLM did not affect the expression of Akt protein in the lungs, the level of phosphorylated Akt in the lungs was elevated in BLM-treated mice (Fig. 5A). BLM-evoked pulmonary Akt phosphorylation was significantly attenuated by calcitriol. The effects of calcitriol on BLM-activated p38 MAPK signaling in the lungs were then analyzed. The level of phosphorylated p38 in the lungs was elevated 24 h after BLM injection and remained increased 7 d after BLM injection (Fig. 5B). Calcitriol attenuated BLM-induced pulmonary p38 MAPK phosphorylation in the lungs.

3.5. Calcitriol inhibits BLM-induced EMT in the lungs

TGF-β1 is a stimulator for BLM-induced EMT. As shown in Fig. 6A, pulmonary TGF-β1 mRNA was up-regulated 14 d after BLM injection. Calcitriol attenuated BLM-induced upregulation of TGF-β1 in the lungs. Alpha-SMA is a hallmark of myofibroblasts and is accepted as a marker of EMT. The effects of calcitriol on BLM-induced pulmonary α-SMA were analyzed. The level of pulmonary α-SMA protein was elevated 14 d after BLM injection (Fig. 6B). IHC showed that α-SMA-positive cells were increased in BLM-treated mice (Fig. 6C). Calcitriol attenuated BLM-induced up-regulation of α-SMA in the lungs (Fig. 6C).

3.6. Calcitriol inhibits BLM-induced Smad phosphorylation in the lungs

The effects of calcitriol on BLM-activated Smad phosphorylation in the lungs were analyzed. As expected, the level of phosphorylated Smad in the lungs was elevated 7 d after BLM injection (Fig. 7). Calcitriol attenuated BLM-induced pulmonary Smad phosphorylation in the lungs.

4. Discussion

Increasing evidence demonstrates that calcitriol, the active form of vitamin D3, has an anti-inflammatory activity (Krishnan and Feldman, 2011). According to an earlier report, calcitriol inhibits neutrophil recruitment in hamster model of acute lung injury (Takano et al., 2011). A recent study showed that calcitriol

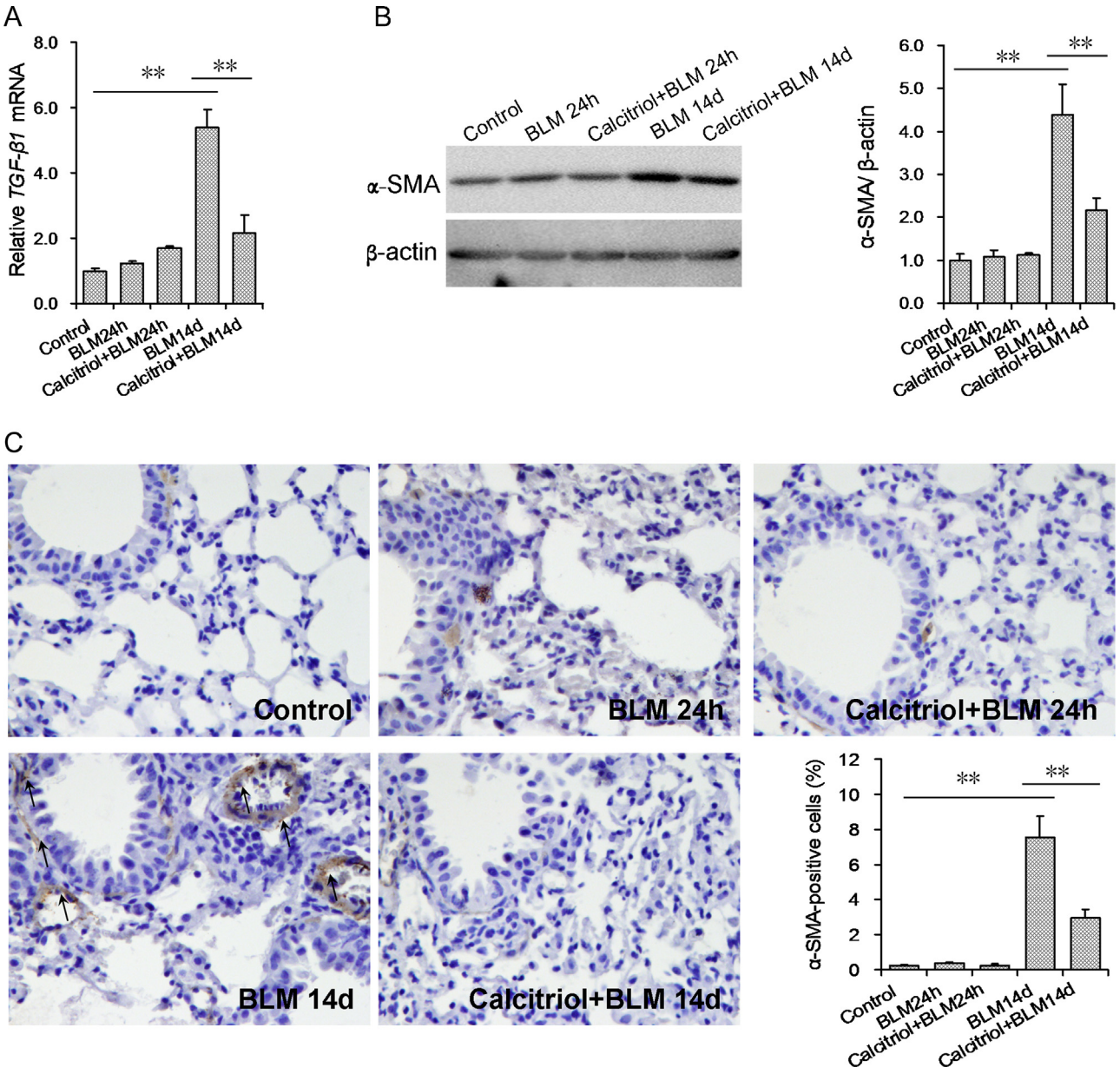

Fig. 6. Calcitriol attenuates BLM-induced EMT in the lungs. In BLM group, mice were intratracheally injected with a single dose of BLM (3.0 mg/kg, dissolved in 50 μL PBS). In BLM + calcitriol group, mice were i.p. injected with calcitriol (1.0 μg/kg) daily, beginning at 48 h before BLM. Lungs were collected 24 h or 14 d after BLM. (A) Pulmonary TGF-β1 mRNA was measured using real-time RT-PCR. (B) The expression of pulmonary α-SMA was detected using immunoblots. All experiments were replicated six times. (C) Pulmonary α-SMA was detected by immunohistochemistry. Representative photomicrographs of pulmonary histological specimens from mice treated with saline, BLM alone (24 h), calcitriol plus BLM (24 h), BLM alone (14 d), calcitriol plus BLM (14 d). Original magnification: 400×. Arrowheads indicate α-SMA-positive cells. Pulmonary α-SMA-positive cells were counted. All data were expressed as means ± SEM ($n = 6$). $^{**}P < 0.01$.

suppressed H1N1-induced transcription of pro-inflammatory cytokines and IL-8 in human lung epithelial cells (Khare et al., 2013). Moreover, an oral administration of calcitriol daily, beginning at 24 h after BLM injection, alleviated BLM-induced lung fibrosis (Zhang et al., 2013). The present study investigated the effects of an intraperitoneal injection with calcitriol daily, beginning at 48 h before BLM injection, on early pulmonary inflammation in mouse model of BLM-induced acute lung injury. We showed that calcitriol markedly alleviated BLM-induced infiltration of inflammatory cells in the lungs. Moreover, calcitriol significantly attenuated BLM-induced pulmonary TNF-α, IL-1β and IL-6, three pro-inflammatory cytokines, and MCP-1, MIP-1α and MIP-2, three chemokines. These results demonstrate that calcitriol inhibits early pulmonary inflammation during BLM-induced acute lung injury.

The MAPK and PI3K/Akt signaling pathways are two of the most important signaling cascades that regulate early inflammatory response in BLM-induced pulmonary injury (Matsuoka et al., 2002; Wei et al., 2010; Russo et al., 2011). Several studies demonstrate that calcitriol modulates inflammatory response through repressing the MAPK pathways (Miodovnik et al., 2012; Choi et al., 2013). According to a recent study, calcitriol reduces LPS-evoked COX-2 expression and proinflammatory cytokines in murine macrophages through inhibiting PI3K/Akt signaling (Wang et al., 2014). The present study investigated the effects of calcitriol on pulmonary MAPK and PI3K/Akt pathways during BLM-induced acute lung injury. As expected, the level of pulmonary phosphorylated p38 MAPK was markedly elevated 24 h after BLM injection and remained elevated one week after BLM injection. In addition, the level of pulmonary phosphorylated Akt was obviously elevated

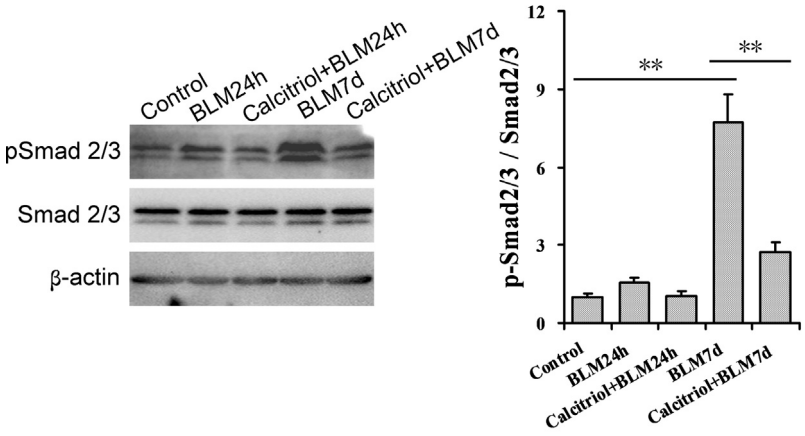

Fig. 7. Calcitriol attenuates BLM-induced Smad phosphorylation in the lungs. In BLM group, mice were intratracheally injected with a single dose of BLM (3.0 mg/kg, dissolved in 50 μL PBS). In BLM + calcitriol group, mice were i.p. injected with calcitriol (1.0 μg/kg) daily, beginning at 48 h before BLM. Lungs were collected 24 h and 14 d after BLM. Pulmonary p-Smad and Smad were measured using immunoblots. All experiments were replicated six times. All data were expressed as means ± S.E.M. ($n = 6$). $^{**}P < 0.01$.

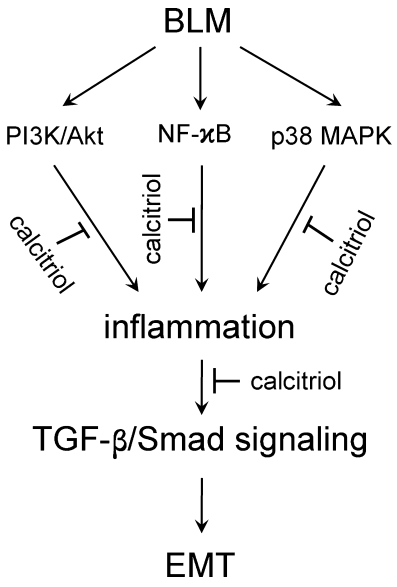

Fig. 8. A flowchart for calcitriol-mediated protection against BLM-induced early inflammatory response and EMT in the lungs.

24 h after BLM injection and remained elevated one week after BLM injection. Interestingly, calcitriol significantly attenuated BLM-induced pulmonary p38 MAPK and Akt phosphorylation. These results suggest that calcitriol inhibits BLM-induced early pulmonary inflammation, at least partially, through suppressing pulmonary p38 MAPK and Akt signaling.

Nuclear factor kappa B (NF-κB) plays a key role on the regulation of inflammatory cytokines and chemokines during BLM-induced pulmonary injury (Zhang et al., 2000). Moreover, NF-κB mediates TNF-α-induced Yin Yang 1 (YY1), a transcription factor that activates the type I and type II collagen and α-SMA gene promoters in fibroblasts (Lin et al., 2011). Indeed, several studies demonstrate that calcitriol exerts its anti-inflammatory effects through repressing NF-κB signaling (Cohen-Lahav et al., 2007; Tan et al., 2008; Talmor-Barkan et al., 2011). The present study investigated the effects of calcitriol on BLM-activated pulmonary NF-κB signaling. As expected, nuclear NF-κB p65 subunit was obviously elevated in BLM-treated mice. Interestingly, calcitriol significantly attenuated BLM-induced nuclear NF-κB p65 translocation. These results suggest that calcitriol inhibits BLM-induced pulmonary inflammation partially, through suppressing pulmonary NF-κB p65 signaling.

Numerous studies demonstrate that EMT of alveolar epithelial cells to myofibroblasts plays an important role in the pathogenesis of idiopathic pulmonary fibrosis (Willis et al., 2005). Indeed, several studies showed that α-SMA, a hallmark of EMT in the lungs, was upregulated during BLM-induced pulmonary fibrosis (Wang et al., 2014; Zhao et al., 2014). The present study showed that pulmonary α-SMA expression was markedly upregulated 14 d after BLM injection. Correspondingly, the number of α-SMA-positive cells in the lungs was significantly elevated one week after BLM injection. Interestingly, calcitriol obviously attenuated BLM-induced EMT in the lungs, as evidenced by its repression of pulmonary α-SMA expression. The mechanism by which calcitriol inhibits pulmonary EMT remains obscure. Alveolar epithelial cells undergo EMT when chronically exposed to TGF-β1 (Kim et al., 2006; Willis et al., 2005). Increasing evidence demonstrates that TGF-β-Smad signal transduction is involved in the pathogenesis of pulmonary EMT (Kim et al., 2009; Chen et al., 2014). The present study showed that the expression of TGF-β1 in the lungs was significantly up-regulated 14 d after BLM injection. Correspondingly, phosphorylated Smad level was obviously elevated 14 d after BLM injection. Interestingly, calcitriol significantly attenuated BLM-induced up-regulation of TGF-β expression in the lungs. Moreover, calcitriol significantly attenuated BLM-induced pulmonary Smad phosphorylation. These results suggest that calcitriol counteracts BLM-induced pulmonary EMT, at least partially, through repressing TGF-β-Smad signal transduction.

A large prospective cohort study demonstrated that there was a negative association between plasma 25(OH)D levels and the risk of chronic obstructive pulmonary disease (Afzal et al., 2014). According to a double-blind randomized placebo-controlled trial, supplementation with vitamin D3 as an adjunctive therapy markedly accelerated resolution of inflammatory responses during tuberculosis treatment (Coussens et al., 2012). Another report found that supplementation with a large bolus dose of vitamin D3 reduced TNF-α and IL-6 in patients with cystic fibrosis (Grossmann et al., 2012). An earlier in vitro study showed that calcitriol inhibited TGF-β1-mediated pro-fibrotic effects in lung fibroblasts and epithelial cells (Ramirez et al., 2010). According to a recent study, calcitriol alleviated BLM-induced lung fibrosis (Zhang et al., 2013). The present study demonstrated that calcitriol attenuated BLM-induced early inflammatory responses and subsequent EMT. These results suggest that calcitriol may be used

as an adjunctive prevention especially in high-risk situations in which the patients are injected with BLM. The present study laid emphasis on the effects of calcitriol on early pulmonary inflammation and subsequent EMT in the pathogenesis of BLM-induced pulmonary injury. However, the present study did not investigate the effects of calcitriol on BLM-induced pulmonary fibrosis. According to a recent report, thymosin b4, an anti-inflammatory agent, failed to prevent lung fibrosis even though it alleviated BLM-induced early pulmonary inflammation (Conte et al., 2015). Thus, additional work is required to determine whether calcitriol has an anti-fibrotic effect and explore the association between its anti-inflammatory and anti-fibrotic effects in different models of pulmonary fibrosis.

In summary, the present study demonstrates that calcitriol, the active form of vitamin D3, inhibits BLM-induced early pulmonary inflammation through blocking activation of NF-κB, p38 MAPK and PI3K/Akt signaling in the lungs. Moreover, calcitriol inhibits BLM-induced EMT through repressing pulmonary TGF-β-Smad signaling (Fig. 8). Additional work is required to determine whether calcitriol alleviates BLM-induced lung fibrosis.

Conflict of interest

The authors have declared that no competing interests exist.

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