Original Article

Inhibition of protein kinase C alpha attenuates lipopolysaccharide-triggered acute lung injury by alleviating the hyperinflammatory response and oxidative stress

Yang Chen[1#], Pengcheng Lin[1#], Wengang Nan[1], Shanshan Su[1], Hong Zheng[2], Hongchang Gao[2], Dan Zhang[1], Yuping Li[1]

[1]Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China; [2]Institute of Metabonomics & Medical NMR, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, China

Contributions: (I) Conception and design: Y Li, D Zhang; (II) Administrative support: Y Li, H Gao, H Zheng; (III) Provision of study materials or patients: Y Chen, P Lin, S Su, W Nan; (IV) Collection and assembly of data: Y Chen, P Lin, S Su, W Nan; (V) Data analysis and interpretation: Y Chen, P Lin; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Yuping Li. Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Wenzhou Medical University, Nanbaixiang Street, Wenzhou 325000, China. Email: wzliyp@163.com.

Background: The currently available treatment methods are ineffective in reducing mortality or improving outcomes in acute lung injury (ALI). The activation of protein kinase C alpha (PKCα) has recently been implicated in ALI development. We explored the potential therapeutic outcomes of PKCα inhibition in cases of ALI and to elucidate the related mechanisms.

Methods: Indexes of lung inflammation and injury were examined in lipopolysaccharide (LPS)-treated C57BL/6J mice (male) and macrophages after pretreatment with a PKCα inhibitor. Tissues were collected to assess lung injury by hematoxylin and eosin (H&E) staining. Bronchoalveolar lavage fluid was used to measure the pulmonary edema, hyperinflammatory response, and oxidative stress by bicinchoninic acid (BCA) method and enzyme-linked immunosorbent assay (ELISA). We tested the effect of PKCα inhibition on LPS-induced proliferation, cytotoxicity, oxidative damage, and the release of inflammatory cytokines in macrophages using the Cell Counting Kit-8 (CCK-8) and lactate dehydrogenase (LDH) cytotoxicity assay kit, flow cytometry, quantitative reverse-transcription polymerase chain reaction (qRT-PCR), and ELISA. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway related proteins were detected by Western blot, immunohistochemistry (IHC), and immunofluorescence staining.

Results: We observed that LPS upregulated PKCα phosphorylation, induced a hyperinflammatory response, and caused lung injury. However, PKCα inhibition effectively attenuated the changes caused by LPS. Moreover, we confirmed that inhibiting PKCα weakened the activity of the NF-κB pathway under LPS-induced ALI. These findings indicated that inhibition of PKCα is protective against LPS-induced hyperinflammatory response in ALI, this effect is likely to attributed to the downregulation of NF-κB signaling pathways.

Conclusions: The results showed that PKCα inhibition could attenuate ALI which may closely related to its anti-inflammatory and anti-oxidative effects.

Keywords: Protein kinase C alpha (PKCα); acute lung injury (ALI); hyperinflammatory response; oxidative stress; NF-κB pathway

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Introduction

Acute lung injury (ALI) is a common acute critical illness associated with serious morbidity and mortality (1). The hyperinflammatory response is at the core of ALI pathogenesis. Once activated, inflammatory cells in lung tissue, including neutrophils and macrophages, release a great number of proinflammatory mediators that destroy the integrity of the epithelial-endothelial barrier, block the clearance of alveolar fluid, and then cause lung injury (2). Lipopolysaccharide (LPS) is the main component of the outer membrane of Gram-negative bacteria, which can cause inflammatory reaction disorder, has been considered one of the important factors leading to ALI. Although treatment strategies such as the administration of nitric oxide (NO), surfactants, and glucocorticoids as well as mechanical ventilation are used for managing ALI, none of these methods are effective in reducing mortality or improving outcomes (3). Thus, novel therapeutic targets and therapies for improving ALI prognosis are warranted.

Protein kinase C (PKC) is a widely distributed family of serine/threonine protein kinases, and it can be divided into 3 categories according to structural variations and biochemical properties (4). A typical representative of classical PKC molecules, PKCα is made up of 672 amino acids; and in contrast to other PKC subtypes which show restricted expression in specific tissues, is widely expressed in all tissues (5). As an important intermediate in the inflammatory, PKCα has been reported to active downstream targets in Toll-like receptor (TLR) pathways, such as MAPKs, RhoA, TAK1 and NF-κB, promotes expression of proinflammatory genes. Phorbol 12-myristate 13-acetate, a classical PKC agonist, can increase inflammatory cytokine levels in the murine epidermis. Moreover, the PKCα-lysine-specific demethylase 1-NF-κB-signaling cascade is known to be a promoter that amplifies the inflammatory response and subsequently aid in the development of inflammatory diseases such as sepsis (6). New evidence has shown that the activation of PKCα is involved in the pathophysiology of ALI. Wang *et al.* demonstrated that PKCα messenger RNA (mRNA) and protein levels showed a significant upregulation in lung tissue 3 days after cerebral ischemia/reperfusion (I/R) injury. The molecular mechanism underlying cerebral I/R-induced ALI, is at least in part, related to PKCα activation (7). However, whether PKCα activation mediates NF-κB pathway contributing to the hyperinflammatory response and oxidative stress in LPS-induced ALI is unclear.

Therefore, we examined the changes in PKCα expression in an LPS-induced murine model of ALI as well as in LPS-treated macrophages. Subsequently, we studied the molecular mechanism underlying the involvement of PKCα in ALI using an inhibitor of PKCα.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6497/rc).

Methods

Reagents

We obtained the following reagents from Sigma (St. Louis, MO, USA): LPS (from *E. coli* O55:B5; L2880) and Calphostin C (from *Cladosporium cladosporioides*; C6303). We obtained the following reagents from Boyun Biotechnology (Shanghai, China): tumor necrosis factor-α (TNF-α) (BP-E20220), interleukin-1β (IL-1β) (BP-E20533), IL-6 (BP-E20012), myeloperoxidase (MPO) (BP-E20262), and the following enzyme-linked immunosorbent assay (ELISA) detection kits; malondialdehyde (MDA) (BYS0001), superoxide dismutase (SOD) (BYS0211) assay kits. Antibodies against PKCα (phospho S657) [EPR1901(2)] (ab180848) and PKCα [Y124] (ab32376) were bought from Abcam (Cambridge, MA, USA). We obtained the following antibodies from Cell Signaling Technology (CST; Danvers, MA, USA): anti-β-actin (13E5) (4970S), anti-phospho-NF-κB P65 (Ser536) (93H1) (3033S), anti-NF-κB P65 (D14E12) (8242S), anti-phospho-IκB (Ser32) (14D4) (2859S), and anti-IκBα (L35A5) (4814S). We also procured an anti-claudin 18 polyclonal antibody (21126-1-AP) from Proteintech (Wuhan, Hubei, China).

Animals and experimental design

Known as lipoglycans and endotoxins, LPS are outer membrane components of gram-negative bacteria and cause severe inflammation and lung damage. Intratracheal administration of LPS is commonly used to establish animal models of ALI, and this model was used in the present study too. We obtained C57BL/6J mice (male; age 6–8 weeks; weight 20–25 g) from Shanghai SLAC Laboratory Animal Co. (Shanghai, China) were bred under specific pathogen-free conditions at temperatures of 22–24 ℃, and were fed standard laboratory pelleted food and sterile water. A protocol was prepared before the study without registration. All experimental procedures performed on animals were

reviewed and approved by Wenzhou Medical University's Institutional Animal Care and Use Committee (No. 2021-0095), and all animal surgeries were strictly performed in accordance with internationally recognized and institutional guidelines for the care and use of animals. Using the random number table, 60 mice were divided into 1 of the following groups (n=12): (I) control group [phosphate-buffered saline (PBS)]; (II) dimethyl sulfoxide (DMSO) alone group (PBS + DMSO); (III) Calphostin C alone group (PBS + DMSO + Calphostin C); (IV) LPS group (LPS); and (V) LPS with Calphostin C group (LPS + DMSO + Calphostin C). Authors were aware of group allocation at the different stages of the experiment. In the LPS and LPS with Calphostin C groups, after the mice were anesthetized, we injected LPS into the trachea of the animals according to their weight (10 mg/kg). Mice in the LPS with Calphostin C group also received an intraperitoneal injection of DMSO + Calphostin C [0.15 mg/kg (according to the previous reports and our preliminary results), Calphostin C dissolved in DMSO (1 mg/mL) and then diluted with PBS to 0.015 mg/mL] 4 h before LPS treatment. No mice died or needed to be terminated 24 h after LPS stimulation. The mice were then euthanized and lung tissue and bronchoalveolar lavage fluid (BALF) samples were collected for further study.

Cell culture and treatment

Murine RAW 264.7 macrophages obtained from Cell Bank of the Chinese Academy of Science (Shanghai, China) were cultured in high-glucose Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 ℃ with 5% CO_2. After stimulation with 1 μg/mL LPS for 0–24 h, the levels of PKCα phosphorylation in the cells were examined. A different group of cells was pretreated with 100 nmol/L Calphostin C (according to the previous reports and our preliminary results) for 2 h and then exposed to 1 μg/mL LPS. After 30 min, the cells were harvested to evaluate reactive oxygen species (ROS) levels. After 1 and 12 h, the expression of NF-κB pathway proteins was examined. After 12 and 24 h, proinflammatory cytokine analysis was performed.

Histopathology

Lung tissue samples were fixed in 4% paraformaldehyde. Then, the samples were dehydrated and embedded in paraffin. Subsequently, 5-μm-thick sections were obtained; these sections were de-paraffinized, rehydrated, stained with hematoxylin and eosin (H&E), and observed using an optical photomicroscope. Alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in the alveolar cavity or vascular wall, and alveolar wall thickening and/or transparent membrane formation were observed under an optical microscope. Lung injury scores were calculated by using a semi-quantitative scoring method in a blinded manner. A score of 0 means no damage; 1 means mild damage; 2 means moderate damage; 3 means severe damage, and 4 means very severe histological damage (8).

Lung edema measurement

Mouse lungs were lavaged thrice with 1 mL PBS via a tracheal tube, and approximately 0.6 mL of BALF was obtained. Subsequently, the BALF samples were centrifuged (3,000 rpm, 15 min, 4 ℃) and the protein levels in the BALF were measured using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Lung tissue was washed twice in ice-cold PBS to remove blood, then wiped dry with a filter paper. Then, we weighed the samples as promptly as possible to estimate the wet weight. The tissue was dried at 60 ℃ for 48 h, after which the dry weight was obtained. The wet/dry weight ratio was obtained to assess the severity of pulmonary edema.

ELISA

Common inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are crucial triggers of ALI. Thus, we tested the secretion of these inflammatory mediators in LPS-treated mice and cells. Tests for the levels of TNF-α, IL-1β, and IL-6 in BALF and the cell supernatant, and the levels of MPO in BALF were performed using the corresponding ELISA kits by following the manufacturer's instructions.

Oxidative stress index measurement

Since oxidative stress is involved in ALI progression, we assessed the levels of oxidation products and the activity of antioxidant enzymes in BALF. The MDA and SOD levels in BALF were detected using commercial kits acquired from Boyun Biotechnology (Shanghai, China).

Immunohistochemical staining

Paraffin-embedded sections were blocked in 5% bovine serum albumin (BSA) and incubated at 4 ℃ with

primary antibodies against claudin-18 and NF-κB P65 (1:200 dilution) overnight. After the sections were stained using an avidin-biotin-peroxidase complex method, they were counterstained with hematoxylin. Protein expression levels and localization were evaluated on the basis of the integrated optical density using Image J version 8 (https://imagej.nih.gov/ij/).

Immunofluorescence staining

First, cells were fixed in 4% paraformaldehyde (30 min), permeabilized in 0.1% Triton X-100 (10 min), and blocked in 1% BSA (30 min) after being pretreated with Calphostin C for 2 h and with LPS for 12 h. The cells were incubated with the anti-P65 primary antibody (1:200) overnight at 4 ℃. A secondary fluorescent dye-conjugated goat anti-rabbit antibody (1:200) was incubated with the cells for 1 h at 37 ℃. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and imaged using a fluorescence microscope (Nikon, Tokyo, Japan).

Western blot

Proteins from the mouse lung-tissue homogenate and cells were isolated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were first incubated in proteinless rapid blocking fluid at 37 ℃ for 10 min in order to block non-specific binding sites and were then incubated with the primary antibody (1:1,000) at 4 ℃ overnight. After this, they were washed several times with tris-buffered saline containing Tween (TBST) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin IgG (1:5,000) for 1 h. Band intensity was analyzed using Image J software after exposure to an X-ray film, and the results were normalized to β-actin expression.

Detection of cell proliferation and cytotoxicity

The RAW264.7 cells were grown in 96-well microplates (1×10^4 cells/well) and incubated in complete medium at 37 ℃ in 5% CO_2 for 12 h. Cells were then pretreated with Calphostin C (100 nmol/L) for 2 h before exposure to LPS (1 μg/mL) for 24 h. We examined cell proliferation using the Cell Counting Kit-8 (CCK-8) reagent (C0038, Beyotime Biotechnology, Shanghai, China). Lactate dehydrogenase (LDH) activity in the cell supernatant was measured with an LDH cytotoxicity assay kit (C0016, Beyotime Biotechnology, China).

Examination of ROS

Intracellular ROS levels were assessed using flow cytometry. Cells pretreated with Calphostin C were challenged with LPS (1 μg/mL) for 30 min. Harvested cells were incubated in 1 mL of FBS-free medium containing 1 μL dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 20 min (37 ℃) under dark conditions. Cells were washed and re-suspended in cold PBS for flow cytometry [FACSCalibur; Becton, Dickinson, and Co. (BD), Franklin Lakes, NJ, USA]. Intracellular ROS levels were evaluated using FlowJo (v. 10.5.3; Ashland, OR, USA).

Quantitative reverse transcriptase-polymerase chain reaction

The RAW264.7 cells (3.5×10^5/well) were added to 12-well plates and treated with Calphostin C for 2 h; they were then with treated with LPS for 12 h. Total RNA isolation was performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcription into first-strand complementary DNA (cDNA) was performed using a HiScript II Q RT SuperMix for qPCR Kit (Vazyme, Nanjing, China). Finally, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using TB Green Premix ExTaq™ II (Takara Bio Inc., Shiga, Japan). The mRNA levels of TNF-α, IL-1β, and IL-6 were quantified via the ΔCt method, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. Each sample was performed in triplicate.

The following primer sequences were used:

- GAPDH: F, 5'-AGGTCGGTGTGAACGGATTTG-3'; R, 5'-TGTAGACCATGTAGTTGAGGTCA-3';
- TNF-α: F, 5'-GCGACGTGGAACTGGCAGAAG-3'; R, 5'-GCCACAAGCAGGAATGAGAAGAGG-3';
- IL-1β: F, 5'-TCGCAGCAGCACATCAACAAGAG-3'; R, 5'-TGCTCATGTCCTCATCCTGGAAGG-3';
- IL-6: F, 5'-TCCATCCAGTTGCCTTCTTG-3';

R, 5'-AAGCCTCCGACTTGTGAAGTG-3'.

Statistical analyses

Data were expressed as mean ± standardized error of the mean (SEM). One-way analysis of variance (ANOVA) was used to analyze differences among the different treatment groups and the control group. All data analyses were performed using GraphPad Prism 8.0 software (San Diego, CA, USA). The cut-off for statistical significance was $P<0.05$.

Results

Inhibition of PKCα alleviates LPS-triggered ALI and pulmonary edema

Our preliminary experiments (Appendix 1) showed that 24 h after the intratracheal injection of LPS (10 mg/kg), alveolar congestion and hemorrhage, wall thickening, and inflammatory cell infiltration became obvious (Figure S1A). Moreover, the levels of PKCα phosphorylation were markedly higher in mice treated with different concentrations of LPS, especially those treated with the highest LPS concentration, than in untreated control mice (Figure S1B). Therefore, we chose 10 mg/kg as the final concentration of LPS (24-h treatment) for establishing an animal model of ALI for follow-up experiments.

The phosphorylation of PKCα was inhibited by Calphostin C in the lungs of LPS-treated mice (*Figure 1A*). Lung injury was assessed using H&E staining and the lung injury score. The lung tissue structure was normal in the control group and there were no histopathological changes. However, alveolar destruction, alveolus collapse, thickened alveolar septum, and extensive inflammatory cell infiltration were detected in the LPS group; these changes were ameliorated after the inhibition of PKCα (*Figure 1B*). Treatment with LPS was associated with an obvious increase in lung injury scores, while Calphostin C pretreatment significantly reduced this score *(Figure 1C*). As demonstrated in *Figure 1D*, the lung wet/dry weight ratio was dramatically elevated after LPS treatment, while the administration of Calphostin C appreciably reduced this change. Additionally, LPS exposure severely disrupted lung barrier function, leading to an increase in the protein content in BALF. The inhibition of PKCα using Calphostin C obviously reduced barrier function impairment and the leakage of proteins into BALF (*Figure 1E*).

Inhibition of PKCα reduces the hyperinflammatory response in mouse lung tissue

The LPS stimulation significantly promoted secretion of TNF-α, IL-1β, and IL-6 into BALF, as observed using ELISA. Further, Calphostin C reduced the levels of these cytokines in BALF (*Figure 2A-2C*).

Inhibition of PKCα alleviates oxidative stress in mouse lung tissue

The levels of MDA and MPO were significantly higher while SOD activity was markedly lower after LPS treatment. However, treatment with Calphostin C significantly reversed the effects of LPS on these oxidative stress-related factors (*Figure 3A-3C*).

Effects of the inhibition of PKCα on the expression of claudin-18 in LPS-triggered ALI mice

Lung epithelial cells are connected by tight junction proteins, which form a physical barrier against the free diffusion of solutes. Claudins, major components of tight junctions, are necessary for maintaining lung epithelial barrier function (9). Previous studies have demonstrated high levels of claudin-3, claudin-4, and claudin-18 in the alveolar epithelium (10). Therefore, IHC was used to detect the expression of claudin-18 in mouse lung tissue. Immunoperoxidase was used to identify the expression pattern of claudin-18 in the alveolar epithelium (*Figure 4*). Unexpectedly, in the LPS group, claudin-18 showed strongly positive expression in lung epithelial cells, with a continuous and undulating distribution along the cell membrane. Furthermore, this change was reversed following the administration of Calphostin C.

The effects of PKCα inhibition on LPS-induced proliferation, cytotoxicity, oxidative damage, and the release of inflammatory cytokines in macrophages

Given the important role of macrophages in the hyperinflammatory response, we assessed the effect of PKCα inhibition on LPS-treated macrophages. The LPS stimulation induced PKCα activation, and the level of PKCα phosphorylation peaked at 30 min and gradually declined to near-normal levels within 0–24 h (*Figure 5A*). Moreover, LPS treatment significantly promoted cell proliferation and cytotoxicity; these indexes, however, were

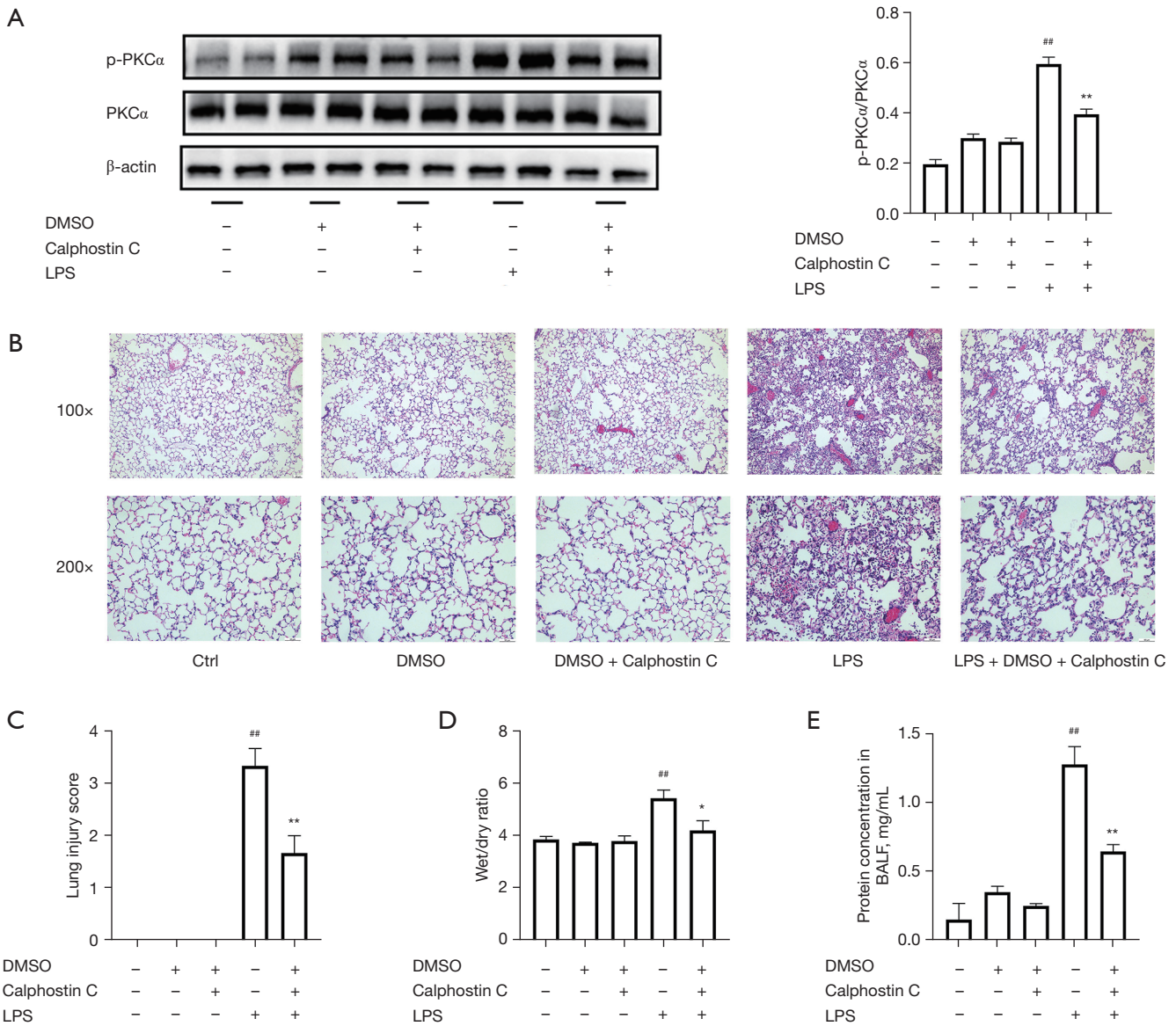

Figure 1 The inhibition of PKCα alleviates LPS-triggered acute lung injury and pulmonary edema. (A) Representative protein levels of PKCα in mouse lung tissue, detected using Western blot. (B) Histopathological analyses of lung tissue carried out using H&E staining (×100 and ×200). (C) Severity of lung injury evaluated using semi-quantitative histological scoring. (D) Wet/dry weight ratio of the lungs. (E) BALF protein levels evaluated using the BCA method. Ctrl: control group; DMSO: DMSO alone group; DMSO + Calphostin C: Calphostin C alone group; LPS: LPS group; LPS + DMSO + Calphostin C: LPS with Calphostin C group. The results are presented as means ± SEM (n=3–6). "–" stands for the corresponding substance is not used; "+" stands for the corresponding substance is used. *, P<0.05; **, P<0.01 *vs.* LPS group. ##, P<0.01 *vs.* control group. PKCα, protein kinase C alpha; LPS, lipopolysaccharide; H&E, hematoxylin and eosin; BALF, bronchoalveolar lavage fluid; DMSO, dimethyl sulfoxide; SEM, standardized error of the mean.

markedly reversed by Calphostin C treatment (*Figure 5B*). Furthermore, PKCα inhibition reduced the LPS-induced production of ROS (*Figure 5C*). Finally, on assessing whether PKCα inhibition affects the release and expression of inflammatory cytokines in LPS-treated macrophages, we found that LPS treatment markedly up-regulated the mRNA and protein levels of TNF-α, IL-1β, and IL-6. However, PKCα inhibition remarkably reversed the

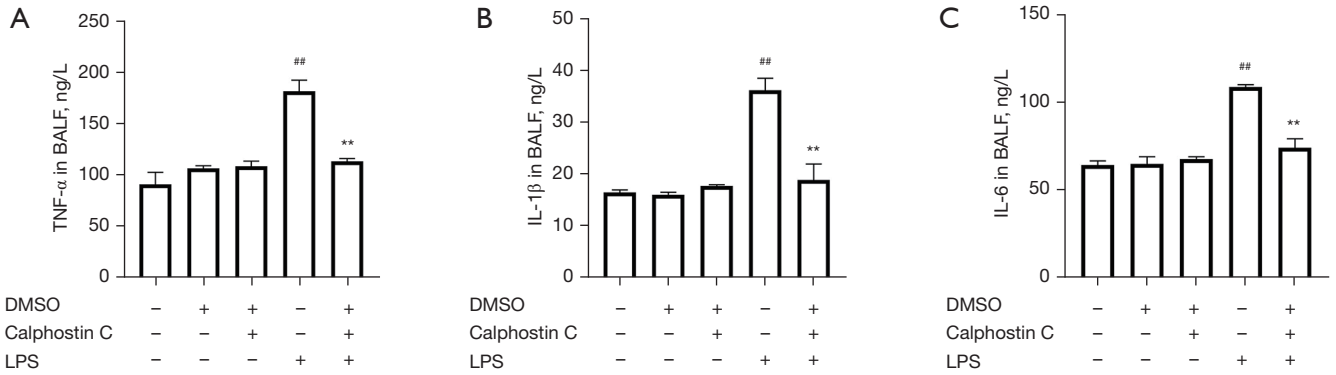

Figure 2 PKCα inhibition reduces the hyperinflammatory response in mouse lung tissue. The levels of TNF-α (A), IL-1β (B), and IL-6 (C) in BALF examined using ELISA. The results are presented as means ± SEM (n=3). "–" stands for the corresponding substance is not used; "+" stands for the corresponding substance is used. **, P<0.01 *vs.* LPS group. ##, P<0.01 *vs.* control group. PKCα, protein kinase C alpha; TNF-α; tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; SEM, standardized error of the mean.

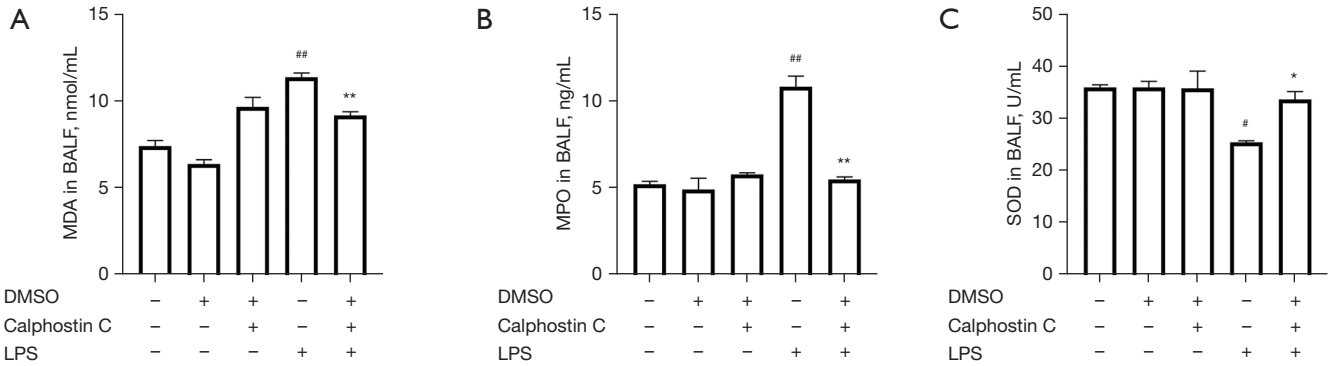

Figure 3 The inhibition of PKCα alleviates oxidative stress in mouse lung tissue. MDA (A), MPO (B), and SOD (C) activity in BALF was detected using commercial kits. The results are presented as means ± SEM (n=3). "–" stands for the corresponding substance is not used; "+" stands for the corresponding substance is used. *, P<0.05, **; P<0.01 *vs.* LPS group. #, P<0.05; ##, P<0.01 *vs.* control group. PKCα, protein kinase C alpha; MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase; BALF, bronchoalveolar lavage fluid; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; SEM, standardized error of the mean.

increase in these inflammatory cytokines (*Figure 5D,5E*).

Inhibition of PKCα affects the viability of the NF-κB signaling pathway

In ALI, NF-κB-related inflammatory signaling is essential for the occurrence and development of hyperinflammation. Therefore, we examined the effect of PKCα inhibition on this pathway. The expression of NF-κB P65 was low in the mouse lung in the control group but it was significantly upregulated after LPS stimulation (*Figure 6A*). However, the increase was inhibited by Calphostin C. *In vitro*, the levels of P65 and IκB phosphorylation were elevated after LPS treatment, and Calphostin C markedly reversed this effect (*Figure 6B*). Similarly, immunofluorescence images revealed that strong nuclear accumulation of NF-κB P65 was observed following LPS exposure. However, no P65 translocation to the nucleus was observed after pretreatment with Calphostin C (*Figure 6C*). These findings suggested that PKCα inhibition downregulated the activity of the NF-κB signaling pathway in LPS-treated mice and macrophages.

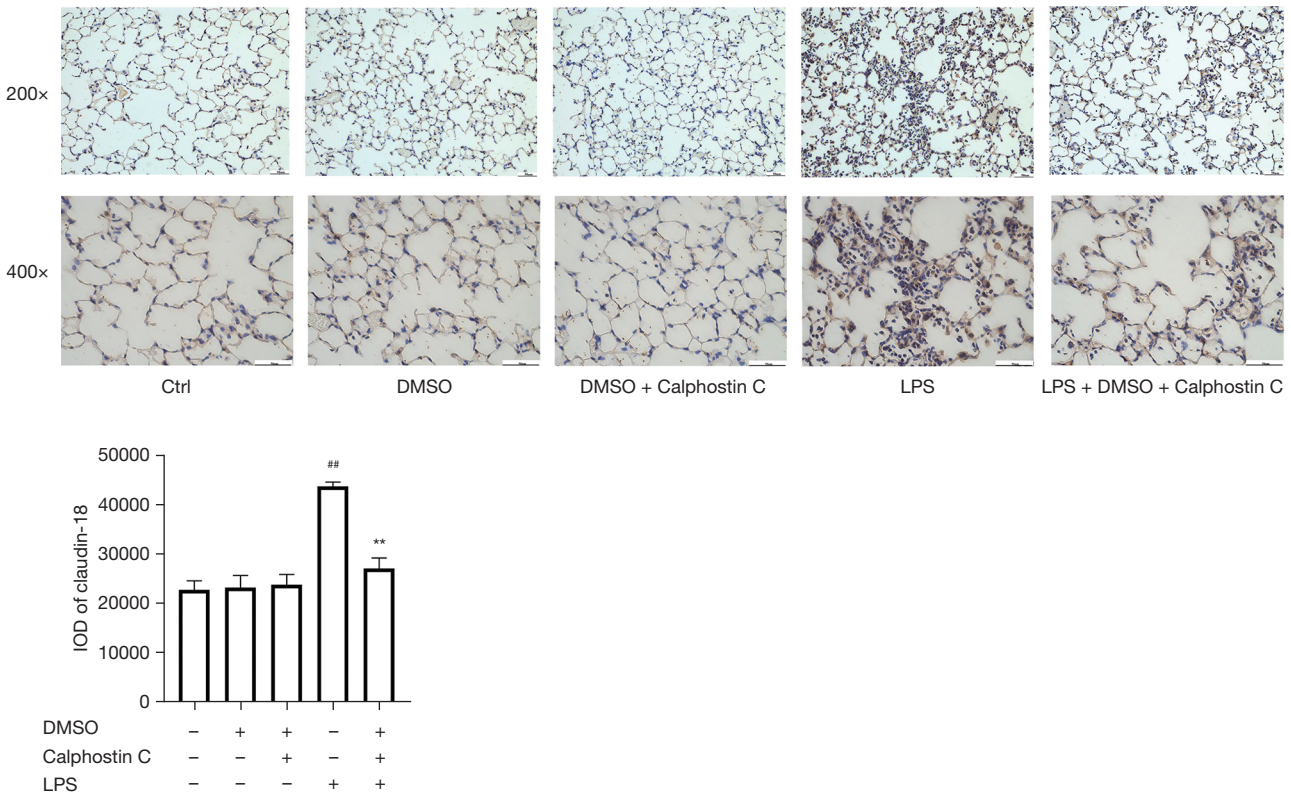

Figure 4 Effect of PKCα inhibition on claudin-18 in mouse models of LPS-triggered ALI. The expression and distribution of claudin-18 analyzed using immunohistochemistry (×200 and ×400). Ctrl: control group; DMSO: DMSO alone group; DMSO + Calphostin C: Calphostin C alone group; LPS: LPS group; LPS + DMSO + Calphostin C: LPS with Calphostin C group. The results are presented as means ± SEM (n=3). "–" stands for the corresponding substance is not used; "+" stands for the corresponding substance is used. **, P<0.01 *vs.* LPS group. ##, P<0.01 *vs.* control group. PKCα, protein kinase C alpha; ALI, acute lung injury; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; SEM, standardized error of the mean.

Discussion

Here, we showed that LPS treatment promoted the expression of phosphorylated PKCα in the mouse lung and RAW264.7 cells. The inhibition of PKCα reduces LPS-triggered ALI. The LPS-induced increase in the inflammatory response and oxidative stress was reversed following the administration of a PKCα inhibitor (Calphostin C), both *in vivo* and *in vitro*. As evidenced by these results, PKCα inhibition may be a novel treatment option for ALI.

Previous studies have demonstrated that PKCα is a key signaling regulator in inflammatory diseases, making it an attractive therapeutic target. Shukla *et al.* found that PKCα and NF-κB levels were upregulated in the kidneys of rat models of diabetic nephropathy, and the increase was linked to the induction of inflammation in the pathogenesis of diabetic nephropathy (11). Zhou *et al.* noted that the ratio of phosphorylated PKCα to PKCα protein expression, which is related to hepatic oxidative stress, markedly increased in a juvenile mouse model of nonalcoholic steatohepatitis (12). Similarly, using a rat model of autoimmune myocarditis, Zhong *et al.* showed that PKCα was significantly activated in the heart and that a PKCα inhibitor (Ro-32-0432) partially limited the production of proinflammatory cytokines such as IL-1β and IL-17 (13). In line with previous studies, we observed that LPS increases the expression of phosphorylated PKCα. Based on this experimental finding, we further explored the involvement of PKCα in LPS-induced ALI through *in vitro* as well as *in vivo* investigations and attempted to clarify the internal mechanism involving its effects.

It is well known that an excessive inflammatory cascade response is at the core of ALI pathogenesis. Current evidence

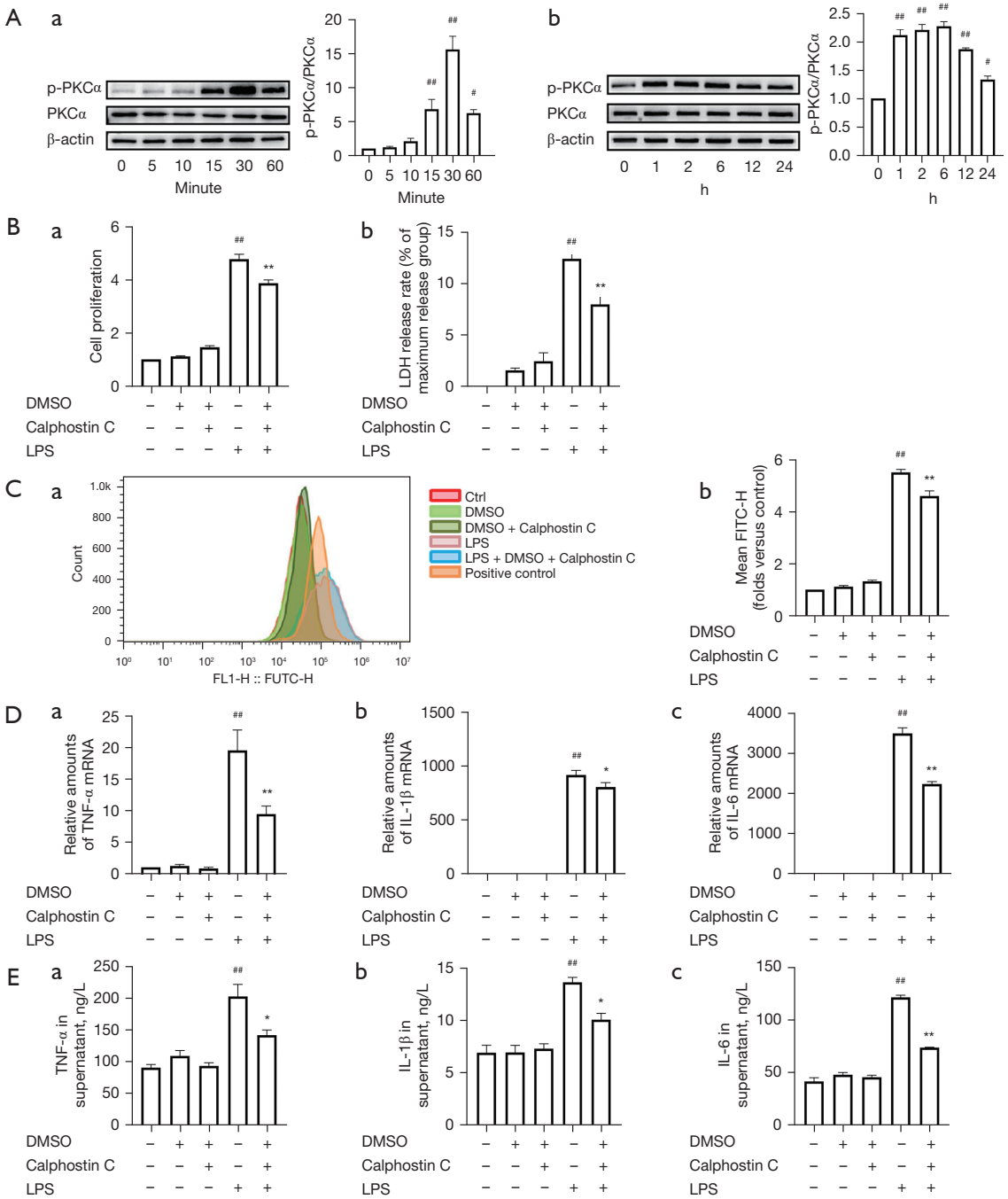

Figure 5 Effect of PKCα inhibition on LPS-induced proliferation, cytotoxicity, oxidative damage, and the release of inflammatory cytokines in macrophages. (A) Levels of PKCα phosphorylation. (B) Cell proliferation (a) and cell death (b) measured using the CCK-8 assay and LDH cytotoxicity assay kit, respectively. (C) Intracellular ROS levels examined using DCFH-DA. (D) The mRNA levels of TNF-α (a), IL-1β (b), and IL-6 (c) evaluated using qRT-PCR. (E) Levels of TNF-α (a), IL-1β (b), and IL-6 (c) secreted into the medium examined using ELISA. The results are presented as means ± SEM (n=3–6). "–" stands for the corresponding substance is not used; "+" stands for the corresponding substance is used. *, P<0.05, **; P<0.01 *vs.* LPS group. #, P<0.05; ##, P<0.01 *vs.* control group. PKCα, protein kinase C alpha; LPS, lipopolysaccharide; CCK-8, Cell Counting Kit 8; LDH, lactate dehydrogenase; ROS, reactive oxygen species; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; mRNA, messenger RNA; ELISA, enzyme-linked immunosorbent assay; TNF-α; tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; SEM, standardized error of the mean.

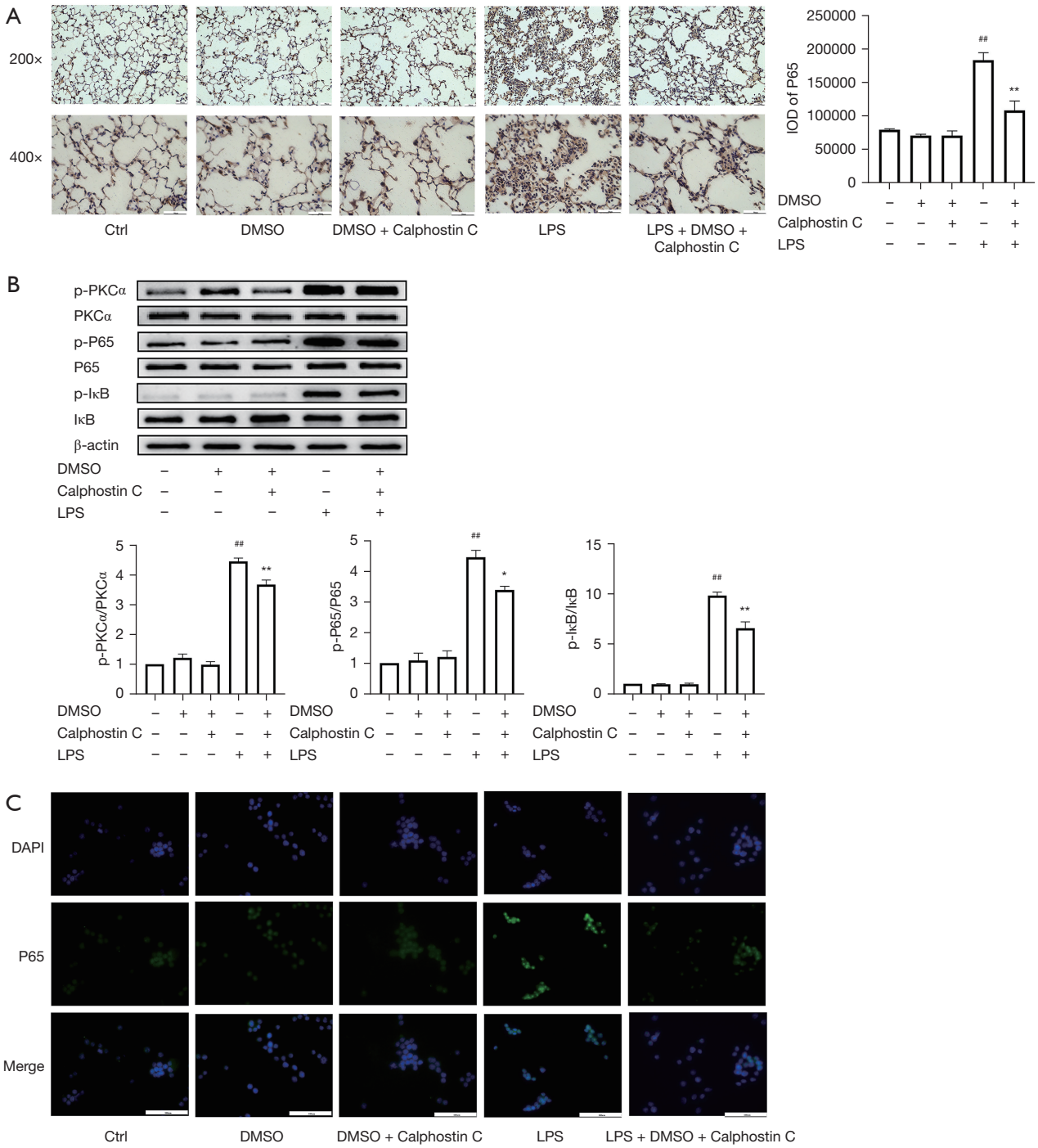

Figure 6 PKCα inhibition affects the viability of the NF-κB signaling pathway. (A) Immunohistochemical staining showing the expression of NF-κB P65 in the lungs of mice (×200 and ×400). (B) p-PKCα, PKCα, p-P65, P65, p-IκB, and IκB protein levels measured using Western blot analysis. (C) Cells immunostained with DAPI and anti-P65 antibodies. Translocation of NF-κB P65 visualized by immunofluorescence staining (×400). Ctrl: control group; DMSO: DMSO alone group; DMSO + Calphostin C: Calphostin C alone group; LPS: LPS group; LPS + DMSO + Calphostin C: LPS with Calphostin C group. The results are presented as means ± SEM (n=3). "–" stands for the corresponding substance is not used; "+" stands for the corresponding substance is used. *, P<0.05, **; P<0.01 *vs.* LPS group. ##, P<0.01 *vs.* control group. PKCα, protein kinase C alpha; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; SEM, standardized error of the mean.

suggests that direct exposure to LPS can significantly activate neutrophils and macrophages in the lungs and cause them to release a large number of inflammatory mediators, leading to a hyperinflammatory response and ultimately lung tissue damage (14). TNF-α, IL-6 and IL-1β are characteristic early cytokines related to the ALI inflammatory process, which play an important role in the triggering of the inflammatory cascade and are also the most common and basic indicators of inflammation level in the experimental model. The activation of PKCα is critical to the initiation of the pulmonary hyperinflammatory response. A previous study demonstrated that a PKCα inhibitor (Gö6976) can attenuate the inflammatory response induced by carbon black nanoparticles by down-regulating ROS, NO, and prostaglandin E2 (PGE2) generation in A549 cells (15). As demonstrated in present study, PKCα inhibition can appreciably reduce the lung wet/dry weight ratio and BALF protein levels, markedly ameliorating pathological changes. Additionally, the expression of LPS-triggered inflammatory mediators including TNF-α, IL-1β, and IL-6 in BALF was remarkably reversed after PKCα inhibition. In an *in vitro* experiment, we further confirmed the anti-inflammatory protective effects of PKCα inhibition. Inhibition of PKCα decreased the proliferation and cytotoxicity of LPS-treated RAW264.7 cells and downregulated TNF-α, IL-1β, and IL-6.

It has been reported that oxidative stress-related damage is closely related to ALI. The production of oxidants and antioxidants is balanced under normal physiological conditions (14). Interestingly, LPS could be an effective stimulator of ROS production. As a marker of oxidative stress, MDA reflects the extent of cell damage induced by reactive oxygen metabolites. The peroxidase enzyme, MPO, that is found in the cytoplasmic granules of neutrophils, is an indicator of neutrophil infiltration into the alveolar or parenchymal space. The intracellular antioxidant SOD can effectively scavenge oxygen free radicals and protect the body against oxidative damages (16). Cho *et al.* reported that PKCα activation is necessary for the expression of the antioxidant enzyme heme oxygenase-1 (HO-1) induced by rosiglitazone in human pulmonary alveolar epithelial cells. In addition, HO-1 protein and mRNA expression were found to be markedly reduced after pretreatment with an inhibitor of PKCα (17). However, in our current study, PKCα inhibition increased SOD activity and decreased MPO activity and MDA content, thereby alleviating oxidative damage in LPS-treated mice. *In vitro*, PKCα inhibition reduced the LPS-induced production of ROS in macrophages. Our results indicate that the protective role of PKCα inhibition in ALI is partly associated with its anti-oxidative effects.

Our experiments showed that the inhibition of PKCα affected the expression of the barrier function protein claudin-18 in LPS-treated mice. Claudin-18 is one of the most interesting proteins in the claudin family; it is expressed in alveolar epithelial cells, particularly in type 1 alveolar epithelial cells (10). A role is played by PKCα in the regulation of alveolar epithelial barrier functions. For example, mechanical ventilation activates PKCα and downregulates occludin expression in a volume-dependent manner, an effect that is reversed by PKCα inhibitor treatment (18). Our data showed that claudin-18 protein levels were significantly increased in the mouse lung 24 h after intratracheal challenge with LPS. In addition, PKCα intervention in combination with Calphostin C treatment restored the levels of claudin-18 to a near-baseline state. Our study is the first to show an increase in the levels of the claudin-18 protein in an LPS-induced mouse model of ALI. This result is in conflict with the downregulated expression of the alveolar epithelium barrier proteins observed in other lung injury models. Based on previous studies, we propose the following hypothesis: (I) the transient modulation of claudin-18 may be beneficial for progenitor cells and may promote regeneration after ALI. (II) Elevated claudin-18 levels could reflect an adaptive response in alveolar epithelial cells aimed at enhancing barrier function and accelerating the resolution of pulmonary edema during ALI. (III) The increase in claudin-18 protein levels may be accompanied by changes in other tight junction structural proteins. Nevertheless, the dynamic balancing regulatory mechanisms between different types of tight junction proteins remain unclear. A limitation of our study is that claudin-18 levels were examined in a semi-quantitative manner and therefore any error associated with the use of this method cannot be ruled out. In the future, we will examine the changes in claudin-18 expression using quantitative analysis.

The NF-κB signaling pathway has been confirmed as a pivotal upstream regulatory pathway for the occurrence and development of inflammation (19). Previously, it has been found that LPS stimulation can activate NF-κB signaling. Furthermore, PKCα is known to be upstream of NF-κB signaling. For example, Wang *et al.* found that the PKCα/NF-κB/c-fos signaling pathway was critical for the development of diabetic cardiomyopathy-related cardiac hypertrophy (20). Other studies showed that high glucose levels promote the activation of PKCα, which can trigger NF-κB signaling, resulting in inflammation and

lipid metabolism disorder in rat models of diabetes (11,21). Therefore, we also investigated the potentially NF-κB-mediated protective mechanism of PKCα inhibition against hyperinflammation and oxidative stress *in vivo* and *in vitro*. Consistent with earlier studies, our results demonstrated that PKCα inhibition inhibited NF-κB activation, IκBα phosphorylation, and NF-κB P65 phosphorylation. It also inhibited the translocation of NF-κB P65 to the nucleus. These data indicate that the protective effects of PKCα inhibition in ALI may partially be dependent on NF-κB signaling.

Our current study still had some limitations. On the basis of our previous experiments, we found that pretreatment of Calphostin C can significantly improve the inhibitory effect of PKCα.Therefore, we inhibited the expression of phosphorylated PKCα in mice by intraperitoneal injection of Calphostin C 4 h before LPS treatment. But this approach may lead to a series of side effects, despite the final satisfactory results. In the future, we will use PKCα gene knockout mice to establish ALI model or use local medication. In addition, we only used RAW264.7 cells to verify the protective effects of PKCα inhibition *in vitro*. Therefore, we will use primary lung epithelial cells and endothelial cells to further explore the regulatory mechanism of PKCα in ALI.

Conclusions

The level of PKCα phosphorylation was upregulated in both LPS-induced mouse models of ALI and LPS-treated RAW264.7 cells. Further, PKCα inhibition alleviated lung injury, weakened the inflammatory response and oxidative stress, regulated tight junction protein levels, and reversed the activation of the NF-κB signaling pathway. Therefore, PKCα could be a promising novel therapeutic target in ALI.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-21-6497/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-21-6497/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6497/coif). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental procedures performed on animals were reviewed and approved by Wenzhou Medical University's Institutional Animal Care and Use Committee (No. 2021-0095), and all animal surgeries were strictly performed in accordance with internationally recognized and institutional guidelines for the care and use of animals.

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Appendix 1

Supplementary materials and methods

Animals and experimental design

A total of 10 C57BL/6J mice (male, 6–8 weeks, 20–25 g) were used in the pre-experiment after random allocation to the following groups: (I) control group (PBS): PBS (same volume as that in the LPS group) was injected into the trachea; (II) LPS group (LPS): different concentrations of LPS were injected into the trachea of mice according to their weight (5 and 10 mg/kg); stimulation was allowed to continue for 6 or 24 h.

Supplementary results

PKCα phosphorylation is upregulated in LPS-treated mice

The pre-experiment showed that lung injury was the most obvious after the intratracheal infusion of LPS (10 mg/kg) for 24 h (*Figure S1A*). Therefore, we used this dose and stimulation duration to establish an LPS-induced ALI model. Earlier research has shown that PKC isoform activity is altered in the event of ALI. In the pre-experiment, the activity of PKCα in mouse lung tissue was detected using Western blots. We found that LPS upregulated p-PKCα/PKCα expression irrespective of concentration (*Figure S1B*). Hence, we hypothesized that PKCα may participate in the pathogenesis of ALI. To test this hypothesis, we used Calphostin C, an inhibitor of PKCα, for subsequent experiments.

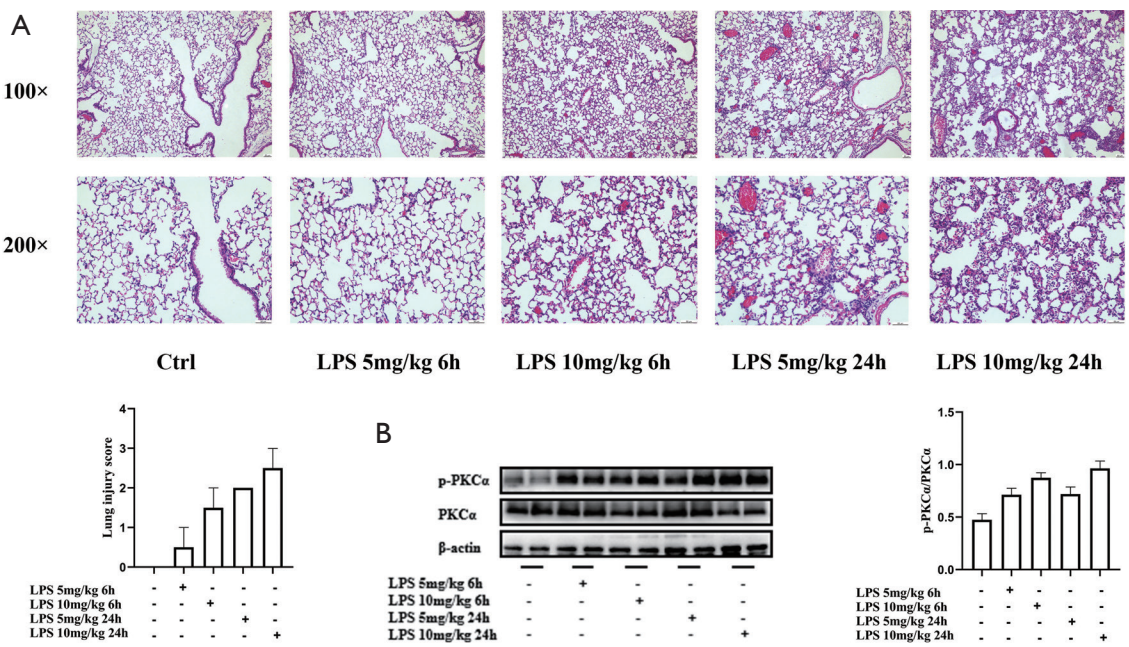

Figure S1 PKCα phosphorylation was up-regulated in LPS-triggered ALI mice. (A) Histopathological analyses of lung tissue (H&E staining; ×100 and ×200). (B) Protein levels of PKCα in lung tissue. "–" stands for the corresponding substance is not used; "+" stands for the corresponding substance is used. PKCα, protein kinase C alpha; ALI, acute lung injury; H&E, hematoxylin and eosin.

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