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Lipoxin A4 reduces hyperoxia-induced lung injury in neonatal rats through PINK1 signaling pathway

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is a common chronic lung disease in premature infants and is mainly caused by hyperoxia exposure and mechanical ventilation. Alveolar simplification, pulmonary vascular abnormalities and pulmonary inflammation are the main pathological changes in hyperoxic lung injury animals. Lipoxin A4 (LXA4) is an important endogenous lipid that can mediate the regression of inflammation and plays a role in acute lung injury and asthma. The purpose of this study was to evaluate the effects of LXA4 on inflammation and lung function in neonatal rats with hyperoxic lung injury and to explore the mechanism of the PINK1 pathway. After 85% oxygen exposure in newborn rats for 7 days, the BPD model was established. We found that LXA4 could significantly reduce cell and protein infiltration and oxidative stress in rat lungs, improve pulmonary function and alveolar simplification, and promote weight gain. LXA4 inhibited the expression of TNF-α, MCP-1 and IL-1β in serum and BALF from hyperoxic rats. Moreover, we found that LXA4 could reduce the expression of the PINK1 gene and down-regulate the expression of PINK1, Parkin, BNIP3L/Nix and the autophagic protein LC3B.These protective effects of LXA4 could be partially reversed by addition of BOC-2.Thus, we concluded that LXA4 can alleviate the airway inflammatory response, reduce the severity of lung injury and improve lung function in a hyperoxic rat model of BPD partly through the PINK1 signaling pathway.

1. Introduction

Bronchopulmonary dysplasia (BPD) is a common chronic lung disease (CLD) in premature infants, especially those with low birth weight (≤1250 g) and low fetal age (≤28 weeks). Alveolar simplification, pulmonary fibrosis (PF) and abnormal angiogenesis are the main pathological changes of BPD in the lungs and impact the effective lung ventilation area and lung function [1,2]. Oxygen toxicity and mechanical ventilation are the major risk factors for BPD in infants. As premature infants cannot tolerate excessive oxidative stress, excess reactive oxygen species (ROS) exposure in lung cells can activate specific inflammatory cells, such as neutrophils and macrophages, and lead to increased levels of inflammatory cytokines and proteins in the airway, resulting in lung injury and abnormal pulmonary cell death, particularly that of alveolar epithelial cells and endothelial cells [3,4].

Pulmonary function analysis is an important tool to evaluate respiratory disease in rat models. Due to the destruction of lung epithelial cells and the proliferation of fibrocytes, various abnormalities in lung function, such as reduced lung compliance (Cst), can produce lung injury in animals. At present, the techniques for measuring lung function mainly include noninvasive and invasive method [5]. The invasive method involves a comprehensive assessment of the lung function status by connecting the tracheal incision and the ventilation device in rats after anesthesia [6]. A FlexiVent animal respiratory function detector (SCIREQ, Montreal, CA) can apply a multifunction intervention model to detect lung capacity (A), airway resistance (Rn), Cst, lung tissue damping (G) and other related parameters and can reflect a comprehensive view of changes in lung function.

Lipoxin A4 (LXA4) is an endogenous anti-inflammatory agent that can promote inflammatory resolution in inflammatory diseases. In humans, LXA4 is produced from arachidonic acids by different lipoxygenases (LO) and plays a regulatory role through binding to lipoxin receptors (ALX) on the cell surface [7]. Currently, LXA4 has been used in a variety of pneumonia diseases in animal models, such as acute lung injury, asthma, and cystic pulmonary fibrosis (CPF) [8–11], to alleviate the severity of lung damage. In a rat model of lipopolysaccharide (LPS)-induced acute lung injury, LXA4 was shown to promote inflammatory regression by enhancing the clearance of pulmonary fluid through the

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PI3K/Akt pathway [12]. In neonatal hyperoxic lung injury mice, Martin et al. reported that LXA4 can promote lung development and reduce inflammation [13]. Chen et al. found LXA4 could inhibit the expression of TGF-beta and alleviate lung injury [14]. However, no studies have examined the effects and mechanisms underlying the actions of LXA4 on lung function and mitochondria.

PTEN-induced putative kinase 1 (PINK1) is one of the major mitochondrial proteins and has been found to be closely related to normal function and integrity. In a recent study, during mitochondrial autophagy, PINK1 was shown to phosphorylate Parkin on the mitochondrial outer membrane and translocate it to the mitochondrial intima and activate autophagy and apoptosis [15,16]. The role of PINK1 in the regulation of lung diseases has been reported in PF, chronic obstructive pulmonary disease (COPD) and lung tumors [17–19]. Several recent studies on PF have shown that PINK1 deletion can depolarize alveolar epithelial cells, cause mitochondrial swelling and promote fibrosis in elderly mouse models [20,21]. In human lung tumors, silencing of PINK1 inhibited the growth and migration of tumor cells, promoted apoptosis and increased the therapeutic effect of drugs on tumors. High expression of PINK1 was significantly correlated with poor prognosis of lung adenocarcinoma [22,23]. However, the expression and role of the PINK1 gene in neonatal hyperoxic lung injury have not been reported.

In our study, we investigated the effect of LXA4 on airway inflammation and lung function in hyperoxic lung injury rats. Our main purpose was to test the hypothesis that LXA4 can inhibit the infiltration of inflammatory cells and proteins in the lungs, thereby reducing alveolar simplification and alleviating lung injury, improving the poor lung function caused by hyperoxia exposure. We added the ALX antagonist N-butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe (BOC-2) to further confirm the protective effect of LXA4 [24]. Our secondary purpose was to investigate the role of the PINK1 pathway in hyperoxic lung injury and the correlation between the reduction in the inflammatory response by LXA4 and the PINK1 pathway.

2. Materials and methods

2.1. Animals and study design

All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the laboratory Animal Ethics Committee of Wenzhou Medical University. Sprague-Dawley (S-D) rats were obtained from the Animal Center of the Chinese Academy of Science (Shanghai, China). Adult rats were crossed to deliver litters for subsequent studies, and all rats were housed in an environment with a constant temperature under a 12 h light/dark cycle and allowed free access to food and water ad libitum.

At full term (d 21–22 of pregnancy), the dams delivered naturally, and the pups were pooled, randomized, and returned to the nursing dams within 6 h, after which the pups were divided into five experimental groups: Normoxia group, Normoxia + LXA4 group, Hyperoxia group, Hyperoxia + LXA4 group, Hyperoxia + LXA4 + BOC-2 group. Each neonatal rat was treated with intraperitoneal injection of 250 μL saline. LXA4 (2 ng/g) (Cayman Chemical Company, Ann Arbor, USA) was given to the rats on postnatal d 0, 2, 4 and 6 for a total of four injections. BOC-2 (10 ng/g) (Phoenix biotech, Beijing, China) was administered to the rats on postnatal d 0, 2, 4 and 6, simultaneously with LXA4 treatment. The dosages of LXA4 and BOC-2 were chosen according to those reported in previous studies and our preliminary experiments [12,25–27]. The preparation of 2 compounds was based on the manufacturer's instructions. Normoxia group rats were placed in room air with 21% oxygen, and hyperoxia-exposed rats were placed in 85% oxygen conditions for 7 days. Continuous exposure to 85% O_2 was achieved in a Plexiglass chamber flow-through system, and the O_2 level inside was monitored continuously with an O_2 analyzer. Nursing dams were rotated between hyperoxic and normoxic litters every 24 h to prevent O_2 toxicity. The chamber was opened once a day for 1 h to weigh the pups and to replace the food and water.

Animals in each group were sacrificed on postnatal d 7 by intraperitoneal injection of 1% pentobarbital (50 mg/kg body weight). For each group, 18 rats were sacrificed for lung tissues and 8 rats were anesthetized and detect lung function. The blood was collected from the right ventricle and centrifuged at 3000 rpm for 10 min at 4 °C, and then, the supernatant was stored at −80 °C. The BALF was collected as described below, and lung tissues were harvested and stored at −80 °C for further analysis.

2.2. Lung histological and morphometric analyses

Six rats from each group were sacrificed, and the right bronchus was ligated. The left lungs were perfused with 4% paraformaldehyde (PFA) at 20 cm H_2O pressure via an intravenous needle inserted into the trachea. The left lungs were postfixed in 4% PFA for 48 h before paraffin embedding and sectioning into 4-micron sections. The sections were stained with hematoxylin and eosin (HE) (Solarbio Science & Technology, Beijing, China) for morphometric analysis by microscopy (Olympus, Tokyo, Japan). Images were acquired at 100× magnification. The radial alveolar count (RAC), mean linear intercept (MLI) and mean alveolar diameter (MAD) were measured to quantify the interalveolar distance, as reported in previous studies [28,29]. MAD was calculated as the average alveolar diameter and performed for at least 20 alveoli per field. MLI was calculated as the volume-to-surface ratio of alveoli and performed by drawing five lines in each field and dividing the length of each line by the number of alveolar intercepts for that line. RAC was obtained by drawing a line from the center of terminal bronchioles to the nearest connective tissue septum and counting the number of the alveoli on this line. All images were assessed by investigators blinded to the experimental groups. A minimum of 20 nonoverlapping fields were examined in each group.

2.3. Collection and analysis of BALF

Eight rats from each group were narcotized, and the right bronchus was ligated. BALF was collected by flushing the left lung, as described previously [30]. Each rat was perfused three times with 200 μL ice-cold sterile saline through the trachea; the rate of recovery was > 80%. BALF was centrifuged at 120 ×g for 10 min at 4 °C. The supernatants were aliquoted and frozen at −80 °C until they were used for further experiments. The cell pellets were resuspended in 20 μL ice-cold sterile phosphate-buffered saline (PBS; Gibco, Invitrogen, NY, USA). Then, 10 μL cell suspension was transferred into a disposable counting slide to measure the total number of cells by using a TC20 automated cell counter (Bio-Rad, Berkeley, USA). Another 10 μL cell suspension was smeared and stained with Wright stain (Solarbio Science & Technology, Beijing, China). The differential white blood cell count was analyzed under the microscope (Olympus, Tokyo, Japan) at 200× magnification. The supernatant was used to measure the protein concentrations by a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Logan, USA).

2.4. Enzyme-linked immunosorbent assays (ELISAs)

The concentrations of TNF-α, IL-1β and MCP-1 in serum and BALF supernatant were assessed by a standardized sandwich ELISA kit (Boyun, Shanghai, China) according to the manufacturer's protocol. The ELISA plate was read at 450 nm and analyzed according to the standards.

2.5. Oxidative stress in lung tissues

Peroxides are the unstable indicators of oxidative stress in cells that decompose to form reactive compounds, such as malondialdehyde (MDA). Superoxide dismutase (SOD) is an essential antioxidant which can scavenge superoxide anion free radicals and protect cells from hyperoxia damage. Frozen lung tissues were homogenized and determined the protein concentrations. Concentrations of MDA and activities of SOD in lung tissues were assessed by commercially available kits (Beyotime Biotechnology, Shanghai, China). To the concentration of MDA, 0.1 mL lung lysates and 0.2 mL MDA operating solution were added in tubes and heated at 100 °C for 15 min. Then, the mixture was cooled down to room temperature, centrifuged at 1000 rpm for 10 min. 200 μL supernatants was used to measure the absorbance at 532 nm and analyzed according to the standards. The concentration of MDA in lung was showed as MDA concentration/per protein concentration (μmol/mg). To SOD activity, 20 μL lung lysates and 180 SOD operating solution were added in 96-well plate and incubated at 37 °C for 30 min. Then, the mixture was read at 450 nm and analyzed according to the standards. The SOD activity in lung was showed as SOD activity/per protein concentration (U/mg).

2.6. Lung function analysis

Lung function was measured within 3 h on postnatal d 7, in accordance with previously reported protocols [30]. Rats were anesthetized as described above. When spontaneous breathing was aborted, the trachea was cannulated with a 12G tubing adaptor and connected to the forced oscillation equipment. The rats were ventilated at 90 breaths/min with a tidal volume (VT) of 3 mL and a positive end-expiratory pressure (PEEP) of 5 cm H_2O using the ventilator (FlexiVent, SCIREQ, Canada). We measured the inspiratory capacity (A) and Cst in Pressure-Volume Loop Salazar Knowles equation mode and detected Rn, G, and tissue elastance (H) in the Quick Prime-3 model.

2.7. PINK1 mRNA expression analysis

Frozen lung tissues were used for PINK1 mRNA expression assays. Total RNA was extracted from lung tissues by using TRlzol reagent (Invitrogen, Carlsbad, USA) and measured by spectrophotometer (Thermo Fisher Scientific, Logan, USA). Two micrograms of RNA was reverse-transcribed by using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Logan, USA) and Oligo (dT) 18 primer (Invitrogen, Carlsbad, USA) to generate cDNA. Then, the cDNA was amplified by mixing the SYBR Green Master Mix (Thermo Fisher Scientific, Logan, USA), specific primers and ddH_2O in a real-time PCR instrument (Light Cycler, Roche Diagnostics, Indianapolis, USA). The RT-PCR amplification reaction was as follows: preincubation at 95 °C for 5 min; 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s. Quantification was performed by using the comparative 2 − (ΔΔCt) method as follows: ΔΔCt = (target gene Ct of the experimental group − reference gene Ct of the experimental group) − (target gene Ct of the Normoxia group − reference gene Ct of the Normoxia group). Expression levels for PINK1 genes were normalized to that of GAPDH. The primers were as follows: PINK1, forward: 5′-GTAGACAGTCACTACCTATGCC-3′, reverse: 5′-TGACAGCTAAGTCATCACAACT-3′; GAPDH, forward: 5′-CAACGGGAAACCCATCACCA-3′, reverse: 5′-ACGCCAGTAGACTCCACGACAT-3′.

2.8. PINK1, Parkin, LC3B and BNIP3L/Nix protein expression analysis

Frozen lung tissues were used for protein assays. Protein lysates were obtained using RIPA lysis buffer supplemented with phosphatase inhibitors and PMSF, ultrasonication for 5 s three times, and centrifugation at 12,000 rpm for 20 min at 4 °C. Protein concentrations were measured by a Pierce™ BCA Protein Assay Kit. Thirty micrograms of protein was subjected to SDS-PAGE on 4–20% Tris-glycine gels (Bio-Rad, Berkeley, USA) and then electro-transferred to PVDF membranes. The membranes were blocked in 5% nonfat milk supplemented with TBST and incubated with primary (overnight at 4 °C) and secondary antibodies (2 h at room temperature). Antibodies against Parkin (1:1000) (Abcam, Cambridge, UK), PINK1 (1:1000), BNIP3L/Nix (1:1000) (Cell Signaling Technology, Boston, USA), LC3B (1:1000) (Sigma Aldrich, St. Louis, USA), and GAPDH (1:3000) (Bioworld Technology, Inc., Nanjing, China) were used. The protein bands were detected by Pierce™ ECL western blotting substrate (Thermo Fisher Scientific, Logan, USA) and visualized by ChemiDoc XRS$^+$ Imaging System (Bio-Rad, Berkeley, USA).

2.9. Statistical analysis

The experiments were performed in triplicate and repeated a minimum of three times. The data are presented as the mean ± SD or SEM and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (equal variance) or Dunnett T3's post hoc test (unequal variance) for multiple comparisons. Correlation analyses were performed by Pearman's rank correlation. Statistical analysis was carried out by SPSS Statistics19.0 (SPSS Inc., Chicago, USA) or GraphPad 6.0 (GraphPad Software, San Diego, USA). Values of $P < 0.05$ versus the indicated group were considered statistically significant.

3. Results

3.1. Body weight of newborn rats

Eight neonatal rats were used to weigh the body weight from postnatal day 0 (P0) to P7. On P0, the mean body weight of rats from each group had no obvious difference. After 2 days of 85% oxygen exposure, the body weight of the Hyperoxia group rats was significantly retarded compared with that of the Normoxia group rats, and this trend continued until P7. There was an increase in body weight between Hyperoxia + LXA4 rats and Hyperoxia rats after P4 and on. In addition, the mean body weights of Hyperoxia + LXA4 + BOC-2 rats was distinctly lower than that of Hyperoxia + LXA4 rats beginning on P5 (Fig. 1). All neonatal rats in each group survived the entire experimental period.

3.2. Effect of LXA4 on alveolar simplification in lung tissue

The lung development is shown in Fig. 2A by HE staining. The lung tissues in the Normoxia group showed intact lung structures with a normal alveolar epithelium, uniform alveolar septum and clear arrangement of airway and blood vessels. The Normoxia + LXA4 group lung tissue had a similar structure to that of the Normoxia group. After 7 days of high oxygen exposure, we observed an obviously simplified alveolar appearance in hyperoxic lungs. In per unit area, the alveolar

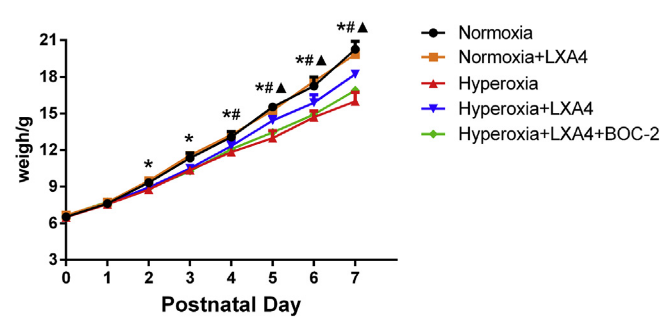

Fig. 1. The body weight of rats in each group by postnatal day. The values are shown as the mean ± SD; n = 8 rats/group. $^*P < 0.05$ Hyperoxia group versus Normoxia group on the same postnatal day, $^\#P < 0.05$ Hyperoxia + LXA4 group versus Hyperoxia group on the same postnatal day, $^\blacktriangle P < 0.05$ Hyperoxia + LXA4 + BOC-2 group versus Hyperoxia + LXA4 group on the same postnatal day.

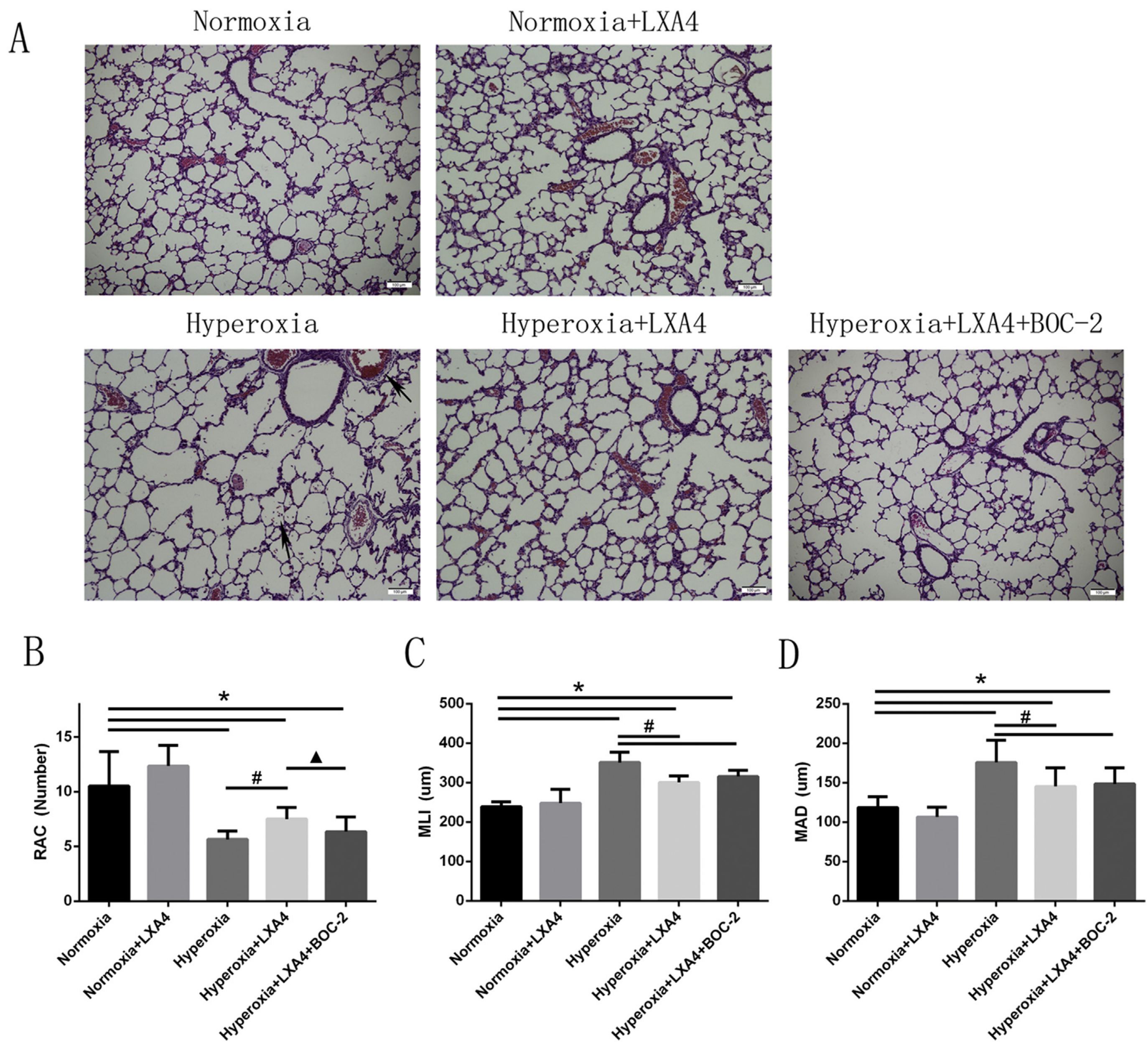

Fig. 2. The effects of LXA4 on pulmonary alveolar simplification in lung. (A) Representative HE staining (light microscopy, ×200) of lung tissue slides of each group. Calibration bars = 100 μm. The arrow indicates the inflammatory cells and red blood cell infiltration in alveoli and erythrostasis in small vessels in lung tissues. (B) Semi-quantitative pathology score of RAC in lung tissues. (C) Semi-quantitative pathology score of MLI in lung tissues. (D) Semi-quantitative pathology score of MAD in lung tissues. The values are the mean ± SD; n = 20 nonoverlapping fields/group. *$P < 0.05$ versus Normoxia group, $^{\#}P < 0.05$ versus Hyperoxia group, ▲$P < 0.05$ versus Hyperoxia + LXA4 group.

volume increased, the alveolar number decreased and the alveolar interval increased. Inflammatory cells and red blood cells could be seen in the alveolar cavity, and marked erythrostasis was observed in the vessels (arrow). These results suggested that high oxygen exposure increases alveolar injury and leads to inflammation, resulting in alveolar simplification and delayed lung development. After LXA4 intervention following hyperoxia exposure, the alveolar simplification was significantly improved, and the alveolar septum was reduced compared with that of the Hyperoxia group rats. Cell infiltration was rarely observed in the alveoli. In the Hyperoxia + LXA4 + BOC-2 group rats, the alveolar development was similar to that in the Hyperoxia group, but the alveolar diameter and alveolar intercept were improved (Fig. 2B–D).

3.3. LXA4 decreased cell and protein infiltration in BALF

Total cells and protein in BALF were distinctly increased in Hyperoxia rats compared with Normoxia rats. Under LXA4 treatment, total cells and protein were decreased in Hyperoxia + LXA4 group compared to Hyperoxia group rats. The effects of LXA4 on protein levels were reversed with BOC-2 treatment, but no obvious difference in the cells in BALF was observed (Fig. 3A–B). The changes in cell count and protein in BALF occurred at the same time, so there was a positive correlation between the cells and protein in BALF (Fig. 3C). Moreover, results from BALF smear via Wright stain showed that hyperoxia exposure increased infiltration of mononuclear cells (such as lymphocytes, monocytes and macrophage cells) and neutrophils in BALF (Fig. 3D–E).

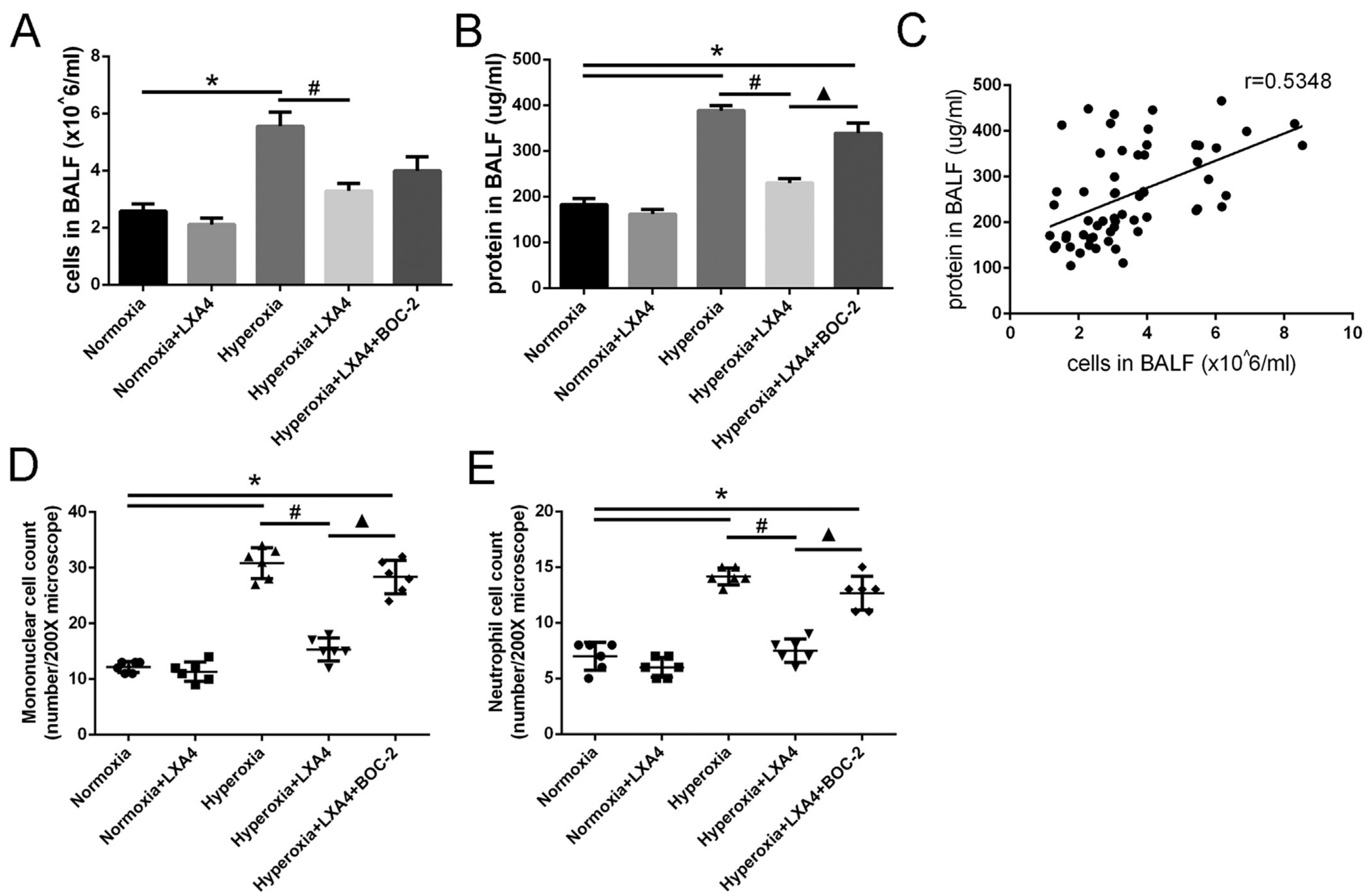

Fig. 3. LXA4 reduced inflammatory infiltration in bronchoalveolar lavage fluid (BALF). (A) Total cell numbers in BALF and (B) protein in BALF, n = 6 rats/group. (C) The correlation of cells and proteins in BALF, n = 60, r = 0.5348, $P < 0.001$. (D) Mononuclear cell count in BALF. (E) Neutrophil cell count in BALF, n = 6 rats/group. The values are the mean ± SEM. *$P < 0.05$ versus Normoxia group, $^{\#}P < 0.05$ versus Hyperoxia group, ▲$P < 0.05$ versus Hyperoxia + LXA4 group.

3.4. LXA4 decreased the pro-inflammatory cytokines and oxidative stress in neonatal rats

The expression of pro-inflammatory cytokines in BALF and blood serum was detected by ELISAs. As shown in Fig. 4A–F, the levels of IL-1β, MCP-1 and TNF-α in BALF and serum were distinctly increased in Hyperoxia rats compared with Normoxia rats. Under LXA4 treatment, most of these parameters were decreased in Hyperoxia + LXA4 group compared to Hyperoxia group rats. The effects of LXA4 on IL-1β (BALF and serum), MCP-1 (serum) and TNF-α (serum) were reversed with BOC-2 treatment. There was also significant decrease in IL-1β (serum) and TNF-α (BALF) between the Hyperoxia + LXA4 + BOC-2 group rats and the Hyperoxia group rats.

The expression of oxidative stress in lung tissues was detected by available kits. As shown in Fig. 4G, the MDA level was distinctly increased in Hyperoxia rats compared with Normoxia rats. In Fig. 4H, the SOD activity was decreased in Hyperoxia rats compared with Normoxia rats. Under LXA4 treatment, MDA level decreased and SOD activity increased in rats lungs. The effects of LXA4 could be reversed with BOC-2 treatment.

3.5. Effect of LXA4 on lung function in newborn rats

We detected lung function indexes in each group rats in 3 h after hyperoxia exposure. The lung function indicators were measured under a forced oscillation technique. As shown in Fig. 5, 7 days of high oxygen exposure decreased A and Cst and increased Rn, G and H compared with those of the Normoxia group rats. With LXA4 treatment, A, Cst, G and H were improved. However, there was no significant difference in Rn between Hyperoxia + LXA4 group and Hyperoxia group rats. With intervention of BOC-2, the A, Cst and H were similar to the rats in hyperoxia + LXA4 group, and significantly lower than hyperoxia group rats. Rn had no obvious change, similar to rats in hyperoxia group, and worse than hyperoxia + LXA4 group rats. G was worse than the rats in Hyperoxia + LXA4 group, but better than the hyperoxia rats.

3.6. Effect of LXA4 on PINK1 pathway proteins in rat lungs

To evaluate the effect of LXA4 on the PINK1 pathway in lung, we examined rat lung tissues by RT-PCR and western blot analyses. First, we measured PINK1 mRNA expression in rat lungs. As shown in Fig. 6A, PINK1 was significantly increased in Hyperoxia rats compared with Normoxia rats, and it was decreased with the administration of LXA4 and was reversed by BOC-2 treatment. We also observed an obvious decrease in this parameter between Hyperoxia rats and Hyperoxia + LXA4 + BOC-2 rats. Then, we detected the protein levels of PINK1, Parkin, BNIP3L/Nix and LC3B in the lung by western blot analyses. We found that PINK1, Parkin, BNIP3L/Nix and LC3BII/I proteins were increased in Hyperoxia group rat lungs, and LXA4 treatment significantly decreased these levels in Hyperoxia rats (Fig. 6B–E). After BOC-2 treatment, the effects on PINK1, BNIP3L/Nix and LC3BII/I could be inhibited, except for that on Parkin protein (Fig. 6B–E). Moreover, there was an obvious decrease in the levels of BNIP3L/Nix and LC3BII/I between Hyperoxia rats and Hyperoxia + LXA4 + BOC-2 rats (Fig. 6C–D).

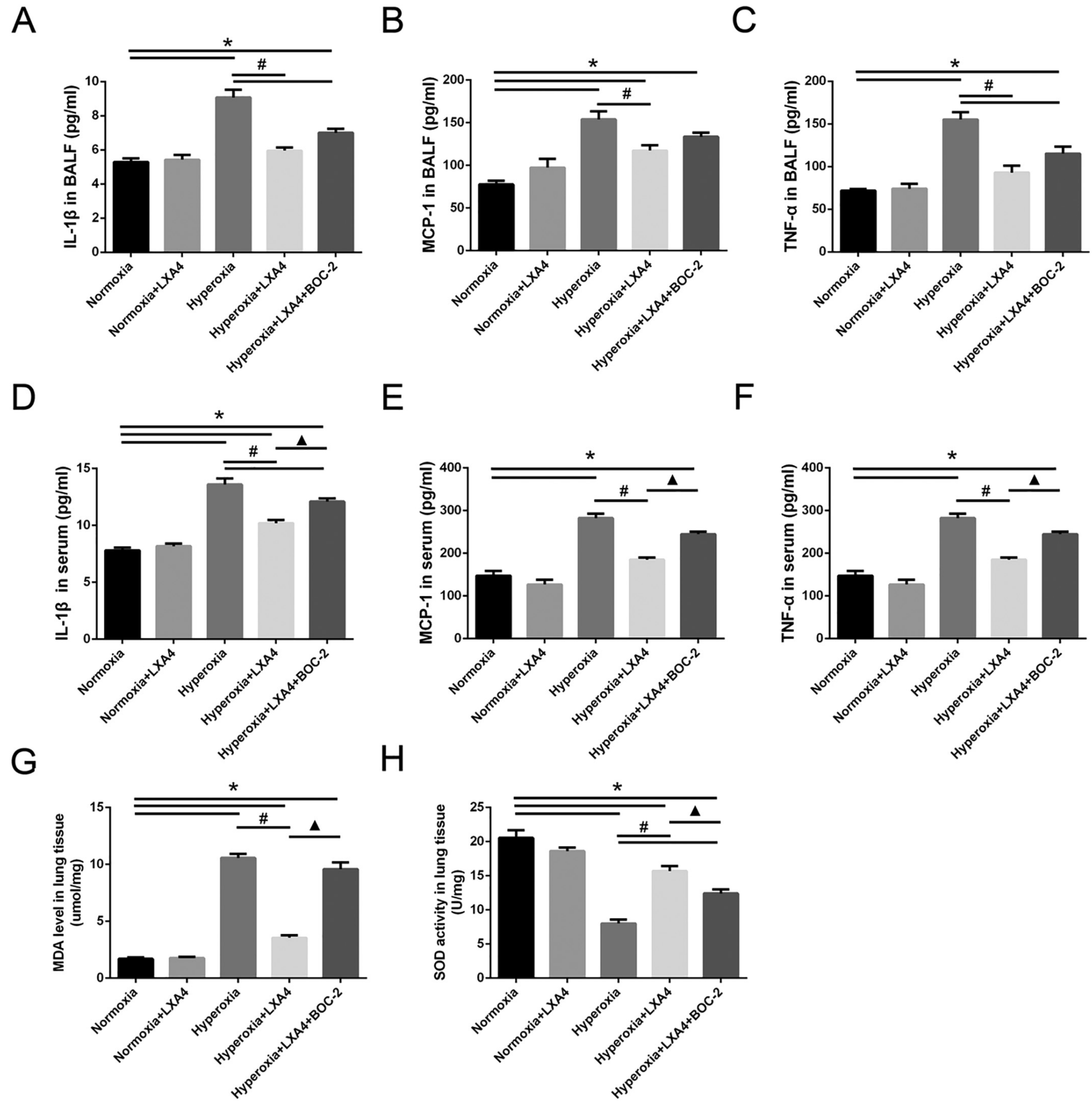

Fig. 4. LXA4 reduced pro-inflammatory cytokines and oxidative stress in neonatal rats. (A) The IL-1β level in BALF. (B) The MCP-1 level in BALF. (C) The TNF-αlevel in BALF. (D) The IL-1β level in serum. (E) The MCP-1 level in serum. (F) The TNF-α level in serum. (G) The MDA level in lung tissue. (H) The SOD activity in lung tissue. *$P < 0.05$ versus Normoxia group, #$P < 0.05$ versus Hyperoxia group, ▲$P < 0.05$ versus Hyperoxia + LXA4 group.

3.7. Correlations among PINK1, pro-inflammatory cytokines and lung function

Finally, we determined the association between the mRNA expression of PINK1 and pro-inflammatory cytokines in BALF and the lung function. As shown in Fig. 7, PINK1 mRNA was positively correlated with IL-1β, MCP-1 and TNF-α in BALF ($P < 0.001$). Moreover, in the analysis of lung function, there was a negative correlation between PINK1 mRNA and A and Cst and a positive correlation between PINK1 mRNA and Rn, G and H ($P < 0.001$).

4. Discussion

We studied the protective effect of LXA4 on hyperoxic lung injury in SD rats and investigated the effects on pulmonary inflammation, lung alveolar development and pulmonary function in hyperoxic rats and the changes in the PINK1 pathway in lung tissues. The results of our study showed that 7 days of hyperoxia exposure resulted in delayed weight growth, significant pulmonary alveolar simplification, a distinct increase in pro-inflammatory factors in BALF and serum, and poor lung function in newborn rats. The intervention of LXA4 could reduce the inflammatory response and inflammatory cytokine infiltration and

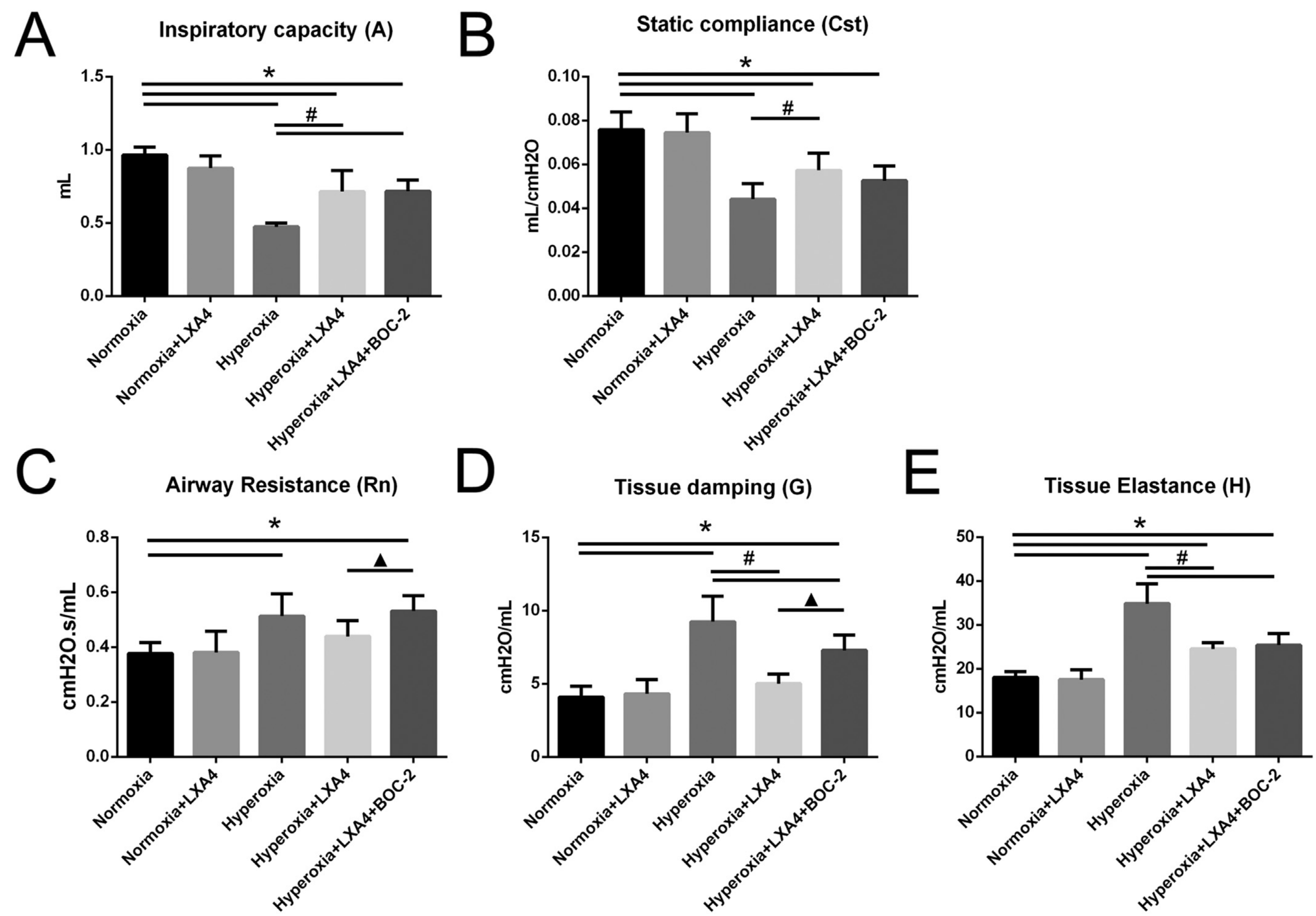

Fig. 5. The effects of LXA4 on rat lung function. (A) Changes in lung inspiratory capacity. (B) Changes in lung static compliance (Cst). (C) Changes in airway resistance (Rn). (D) Changes in lung tissue damping (G). (E) Changes in lung tissue elastance (H). The lung function indicators were detected by the forced oscillation technique after the rats were anesthetized and ventilated with flexiVent. *$P < 0.05$ versus Normoxia group, $^{\#}P < 0.05$ versus Hyperoxia group, ▲$P < 0.05$ versus Hyperoxia + LXA4 group.

improve lung alveolar development and function. In addition, the ALX antagonist BOC-2 reversed the effects on the expression of LXA4, PINK1, Parkin, BNIP3L/Nix and LC3B in hyperoxic rats, and these molecules could be down-regulated after LXA4 treatment, which was positively correlated with pulmonary reaction and lung function. These data suggested that LXA4 could reduce the severity of hyperoxic lung injury, which may be related to the PINK1 pathway.

The hyperoxic lung injury model in newborn rats is a classic model of BPD. High oxygen exposure can cause specific pathological changes, such as lung alveolar simplification, vascular malformation and PF, which have been reported in many studies [2,31]. In our study, hyperoxia exposure delayed weight gain in newborn rats (Fig. 1) and induced simplified alveolar development, as shown in lung HE staining (Fig. 2), which was consistent with the results of our study, suggesting the establishment of a hyperoxic lung injury model. RAC, MAD and MLI are important indices of alveolar growth. Following LXA4 treatment, the alveoli number increased, and the alveolar diameter and alveolar intercept were reduced, which suggested that LXA4 can promote lung alveolar development through its positive effect and reduce lung growth disorders caused by high oxygen, being consistent with the results of Martin et al. [13].

Inflammatory cells and proteins play an important role in BPD. In animal models of hyperoxic lung injury, we clearly observed infiltration of inflammatory cells and proteins in lung tissue and aggravated damage and necrosis in lung cells [3]. In Zhang's study, high oxygen could increase mononuclear and multinuclear cell infiltration in the lungs [32]. LXA4 is an anti-inflammatory and pro-regression factor that can promote lung water clearance and inflammatory regression in acute lung injury models and is closely related to macrophages [33]. In our study, we found that LXA4 could significantly reduce the number of cells and proteins in BALF in Hyperoxia + LXA4 group rats (Fig. 3). Studies have shown that neutrophils and macrophages are the main infiltrating cells in the BALF of high-oxygen mice [34,35]. We classified the cells in BALF and found that mononuclear and neutrophil were both increased in lung BALF and LXA4 could reduce their infiltration. All these data proved that LXA4 can reduce pulmonary inflammatory reactions in BPD (Fig. 3).

ROS is a product of cellular respiration and metabolism and produced in lung through membrane NAPDH oxidase system. In the process of hyperoxic exposure, the excessive production of ROS will damage DNA and respiratory chain proteins, leading to cell oxidative injury and the infiltration of inflammatory cells and cytokines in lung tissues. Our results showed that LXA4 could reduce oxidative stress in lung tissues (Fig. 4). Pro-inflammatory cytokines are proteins produced in the cellular inflammatory response, and TNF and the interleukin family are the major cytokines. When normal lung cells are stimulated by inflammatory agents, they can activate neutrophils and macrophages to produce many pro-inflammatory mediators, regulate and expand the inflammatory response and participate in the cell's immune process. In the BALF of BPD patients, pro-inflammatory factors, such as TNF-α, IL-1, IL-6, and IL-8, were distinctly increased, similar to those in the neonatal rat model of hyperoxic lung injury [2,32,36,37]. In the

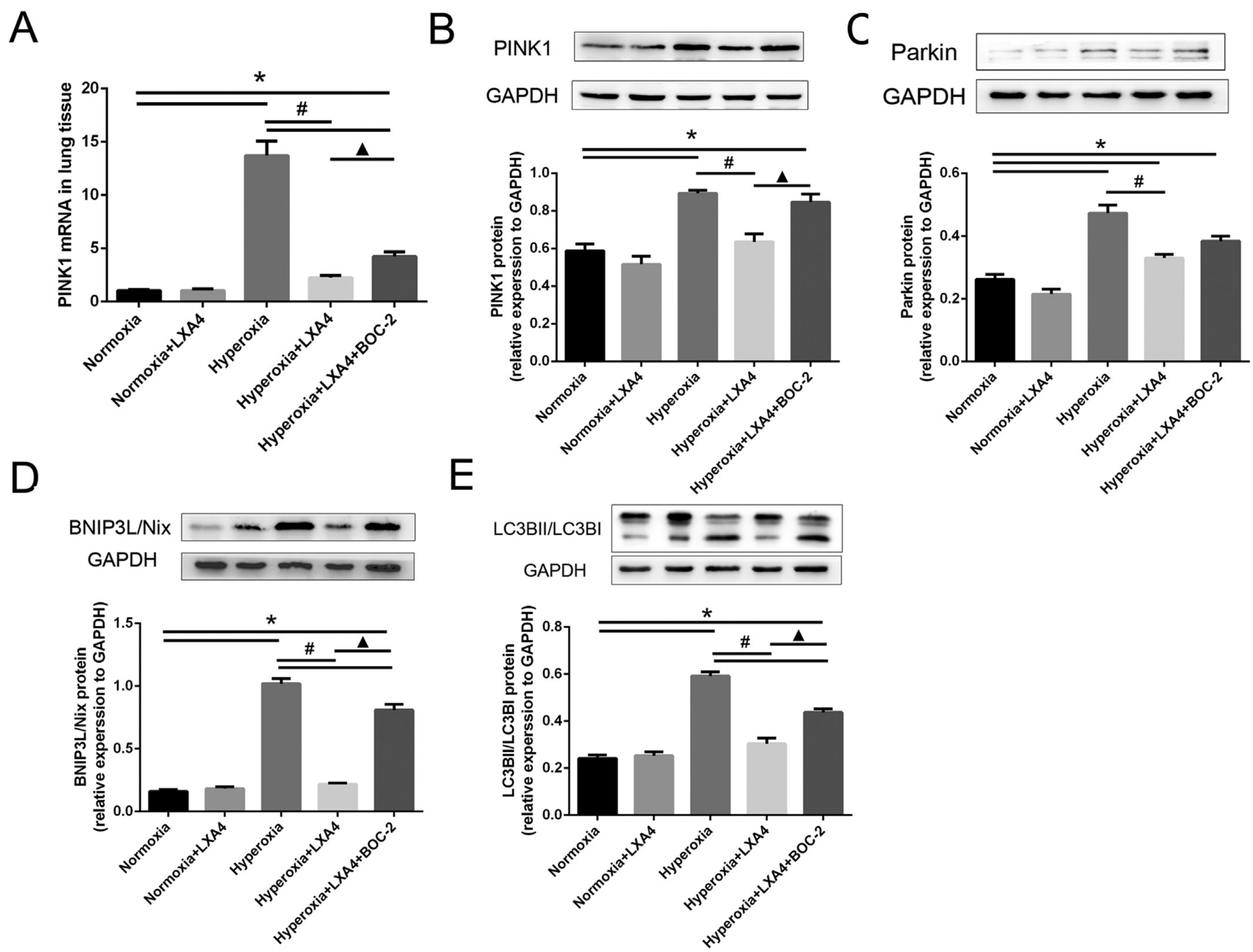

Fig. 6. LXA4 reduced the activation of the PINK1 pathway in rat lung. (A) Relative expression of PINK1 mRNA in lung tissue. (B) Representative image and semi-quantitative analysis of PINK1 protein expression in lung tissue. (C) Representative image and semi-quantitative analysis of Parkin protein expression in lung tissue. (D) Representative image and semi-quantitative analysis of BNIP3L/Nix protein expression in lung tissue. (E) Representative image and semi-quantitative analysis of LC3B protein expression in lung tissue. GAPDH was the loading control. *$P < 0.05$ versus Normoxia group, $^{\#}P < 0.05$ versus Hyperoxia group, $^{▲}P < 0.05$ versus Hyperoxia + LXA4 group.

results by Martin et al., LXA4 could decrease the gene expression of CD46 and CXCL2 [13]. High oxygen can lead to inflammatory responses in the lungs, altered vascular permeability, and increased levels of inflammatory factors in the blood. In our study, we used ELISAs to detect the levels of the pro-inflammatory cytokines TNF-α, IL-1β and MCP-1 in BALF and serum. Our results showed that hyperoxia exposure significantly increased TNF-α, IL-1β and MCP-1 in serum and BALF, and these increases were mostly inhibited by LXA4 treatment. With the intervention of BOC-2, IL-1β, MCP-1 and TNF-α were increased (Fig. 4). These results suggested that LXA4 could reduce pro-inflammatory cytokines in the lungs and serum in a hyperoxic environment, and its role may be to reduce the secretion of inflammatory cytokines or promote the regression of inflammatory cytokines.

Lung function is one of the most direct and specific indicators of lung cell death and injury. When immature lungs are exposed to high oxygen levels, lung function indicators can be damaged [38,39]. Recently, studies on lung function have mainly focused on respiratory function indicators (total A and Cst) and lung mechanics indicators (Rn, G and H). In the study by Richter and Jimenez in newborn rabbits, the inhalation of 95% oxygen for 5–7 days reduced total A and increased G and H, but no significantly change on Rn [38,40]. In the study by Sozo et al. in newborn male mice, the inhalation of 65% oxygen for 7 days increased G and H, and the greatest increase was observed with increasing doses of methacholine [41], similar to the findings in a report by Ramani et al. [42]. Our study was the first to use the invasive pulmonary function test to assess changes in airway and parenchymal lung function on 7 day rats. Pulmonary respiratory function was measured in Pressure-Volume Loop Salazar Knowles equation mode, and we found that after 7 days of high oxygen exposure, the respiratory capacity and Cst were significantly decreased, which could be reversed by the LXA4 treatment. We measured the mechanical indexes under the Quick Prime-3 model and found that the Rn, G, and H were increased in hyperoxic rats and could be decreased by the LXA4 treatment. As LXA4 improved lung function, we speculated that LXA4 could relieve the infiltration of inflammatory cells and proteins in the rat lung, reduce pulmonary intravascular deposition, increase the development of alveoli and block the progression of PF to alleviate the impairment of lung function caused by high oxygen (Fig. 5).

As tissues are exposed to a high oxygen environment, excessive ROS can be produced in cells and cause mitochondrial damage and promote apoptosis [43]. The PINK1 gene is closely related to the normal function and integrity of mitochondria [16]. In the presence of excess ROS or inflammatory factors in cells, PINK1 can be transferred to the mitochondrial outer membrane, bind with Parkin on the mitochondrial

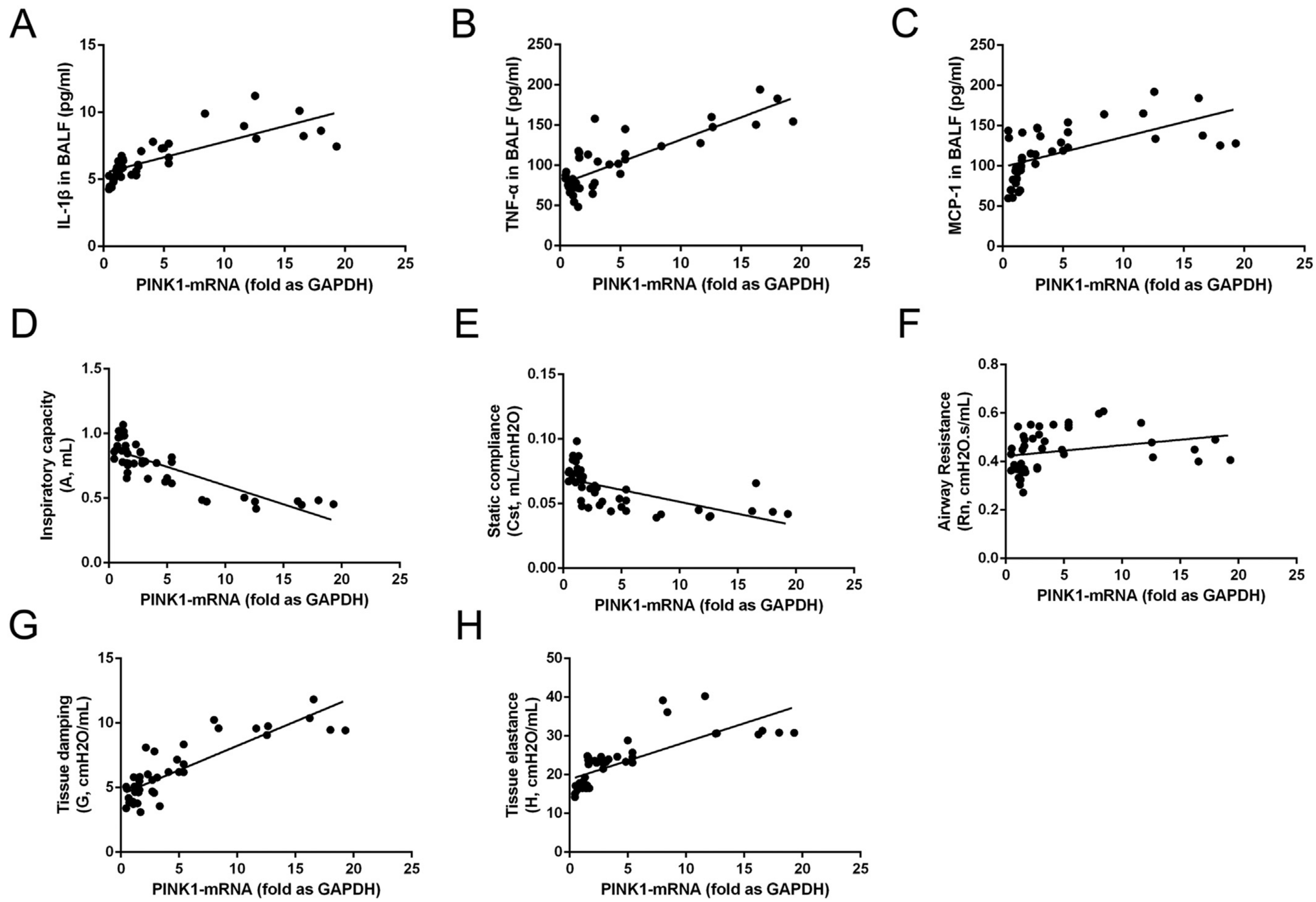

Fig. 7. The correlations among PINK1, inflammatory cytokines and lung function. (A) The correlation of PINK1 mRNA and IL-1β in BALF, n = 40, r = 0.8696, $P < 0.001$. (B) The correlation of PINK1 mRNA and TNF-α in BALF, n = 40, r = 0.7245, $P < 0.001$. (C) The correlation of PINK1 mRNA and MCP-1 in BALF, n = 40, r = 0.7069, $P < 0.001$. (D) The correlation of PINK1 mRNA and inspiratory capacity, n = 45, r = −0.8077, $P < 0.001$. (E) The correlation of PINK1 mRNA and compliance, n = 45, r = −0.8228, $P < 0.001$. (F) The correlation of PINK1 mRNA and airway resistance, n = 45, r = 0.5255, $P < 0.001$. (G) The correlation of PINK1 mRNA and tissue damping, n = 45, r = 0.7884, $P < 0.001$. (H) The correlation of PINK1 mRNA and tissue elastance, n = 45, r = 0.8753, $P < 0.001$.

inner membrane, and then activate downstream mitochondrial membrane-related proteins, such as BNIP3L/Nix [44]. These changes lead to mitochondrial atrophy and the formation of autophagosomes recognized by lysosomes. LC3 is a key factor in intracellular autophagy that can represent the level of autophagy [45]. Some studies have shown that in PF, emphysema and mechanical lung injury models, pulmonary inflammation and oxidative stress can activate the PINK1 pathway and autophagy, promote pulmonary epithelial cell dysfunction or even death, and lead to lung injury [21,46–49]. In our study, we found that PINK1, Parkin, BNIP3L/Nix and LC3B were significantly activated after the newborn rats were exposed to 85% oxygen for 7 days (Fig. 6), suggesting that the PINK1 pathway is involved in hyperoxic lung injury in newborn rats and can aggravate the level of autophagy in the lungs. With the LXA4 treatment, the activation of the PINK1 pathway could be reversed and autophagy was decreased, but addition of BOC-2 could reactivate the PINK1 pathway and promote autophagy. These results proved that LXA4 could bind to ALX receptors on the cell membrane surface in cells, inhibit the signal factors in the PINK1 pathway, and finally play a role in inhibiting autophagy.

To the effect of ALX antagonist (BOC-2), we found its reverse effect is only about 50% in our results. The mechanisms may be as follows. First, BOC-2 could not completely inhibit the activity of ALX in lung cells, so LXA4 played partial protective roles on hyperoxia lung injury. Second, Martin et al. [13] reported that the hyperoxia lung injury could obviously be reversed by the combination use of LXA4 and RvD1.There may have other receptors except ALX which LXA4 could combine. Inhibition of ALX could not completely reverse LXA4's protective effect. Further studies may be needed for dissecting the specific mechanism of LXA4 and BOC-2.

Our study has some limitations. First, we did not target specific lung cells, such as the most widely distributed cell-type I alveolar epithelial cells, which needs to be further explored in BALF and lung tissues. Second, we used LXA4 by intraperitoneal injection as the neonatal rats were hard to intravenous injection, but intratracheal administration may be a better choice in newborn rats to reflect effective concentration of LXA4 in rats lung and easy to regulate in future studies.

In conclusion, LXA4 can reduce airway and serum inflammatory reactions, improve pulmonary function and alveolar simplification, and promote weight gain in a rat hyperoxic lung injury model. LXA4 may reduce the severity of hyperoxic lung injury by inhibiting the PINK1 pathway in lung cells. Thus, this study suggests that LXA4 may be further studied or used as a new drug preparation for the treatment of hyperoxic lung injury.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions

QW, conceived and designed the experiments; QW and LC, performed the experiments; QW and YS, analyzed the data; SC and CL, contributed reagents, materials, and analysis tools; QW, wrote the paper; CL, edited and approved final draft.

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