The Effect and Mechanism of Lipoxin A4 on Neutrophil Function in LPS-Induced Lung Injury

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Abstract—Excessive inflammatory response caused by infiltration of a large number of neutrophils is one of the important features of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Lipoxin A4 (LXA4) is an important endogenous mediator in the process of inflammation resolution, which has a strong role in promoting inflammation resolution. In this study, we examined the impact of LXA4 on the pulmonary inflammatory response and the neutrophil function in ARDS rats. Our results indicated that exogenous administration of LXA4 could reduce the degree of lung injury in ARDS rats and inhibit the release of pro-inflammatory factors TNF-α and IL-1β in lung tissue homogenate. However, LXA4 has no lung protective effect on ARDS rats of neutropenia, nor can it inhibit the levels of pro-inflammatory factors TNF-α and IL-1β in lung tissue homogenate. LXA4 can inhibit the production of reactive oxygen species (ROS) and neutrophil extracellular traps (NETs) in peripheral blood neutrophils of ARDS rats. At the same time, LXA4 can promote the phagocytosis of neutrophils in ARDS rats in vitro and can also promote the apoptosis of neutrophils in ARDS rats. In addition, the effect of LXA4 on the function of neutrophils in ARDS rats is mediated by its receptor ALX. LXA4 can inhibit the release of NE and MPO from neutrophils, thereby reducing the production of NETs. In summary, these findings indicate that LXA4 has a protective effect on LPS-induced ARDS rats by affecting the function of neutrophils.

KEY WORDS: lipoxin A4 (LXA4); neutrophils; ARDS; inflammatory resolution.

INTRODUCTION

ARDS is a common clinical emergency. In recent years, although the research on the pathogenesis of ARDS has made great progress, there is still a lack of specific treatment. Therefore, it is urgent to study the pathogenesis and treatment of ARDS [1–4].

Neutrophils are the most abundant leukocyte human circulation, participate in a variety of immune and inflammatory processes, and play a vital role in the process of immune defense against pathogenic
microorganisms. When the body is invaded by foreign microorganisms, neutrophils are first recruited to the inflammatory injury site in a cascade-like manner leading to activation of specific effector functions such as the release of reactive oxygen species (ROS), phagocytosis, degranulation, and the formation of neutrophil extracellular traps (NETs) [5]. Imbalance in the number, recruitment, or functionality of neutrophils during this process results in clinical maladies, presenting with uncontrolled infections, immunopathology, or autoimmunity [6].

Activated neutrophils play an important role in the clearance of ARDS inflammation by gathering from the peripheral circulation to the lung tissue, and eliminating potential harmful stimuli in ARDS patients [7, 8]. However, neutrophils are also highly histotoxic cells. Excessive accumulation of neutrophils in pulmonary microcirculation, pulmonary interstitium, and alveolar spaces in ARDS patients will cause neutrophil-mediated tissue damage [9, 10]. Therefore, how to effectively promote the antimicrobial activity of neutrophils and accelerate the resolution of dead neutrophils has always been our concern.

LXA4 is an important endogenous anti-inflammatory mediator produced in the process of inflammatory resolution, which can inhibit the recruitment of inflammatory cells, regulate the balance of pro-inflammatory/anti-inflammatory factors, limit inflammatory injury, and prevent injured tissue fibrosis [11, 12]. The unique role of promoting timely apoptosis of neutrophils and improving the phagocytosis of macrophages to neutrophils to promote the timely resolution of inflammation is regarded as the “stop signal” and “brake signal” of inflammatory response [13–17].

In this study, we used the ARDS rat model to further verify that LXA4 can promote the resolution of inflammation in ARDS rats by affecting the function of neutrophils.

**MATERIALS AND METHODS**

**Reagents**

LXA4 (Cayman Chemical Company), LPS (Escherichia coli serotype 055: B5), formyl methionyl leucyl phenylalanine (fMLP), interleukin-8 (IL-8), phorbol ester (PMA), cell chromatography C (Cytochrome C), superoxide dismutase (superoxide dismutase, SOD), elastase, hydroxyethylpiperazine ethylsulfonic acid (HEPES), and emodin were obtained from Sigma-Aldrich (St Louis, MO, USA). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) ELISA kits were obtained from R&D Systems (Minneapolis, MN). MNase, RP-1 antibody (BD 550,002), anti-myceloperoxidase antibody (abcam ab65871), SYTOX Green, and Annexin V-FITC were obtained from eBioscience (San Diego, CA). pHrodo Red E. coli (Cat.No.4615) and pHrodo Green S. aureus (Cat. No. 4620) were obtained from Sartorius (Göttingen, Germany). RPMI 1640, fetal bovine serum (FBS), trypsin, and enzyme-free cell dissociation buffer were purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin in saline citrate buffer were from Invitrogen (Carlsbad, CA, USA). Other chemical reagents are of analytical grade.

**Establishment of ARDS Rat Models**

Experiments were performed on adult male Sprague Dawley rats (250–300 g; Shanghai Experimental Animal Center of China). Rats were provided with water and food ad libitum. The use of animals in this study was approved by the Animal Studies Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

Follow the methods of our lab [18]. Rat received LPS (14 mg/ kg) via tail vein for 12 h, 24 h, 48 h, or 72 h to elicit peak inflammation. Then, for the LPS and LPS + LXA4 group, rat received LPS 6 h later with or without LXA4 (200 ng/kg, i.v.). Twenty-four hours later, the bronchoalveolar lavage fluid (BALF), lung tissue samples, and peripheral venous blood were collected.

Criteria for successful modeling are as follows: ① Increased respiratory rate and respiratory distress, ② cyanosis of the skin and mucous membrane of the lips, ③ upright hair and reduced activity, ④ visual observation of lung tissue swelling and bleeding spots on the surface after chest opening, and ⑤ PaO2 decreased.

Exclusion criteria are as follows: Although the rats have respiratory distress and other symptoms after the model is successfully prepared, rats with one of the following conditions are not included in this research: ① After opening the chest, the lung tissue is visually observed without congestion or swelling, ② lung tissue section HE staining showed no pathological changes such as alveolar edema and structural damage, ③ death before the observation time point. The death of rats in each experimental group due to various reasons should be supplemented by the principle of random sampling.

**Establishment ARDS Rat Model of Neutropenia**

Cyclophosphamide (CTX) is a kind of non-specific chemotherapeutic drug in cell cycle. It can
kill the cells in each phase of the proliferation cycle and inhibit the number of leukocytes in bone marrow. Therefore, it is often used to prepare animal models of neutropenia.

In this experiment, rats were injected intraperitoneally with cyclophosphamide (75 mg/kg) 4 days before and 1 day before the ARDS induced by LPS. And 1 day before ARDS rat model was prepared and 1 day after the model was prepared, the number of neutrophils in rat tail vein blood was less than $2 \times 10^5$/mL by using the blood cell count version technology. Therefore, the ARDS model of neutrophil deficiency rats was prepared. Rats were randomized into three groups ($n = 6$): control group, LPS group, and LPS + LXA4 group.

**Pathological Studies**

Rats were anesthetized with chloral hydrate (7 mL/kg, intraperitoneally) and intubated and connected to the animal ventilator (respiratory parameters are tidal volume: 10 mL/kg and respiratory rate: 40–60 bpm) 6 h after the injection of LPS. After anesthesia and mechanical ventilation with pure oxygen for 1 h, rats were killed by cutting off the abdominal aorta and bloodletting. Rats were subjected to thoracotomy and PBS (25 mL/min) was injected into the right ventricle to flush the pulmonary vessels. Finally, the right lower lung lobe of rats was cut and fixed in 4% paraformaldehyde for 24 h at room temperature, and 4-μm sections were embedded in paraffin and stained with hematoxylin and eosin (H&E) for light microscopy analysis. The rest of the lung tissue was frozen in liquid nitrogen for 48 h and stored in a freezer at −80 °C.

A semi-quantitative scoring system was adopted to evaluate lung injury, which included alveolar congestion, alveolar hemorrhaging, neutrophil infiltration or aggregation in the airspace or vessel wall, and alveolar wall/hyaline membrane thickness and inflammatory cell infiltration. The grading scale for the light microscopy pathologic findings was as follows: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%). The results were graded from 0 to 4 for each item, as described previously. The four variables were summed to represent the lung injury score (total score: 0–16). All histology grades were performed by the same pathologist.

**Determination of Inflammatory Cytokines in Lung Homogenate by Enzyme-Linked Immunosorbent Assay (ELISA)**

Part of the right lung from individual rats was homogenized and centrifuged, and the levels of TNF-α and IL-1β in the resulting tissue supernatants were determined using TNF-α and IL-1β ELISA kits.

After ultrasonic lysis of lung homogenate, the supernatant was obtained by centrifugation at 4 °C for 5000 r/min for 15 min. Follow the reagent instructions. A total of 100 μL of standard or sample to be tested was added to each hole, and the reaction plate was fully mixed and placed at 37 °C for 30 min. Wash the reaction plate fully with washing solution 4 times and print it on the filter paper 6 times. And 100 μL of enzyme-labeled antibody working solution was added to each hole. Put the reaction plate at 37 °C for 30 min. The washing process is the same as before. Then, 100 μL of substrate working solution was added to each hole and reacted in the dark at 37 °C for 15 min. Add 100 μL terminating liquid to each hole and mix the reaction plate fully. The absorbance value of 450 nm was measured by an enzyme-labeling instrument within 30 min.

**Separation and of Rat Neutrophils**

Twenty milliliters of fresh rat blood was treated with dextran to induce sedimentation of the red blood cells. Prepare a Percoll gradient in a 15-mL Falcon tube by first pipetting 5 mL 56% Percoll, then put the Pasteur pipette to the bottom of the tube, and slowly pipette 2.5 mL 80% Percoll to the bottom. Then carefully draw up the plasma and white blood cell suspension from the blood sample with a pipette and slowly layer them on top of the Percoll gradient. 4 °C, 220 g, centrifuge for 20 min, accelerate to 1, decelerate to 0, remove the top layer of serum, aspirate the neutrophil layer, and add PBS to wash twice. Cells were resuspended with RPMI-1640 medium containing 5% FBS and then counted so that the cell concentration was $1 \times 10^6$/mL.

Neutrophils were divided into four groups: control group, LPS group (100 ng/mL), LXA4 (100 nM), and LPS + LXA4 group.
Cell Counting Kit-8 (CCK8 Assay)

We isolated neutrophils from rat peripheral blood and adjusted the cell concentration to 1×10^6/mL. Neutrophils were added to a 96-well plate (100 μL per hole). Three multiple holes and blank control holes were set up at the same time (no cells). LXA4 was added into test holes as a stimulant. PBS was used as a negative control. After 4 h of LXA4 intervention, 10 μL of CCK-8 reagent was added into each hole. The plate was cultured in a 5% CO₂ incubator at 37°C for 4 h, and the OD value of each hole of wavelength of 450 nm was detected by enzyme labeling instrument. Cell inhibition rate (IC) can be calculated according to the formula:

\[
\text{Cell inhibition rate (IC)} = \frac{[(\text{control group OD value} - \text{experimental group OD value}) - \text{zeroing group OD value}] \times 100}{\text{control group OD value}}
\]

Respiratory Burst Detection

The reactive oxygen species released by the activated inflammatory cells can reduce the membrane non-penetrating cytochrome C. The reduced cytochrome C has an absorption peak at 550 nm. Therefore, the amount of reduced cytochrome C is measured using a spectrophotometer. The amount of active oxygen produced can be inferred from this data. ① Set control group, LPS group, and LXA4 groups, and the appropriate amount of LXA4 was added in each group; ② 10 μL SOD (5000 U/mL) was added, and the corresponding dose was added to the test group, equilibrated in a 5% CO₂ incubator at 37°C for 10 min; ③ 10 μL cytochalasin B (1 mmol/L) was added to each group and after 3 min, 10 μL fMLP (0.1 mmol/L) was added for a total of 1 mL and each group was incubated in a 5% CO₂ incubator at 37°C for 30 min; ④ each group was removed and centrifuged at 2000r/min for 10 min; ⑤ supernatant was collected and the OD value was measured with a spectrophotometer. Since the production of O₂^- and the decrease in cytochrome C are in a 1:1 molar stoichiometric relationship, the yield of O₂^- is easily calculated. The millimolar extinction coefficient of the 1 cm optical path is 21.1, and the amount of O₂^- produced by 2×10^6/mL of cells in 1 mL of the solution with a diameter of 1 cm can be directly calculated according to the formula:

\[
\text{OD} \times 47.4 = \text{nmol O}_2^-/2 \times 106\text{cells/time unit test group O}_2^-\text{inhibition rate} = (\text{control O}_2^-\text{content} - \text{test group O}_2^-\text{content})/\text{control group O}_2^-\text{content} \times 100\%
\]

Measuring ROS Production by Isolated Neutrophils

Following isolation, cells were resuspended at 1×10^6/mL in HBSS (with Ca²⁺ and Mg²⁺) (4.5 mL total) in 15 mL Falcon. A total of 100 μL of neutrophils was added to each hole of a 96-well plate. Cells were stimulated with IL-8, fMLP, and PMA. The concentration of IL-8, fMLP, and PMA was shown in the following table for 1 h. The luminometer was set up and the ROS level was tested on the instrument.

| Dilution | Volumes | Concentrations |
|----------|---------|----------------|
|          | 1 mL    | 30 mM          |
|          | into 9 mL | 3 mM          |
|          | pH to 7.3 | 0.5 mM       |
| Luminol  | 1:10    |                |
| IL8      | 1:625   | 6.25 μM         |
|          | 1 μL into 624 μL | 10 nM         |
|          | 6.25 μM | 1.25 nM        |
| fMLP     | 1:500   | 10 nM          |
|          | 12 μL into 5922 μL | 20 μM        |
|          | 10 nM | 2.5 μM         |
| PMA      | A:800   | 1620 μM         |
|          | 1 μL into 799 μL | 200 nM        |
|          | 1620 μM | 25 nM          |
|          | B:10    | (1 mg/mL)       |
|          | 55 μL into 495 μL |                |

Measuring Neutrophil NETs Production

Clear 96-well flat-bottomed plates were prepared, and 100 μL of neutrophils was added to the relevant holes. Lipopolysaccharide (LPS, 100 ng/mL), interleukin-8 (IL-8, 100 ng/mL), phorbol ester (PMA, 1.5 ng/mL), and N-formylthionyl-leucyl-phenylalanine (fMLP, 1000 ng/mL) was used to treat the cells respectively. The control group was treated with an equal volume of medium. They were incubated for 3 h at 37°C in a 5% CO₂ incubator. SYTOX Green was diluted 1:500 (5 mM Stock; 1ul SYTOX Green into 499 μL PBS) and then stored in the dark. Twenty
microliters of diluted SYTOX green was added to each well using a fresh tip for each hole. One microliter of MNase was added to each well using a fresh tip for each well. They were then incubated at room temperature for 10 min in the dark. Samples were transferred to 0.5 mL micro-centrifuge tubes without any pipetting of the liquid up and down. They were immediately centrifuged at 5000 rpm for 10 min in the micro-centrifuge before 160 µL of the supernatant was removed and transferred to a black 96-well flat-bottomed plate. Fluorescence was measured immediately (Programme: Gen5; excitation 485 nm, emission 528 nm with optics position in top 50% of well with a 10-s “medium” shake immediately prior to read).

**Protein Extraction and Western Blot Analysis**

Proteins were extracted using a protein extract kit (Thermo Scientific) according to the manufacturer’s instruction. Whole protein samples were prepared by suspending cells directly in RIPA lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mg/mL leupeptin sodium orthovanadate, sodium fluoride, and EDTA) and 1 mM PMSF for 20 min on ice. This was followed by centrifugation for 20 min at 12,000 g.

Protein lysates were electrophoresed via 10% SDS-PAGE, and the separated proteins were transferred to a polyvinyl difluoride membrane. After the membranes were blocked in 5% dry skim milk/TBST buffer (TBS containing 0.1% Tween-20) for 2 h at room temperature, they were incubated with primary Abs for NE, MPO, and b-actin overnight at 4 °C. This was followed by treatment with the appropriate secondary Abs for 1 h. The proteins were detected using chemiluminescence reagents (Thermo Scientific). Images were scanned with a UVP imaging system and analyzed using an Image Quant LAS 4000 mini system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

**Measuring the Phagocytosis of Neutrophils**

Neutrophils were isolated from control group and ARDS group and adjusted concentration to 1 × 10⁶/mL. Following LPS and LXA4 treatment, neutrophils were inoculated into 96-well plates at 100 µL/hole. pHrodo Red E. coli (Sartorius, Germany) and pHrodo Green S. aureus (Sartorius, Germany) were added to neutrophils respectively to stimulate neutrophils for 30 min, 45 min, and 60 min (Mark as shown in the figure below). Except for “negative Sa” and “negative EC” holes, add 100 μL neutrophils to each hole. Add 50 μL phrodo E. coli or S. aureus to the corresponding holes in reverse order of time. Neutrophils were incubated at 37 °C in a 5% CO₂ incubator in the dark and then centrifuged at 250 g and 4 °C for 5 min to remove the supernatant. The cells were resuspended with 100 µL of 2% PBS/BSA, and this was repeated twice before the cell suspension from each well was transferred into flow tubes. A total of 100 µL of 2% PBS/BSA was added to each tube, gently mixed, and placed on ice. Finally, the phagocytosis of neutrophils was measured using flow cytometry.
Measuring the Rate of Apoptotic Neutrophils

Neutrophils were isolated and inoculated into six well plates at an adjusted concentration of $1 \times 10^6$/mL. Neutrophils were divided into groups and treated for 4 h and 24 h. Cells were harvested as normal and cells were transferred to the appropriate FACS tubes. They were centrifuged at 600 g for 4 min before the supernatant was poured off. Cells were resuspended in 200 µL Annexin V buffer to wash the cells and then pelleted again. The cells were incubated in 100 µL Annexin V-FITC diluted 1:100 in Annexin V buffer for 15–20 min on ice and protected from the light. A total of 200 µL Annexin V buffer was added to each tube. SYTOX was removed from the freezer and defrosted while being protected from the light. A SYTOX stock diluted 1:500 in Annexin V buffer was prepared. Immediately prior to running the sample on the CyAN, 30 µL of the SYTOX solution was added to each tube and they were vortexed well to mix. The FITC and Violet 1 channels on the FACS machine were used to measure.

Statistical Analysis

The data represents the mean ± SEM. There was no missing, lost, or excluded data. Based on previous experience, no prior power analysis was conducted; all data was analyzed by one-way ANOVA followed by Tukey’s post-hoc test for multiple comparisons. All tests were two-sided, and the significance was determined at the $P < 0.05$ level. Statistical analyses were performed using Prism 6.0 software (GraphPad Software, San Diego, CA).

RESULTS

LXA4 Protected Lung Tissues from LPS-Induced ARDS Rats

Tail vein injection of 14 mg/kg LPS induced an increase in neutrophils in a time-dependent manner, rising to a peak at 20 h, then declined to nearly normal at 72 h (Fig. 1B). We evaluated the impact of LXA4 on LPS-induced ARDS rats. Control group showed normal pulmonary histology (Fig. 1C). In contrast, lung tissues in LPS group were significantly damaged, with interstitial edema, hemorrhaging, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitium and alveolar spaces, as evidenced by an increase in lung injury score ($P < 0.001$). Compared with control group, all the morphologic changes observed were less pronounced in LPS + LXA4 group ($P < 0.01$). LXA4 significantly attenuated LPS-induced pathologic changes as shown by the decrease in lung injury score ($P < 0.001$) (Fig. 1D).

As expected, the concentrations of TNF-α and IL-1β in the lung tissue homogenate were significantly higher in LPS group than in control group ($P < 0.001$). But after the administration of LXA4, the concentrations of TNF-α and IL-1β in the lung tissue homogenate in LPS+LXA4 group were significantly reduced, much lower than those in LPS group ($P < 0.001$) (Fig. 1E, F).

LXA4 Has No Protective Effect on ARDS Rats of Neutropenia

We established an ARDS rat model of neutropenia by intraperitoneal injection of cyclophosphamide and intravenous injection of LPS (Fig. 2A). On the second day after receiving cyclophosphamide immunosuppression, the rats displayed poor mental state, reduced diet and activity, and gray and yellow hair, and the above performance was worsened after the injection of LPS.

From the H&E staining samples of rat lung tissue, we can see that the lung histology of the control group is normal (Fig. 2B). In the LPS group, the lung tissue was still damaged, but the degree of damage was not obvious. We can see slight edema and hemorrhage in the interstitium, insignificant thickening of the alveolar wall, and a small amount of inflammatory cells infiltrating the interstitial and alveolar spaces. Compared with the control group, the lung injury score of the LPS group still increased ($P < 0.05$) (Fig. 2C).

Similarly, we administered exogenous LXA4 to the LPS group, and found that LXA4 did not ease the lung tissue damage in the LPS group nor did it reduce the lung injury score ($P > 0.05$) (Fig. 2C). And LXA4 cannot reduce the levels of TNF-α and IL-1β in lung tissue homogenate ($P > 0.05$) (Fig. 2D, E).

100 nM LXA4 Has the Best Effect on Neutrophils in Peripheral Blood of Rats

Neutrophils were isolated from peripheral blood of healthy control rats. We examined the effect of different concentrations of LXA4 (10 nM, 50 nM, 100 nM,
200 nM, and 400 nM) on neutrophil cytotoxicity. As shown in Fig. 3A, the LXA4 of 200 nM and 400 nM had certain cytotoxic effects on neutrophils, while the lower concentration of LXA4 had less cytotoxic effect on neutrophils. Hence, we select the LXA4 of 10 nM, 50 nM, and 100 nM for further study.

The LXA4 of 10 nM, 50 nM, and 100 nM was used to act on neutrophils, and the reduction of cytochrome C was detected by spectrophotometer to infer the production of $O_2^-$. The results are shown in Fig. 3B: LXA4 inhibits the production of $O_2^-$ in a concentration-dependent manner. Figure 3C shows that the concentration of ROS in the LPS group was significantly higher than that in the control group ($P < 0.05$). Compared with the LPS group, both 50 nM LXA4 and 100 nM LXA4 could reduce the production of ROS, and the effect of 100 nM LXA4 was stronger ($P < 0.01$), while 10 nM LXA4 could not effectively reduce the production of ROS.

Thus, we picked 100 nM as the best dose of LXA4 to stimulate peripheral blood neutrophils.
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Fig. 2  LXA4 has no protective effect on ARDS rats of neutropenia. A The time line of giving CTX, LPS and LXA4 to SD rats. B The lung tissues were obtained immediately after exsanguination (6 h after LPS), and the effect of LXA4 was evaluated histologically in H&E-stained sections (original magnification × 200). C Lung injury scores were recorded from 0 to 16 according to the criteria described in Materials and Methods. D The lung tissue homogenate TNF-α protein expression. E The lung tissue homogenate IL-1β protein expression. (The data is presented as the mean ± SEM. n = 6. *P < 0.05.).

Fig. 3  100 nM LXA4 has the best effect on neutrophils in peripheral blood of rats. A CCK8 assay. The impact of different concentrations of LXA4 on neutrophil Cytotoxicity in rats was examined. B Effects of different concentrations of LXA4 on respiratory burst of neutrophils. C Effects of different concentrations of LXA4 on ROS of neutrophils. (The data is presented as the mean ± SEM. n = 10. *P < 0.05, **P < 0.01, and ***P < 0.001.).
LXA4 Inhibits the Production of Reactive Oxygen Species (ROS) of Neutrophils During Inflammatory Resolution in ARDS Rats

The expression of ROS in neutrophils was measured by luminometer. According to the literature, we found that there are four groups of agents that can induce ROS production. The first group of priming agents is composed of physiological inflammatory agents, such as C5a, or formylated peptides/proteins such as fMLP. The second group of priming agents is composed of proinflammatory cytokines and adipokines, such as tumor necrosis factor (TNF-α), IL-8. The third group of priming agents is composed of TLR agonists, such as lipopolysaccharide (LPS or endotoxin). The four group of priming agents is Phorbol ester (PMA). Therefore, we chose three agents IL-8, fMLP, and PMA to stimulate neutrophils to produce ROS.

Neutrophils were isolated from peripheral blood of ARDS rats during inflammatory resolution. The expression of reactive oxygen species (ROS) in neutrophils was detected after neutrophils were treated with IL-8, fMLP, and PMA, respectively. The results showed that IL-8, fMLP, and PMA could induce the production of reactive oxygen species (ROS) in peripheral blood neutrophils of ARDS rats ($P < 0.05$), and the production of ROS in peripheral blood neutrophils after PMA stimulation was significantly higher than that in IL-8 group and fMLP group ($P < 0.001$) (Fig. 4A). Therefore, in the following experiments, PMA was used as an inducer to stimulate neutrophils to produce ROS.

Neutrophils were isolated from peripheral blood of healthy control rats and ARDS rats, and ROS content of neutrophils was detected after PMA intervention. The results suggest that the content of ROS in the ARDS group was higher than that in the control group without PMA stimulation ($P < 0.001$). After stimulation with PMA, the production of ROS increased significantly in both the control group and the ARDS group ($P < 0.001$). However, after PMA stimulation, the production of ROS in the ARDS group was still higher than that in the control group, and the trend was consistent with the absence of PMA stimulation ($P < 0.001$) (Fig. 4B).

![Fig. 4](image-url) Impact of LXA4 on ROS production and NETs production of neutrophils in ARDS rats during inflammatory resolution. A Comparison of the ROS level produced by neutrophils after stimulation with IL-8, fMLP, and PMA. B Comparison of ROS production of neutrophils between Control rats and ARDS rats. C LXA4 inhibits the production of ROS in neutrophils of ARDS rats by binding to ALX receptor. D Comparison of NETs production of neutrophils after stimulation with various stimulants. E Comparison of NETs production of neutrophils between Control rats and ARDS rats. F LXA4 reduces the production of NETs in neutrophils of ARDS rats by binding to ALX receptor. (The data is presented as the mean±SEM. $n=10$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.)
Then, neutrophils were isolated from peripheral blood of ARDS rats during inflammatory resolution and neutrophils were divided into four groups: (1) control group, (2) LXA4 group, (3) BOC-2 group (LXA4 receptor antagonist), and (4) LXA4 + BOC2 group. The production of reactive oxygen species (ROS) was detected after PMA stimulation. The results showed that compared with the control group, the production of ROS by neutrophils severely downregulated after the treatment of LXA4 ($P < 0.01$). After adding BOC-2, the production of ROS by neutrophils in the LXA4 + BOC-2 group was higher than that in the LXA4 group ($P < 0.01$) (Fig. 4C). That is to say, the inhibitory effect of LXA4 on ROS production was significantly attenuated by BOC-2.

**LXA4 Reduces NETs Production of Neutrophils During Inflammatory Resolution in ARDS Rats**

The literature also confirmed that four groups of reagents that induce ROS production can also stimulate the production of NETs, so we also choose four agents LPS, IL-8, fMLP, and PMA to induce the production of NETs. Neutrophils were isolated from the peripheral blood of ARDS rats during inflammatory resolution. Neutrophils were stimulated with IL-8 (100 ng/mL), LPS (100 ng/mL), fMLP (1000 ng/mL), and PMA (1.5 ng/mL) for 3 h, and NETs were detected. The results showed that IL-8, fMLP, and PMA could induce the production of NETs in peripheral blood neutrophils of ARDS rats ($P < 0.05$ and $P < 0.001$), and the production of NETs in peripheral blood neutrophils after PMA stimulation was significantly higher than that in IL-8 group, LPS group, and fMLP group ($P < 0.001$), indicating that PMA has the best effect on NET production in peripheral blood neutrophils (Fig. 4D). As a result, PMA was used as an inducer.

Then, neutrophils were isolated from peripheral blood of healthy control rats and ARDS rats during inflammatory resolution, and NETs production of neutrophils was detected after PMA intervention. Our study found that without PMA stimulation, the content of NETs produced by neutrophils in ARDS rats was higher than that in control rats ($P < 0.05$). After stimulation with PMA, not only the production of NETs in the control group was significantly increased, but also the production of NETs in the ARDS group ($P < 0.001$). However, as same as without PMA stimulation, the production of NETs in the ARDS group was significantly higher than that in the control group after PMA stimulation ($P < 0.01$) (Fig. 4E). Finally, neutrophils were isolated from peripheral blood of ARDS rats during inflammatory resolution and divided into four groups: (1) control group; (2) LXA4 group; (3) BOC-2 group (LXA4 receptor antagonist); and (4) LXA4 + BOC2 group. Neutrophils were stimulated with PMA (1.5 ng/mL) for 3 h, and NETs were detected. The results showed that the production of NETs in the LXA4 group was significantly lower than that in the control group ($P < 0.01$). After adding BOC-2, the inhibitory effect of LXA4 on the increase of NETs production was significantly attenuated by the BOC-2 group. The production of ROS in the LXA4 + BOC-2 group was higher than that in the LXA4 group ($P < 0.05$) (Fig. 4F).

**LXA4 Reduces the Release of Neutrophil Elastase (NE) and Myeloperoxidase (MPO) Production of Neutrophils**

Neutrophils were isolated from peripheral blood of healthy control rats and divided into four groups: (1) control group, (2) LPS group, (3) LPS + LXA4 group, and (4) LPS + NEi group. As shown in Fig. 5A and B, the amount of NE released by neutrophils in the LPS group was significantly higher than that in the control group ($P < 0.01$). The addition of LXA4 can decrease the release of neutrophil NE. The production of NE in the LPS + LXA4 group was lower than that in the LPS group ($P < 0.01$). The effect is similar to NE inhibitor (NEi), but the effect is not as strong as NEi.

Similar to the result in Fig. 5A and B, the result in Fig. 5C and D shows that the amount of myeloperoxidase (MPO) released by neutrophils in the LPS group was significantly higher than that in the control group ($P < 0.01$). After adding exogenous LXA4, the amount of MPO released by neutrophils decreased. The amount of MPO in the LPS + LXA4 group was lower than that in the LPS group ($P < 0.05$). MPO inhibitors (MPO inhibitor I) also have the same effect of inhibiting MPO release but the effect is stronger than LXA4 ($P < 0.05$).

**LXA4 Reduces the Production of NETs by Inhibiting the Release of Neutrophils NE and MPO**

Figure 5E–F demonstrates that LPS can stimulate the release of NETs. After adding NEi and MPO inhibitor I, the production of NETs in LPS + NEi group and
LPS + MPO inhibitor I group decreased significantly compared with LPS group, indicating that both NEi and MPO inhibitor I can significantly inhibit the production of NETs \((P < 0.001)\). Interestingly, exogenous LXA4 could also inhibit the production of NETs, resulting in a decrease in the production of NETs in LPS + LXA4 group compared with LPS group \((P < 0.05)\).

**LXA4 Promotes Phagocytosis of Neutrophils During Inflammatory Resolution in ARDS Rats**

Neutrophils were isolated from peripheral blood of control rats and ARDS rats during inflammatory resolution. PE-labeled *E. coli* (EC) and FITC-labeled *Staphylococcus aureus* (SA) were co-cultured with neutrophils for 30 min, 45 min, and 60 min. The phagocytosis of neutrophils was detected by flow cytometry. The results showed that in healthy control rats, the average fluorescence intensity of SA phagocytized by neutrophils increased from 2749.449 \(\pm\) 469.95 in 30 min to 12,305.01 \(\pm\) 1425.02 in 60 min. The average fluorescence intensity of EC phagocytized by neutrophils increased from 4159.299 \(\pm\) 357.72 in 30 min to 7340.257 \(\pm\) 597.80 in 60 min. In ARDS rats, the average fluorescence intensity of SA phagocytized by neutrophils increased from 4979.964 \(\pm\) 4336 in 30 min to 24,116.71 \(\pm\) 1377.896 in 60 min. The average fluorescence intensity of EC phagocytized by neutrophils increased from 6392.38 \(\pm\) 910.25 in 30 min to 12,618.055 \(\pm\) 1303.405 in 60 min. It is suggested that the phagocytosis of neutrophils to SA and EC increases with time and reaches the peak at 60 min. The phagocytosis of neutrophils to SA in ARDS rats was higher than that of control rats at 30 min, 45 min, and 60 min \((P < 0.05)\), while the phagocytosis of neutrophils to EC in ARDS rats was higher than that of control rats only at 45 min and 60 min \((P < 0.05)\). There was no significant difference in phagocytosis between the two groups at 30 min (Fig. 6B, D).

Neutrophils were isolated from peripheral blood of ARDS rats during inflammatory resolution and divided into four groups: (1) control group; (2) LXA4 group; (3) BOC-2 group (LXA4 receptor antagonist); and (4) LXA4 + BOC-2 group; PE-labeled EC and FITC-labeled SA were co-cultured with neutrophils for 30 min, 45 min,
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and 60 min, and the phagocytosis was detected by flow cytometry. The results showed that the phagocytosis of SA and EC by neutrophils in the LXA4 group was significantly higher than that in the control group \((P < 0.05)\). The phagocytosis of neutrophils to SA and EC in the LXA4 + BOC-2 group was lower than the LXA4 group, indicating that BOC-2 could weaken the promoting effect of LXA4 on neutrophil phagocytosis \((P < 0.05)\) (Fig. 6F, H).

**LXA4 Promotes the Apoptosis of Neutrophils in ARDS Rats During Inflammatory Resolution**

Neutrophils were isolated from peripheral blood of healthy control rats and ARDS rats during inflammatory resolution. After LXA4 100 nM treatment for 4 h and 24 h, the apoptosis of neutrophils was detected by flow cytometry. The results showed that at 4 h, the proportion of living cells in the ARDS group was lower than that in the control group \((P < 0.001)\), and the proportion of apoptotic cells was higher than that in the control group \((P < 0.01)\). After adding LXA4, the proportion of living cells in the ARDS group was also lower than that in the control + LXA4 group \((P < 0.001)\), but the proportion of apoptotic cells was still higher than that in the control group \((P < 0.001)\) (Fig. 7A, C). At 24 h, the proportion of living cells in the ARDS group was higher than that in the control rat group \((P < 0.001)\), and the proportion of apoptotic cells was lower than that in the control group \((P < 0.001)\). After adding LXA4, the situation was reversed. The proportion of living cells in the ARDS + LXA4 group decreased, which was lower than that in the control + LXA4 group \((P < 0.001)\), but the proportion of apoptotic cells increased, which was higher than that in the control group \((P < 0.01)\) (Fig. 7B, D). After treatment with LXA4 100 nM for 24 h, neutrophils of ARDS rats were divided into four groups: (1) control group; (2) LXA4 group; (3) BOC-2 group; and (4) LXA4 + BOC-2 group; the apoptosis of neutrophils was detected by flow cytometry. The results showed that: compared with the control group, the proportion of apoptotic neutrophils in the LXA4 group was significantly increased \((P < 0.01)\), but BOC-2 could significantly inhibit the effect of LXA4 on promoting neutrophil apoptosis \((P < 0.05)\) (Fig. 7H). Compared with the control group, the proportion of dead cells in the LXA4 group was significantly declined \((P < 0.01)\), but BOC-2 could also inhibit the effect of LXA4 on reducing dead cells \((P < 0.01)\) (Fig. 7G). The results showed that LXA4 promoted the apoptosis of neutrophils in ARDS rats cultured for 24 h *in vitro*, and the effect of reducing dead cell induced by NETosis was mediated by ALX receptor.

**DISCUSSION**

ARDS is a rapid non-cardiogenic bilateral lung infiltration syndrome characterized by alveolar vascular injury and neutrophil infiltration and accompanied by the release of pro-inflammatory factors [19]. Neutrophils and macrophages play an important role in the process of lung injury, in which neutrophils are the first line of defense against the invasion of pathogens [20]. Macrophages play a key role in the subsequent clearance of apoptotic neutrophils and promoting the regression of inflammation. This research focuses on the impact of LXA4 on the function of neutrophils in ARDS, so as to explore the anti-inflammatory mechanism of LXA4.

Neutrophils are the largest number of white blood cells in human circulation, and they play an important role in the process of immune defense against pathogenic microorganisms [21]. As effector cells of the innate immune system, neutrophils participate in a variety of immune and inflammatory processes and play an important role in coordinating the overall immune and inflammatory response. In the last 5 years, there have been numerous reports on the anti-inflammatory effects of LXA4 [22, 23]. As the first kind of regression medium, LXA4 has a strong anti-inflammatory and regression effect [7]. Recently, some studies have demonstrated that LXA4 and synthetic analogues can protect tissues from acute and chronic inflammation. Its mechanism includes downregulation of proinflammatory cytokines and chemokines (such as IL-1β and TNF-α), inhibition of the activation of major pro-inflammatory pathways, and increasing the release of proinflammatory cytokines.
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A

4H

Untreated Control

LXA4 Control

Untreated ARDS

LXA4 ARDS

24H

Untreated Control

LXA4 Control

Untreated ARDS

LXA4 ARDS

B

C

D

E

F

G

24H apoptosis cells

24H Dead cells

ARDS
Neutrophils are over-activated during ARDS, which releases neutrophil respiratory burst and ROS to damage the surrounding tissues. In this study, we measured the level of ROS released by neutrophils during the resolution of ARDS inflammation and observed the effect of LXA4 on ROS level released by neutrophils. Our experimental results support this conclusion. The results showed that IL-8, fMLP, and PMA could stimulate the production of reactive oxygen species (ROS) in neutrophils, and PMA was the best. In the absence of PMA stimulation, the ROS production of neutrophils in ARDS rats is higher than that of normal control rats, because after LPS induction, a large number of neutrophils will migrate to the inflammatory site under the attraction of chemokines and eliminate the infection by releasing ROS, NE, and proteolytic enzyme and other substances. Therefore, the ROS production of neutrophils in ARDS rats was higher than that in normal control rats. After PMA stimulation, it can not only stimulate the production of ROS in neutrophils of ARDS rats but also stimulate the production of ROS in neutrophils of normal control rats. Moreover, the production of ROS in ARDS rats after PMA stimulation is still higher than that in normal control rats. The trend is consistent with that without PMA stimulation. This shows that the method of PMA stimulating neutrophils can be used to compare the ROS production by neutrophils in different models. In the inflammatory resolution period of ARDS, excessive release of ROS by activated PMN will cause lung injury [24]. Therefore, we detected the effect of LXA4 on production of ROS in neutrophils of ARDS rats in the inflammatory resolution period of ARDS. The results showed that LXA4 could reduce the production of ROS in ARDS rats during inflammatory resolution. After adding LXA4 receptor antagonist BOC-2, the inhibitory effect of LXA4 on ROS was significantly weakened. This may be one of the mechanisms of the protective effect of LXA4 on ARDS rats: LXA4 weakens the damage of activated neutrophils to surrounding tissues and promotes the resolution of inflammation by reducing the production of ROS, and LXA4 plays a role by binding to its receptor-ALX. NETs are a kind of outer network structure, which is composed of complex DNA triple network structure. It contains major proteins, elastase (NE), myeloperoxidase (MPO), cathepsin G, and other antibacterial proteases. This is a special bactericidal mechanism of neutrophils [25]. Some studies have shown that in the acute stage of inflammation, NETs can capture microorganisms, limit the spread of cytotoxic antibacterial proteins, reduce the damage of host tissue, and play an important role in inflammation.
inhibiting the spread of inflammation [26]. But in the process of inflammation, NETs are a double-edged sword. A large number of studies have shown that a large number of extensive NETs can damage epithelial cells and endothelial cells, leading to the spread of inflammation [27].

In this research, we investigated the impact of LXA4 on the production of NETs by neutrophils of ARDS rats in the resolution period of ARDS inflammation. Firstly, we discovered that PMA had the best effect among the four stimulants (IL-8, LPS, fMLP, and PMA) that can provoke the production of neutrophil NETs. The phenomenon that PMA stimulates the production of NETs is consistent with that of ROS. In the absence of PMA stimulation, the production of NETs in ARDS rats is higher than that in control rats. PMA can not only stimulate the production of NETs in ARDS rats but also stimulate the production of NETs in control rats. After stimulation, the production of NETs in ARDS rats is still higher than that in control rats, and the trend is the same as that without PMA stimulation. LXA4 can decrease the production of NETs in ARDS rats. After adding LXA4 receptor antagonist BOC-2, the inhibitory effect of LXA4 on NETs was significantly diminished. Studies have verified that the mechanism of lung injury caused by excessive formation of NETs is as follows: (1) NETs induce lung injury by hydrolyzing protease and protease-induced inflammatory response; (2) NETs cause inflammatory reaction by activating innate immunity, resulting in lung injury; (3) NETs cause lung injury by disturbing the function of macrophages; (4) NETs activate coagulation pathway, inhibit anticoagulation and fibrinolysis function, and promote microthrombosis; (5) excessive deposition of NETs leads to small airway blockage and airflow restriction. Thus, which aspect does LXA4 reduce the production of NETs during the resolution of ARDS inflammation? In addition, we examined the impact of LXA4 on the production of NE and MPO of neutrophils in ARDS rats during the inflammatory resolution. The results showed that LXA4 can reduce the production of NE and MPO of neutrophils in ARDS rats. Combined with the confirmed research results that NE and MPO are involved in the generation of NETs, neutrophil chromatin densification requires neutrophil elastase (NE), NE-deficient mice unable to form NETs and show immunodeficiency [28–30]. The formation of NETs also requires myeloperoxidase (MPO) to act on histones in the transitional nucleus, and hypochlorite, a product produced by MPO, is necessary for the release of NETs, so patients without MPO activity cannot generate NETs [31, 32]. This shows that LXA4 can decline the production of NETs and alleviate lung injury by reducing the release of NE and MPO during the inflammatory resolution in ARDS rats.

During the inflammatory injury, neutrophils gather at the injury site and play a phagocytic role, which is an important mechanism to kill pathogenic microorganisms. So what about the phagocytosis of neutrophils during the inflammatory resolution in ARDS rats? Our study found that the phagocytic ability of neutrophils to S. aureus (SA) and E. coli (EC) increased with time, reaching the peak at 60 min. In 45 min and 60 min, the phagocytic ability of neutrophils in the ARDS group was higher than that in the healthy control group. LXA4 can promote the phagocytosis of SA and EC by neutrophils in ARDS rats, which is completed by the combination of LXA4 and ALX receptor. This shows that even in the inflammatory resolution period, the phagocytosis of activated neutrophils to bacteria is still higher than that of healthy control group, and LXA4 can further promote the phagocytosis of neutrophils to bacteria in ARDS rats, which is conducive to further clearing harmful substances from inflammatory sites. As we all know, the life span of neutrophils is very short, mainly because with the passage of time, neutrophils in the circulatory system will undergo apoptosis. The resolution of acute inflammation requires macrophages to phagocytize apoptotic neutrophils. Delayed apoptosis of activated neutrophils can lead to persistent acute pneumonia and eventually develop into ARDS. Our experimental results confirmed that the in vitro survival rate of normal neutrophils will gradually decrease with the extension of time during inflammatory resolution, and the in vitro survival rate of neutrophils will decrease to 15.6% after 24 h. Most neutrophils isolated from ARDS rats are activated neutrophils, and the 24-h survival rate is much higher than that of healthy control rats. LXA4 can promote the apoptosis of neutrophils, reduce cell death caused by necrosis, and contribute to the resolution of inflammation.

In summary, this research demonstrates that LXA4 alleviates lung injury and reduces the release of inflammatory cytokines in ARDS rats induced by LPS but has no protective effect on ARDS rats with neutrophil deficiency. In the inflammatory resolution period, LXA4 also downregulated neutrophil respiratory burst and the production of ROS and NETs in neutrophils of ARDS rats, thus reducing the damage of neutrophils to the surrounding tissues. LXA4 can also upregulate the ability of neutrophils to phagocytize bacteria, thereby enhancing the bactericidal ability of neutrophils. In addition, LXA4 can promote the apoptosis
of neutrophils and accelerate the resolution of inflammation. Our findings reveal a novel mechanism for LXA4 to attenuate the inflammatory reaction and show that LXA4 could be exploited therapeutically for ARDS.

CONCLUSION

LXA4 has a protective effect on LPS-induced ARDS rats. It can alleviate lung injury and reduce the release of inflammatory cytokines, by affecting the various functions of neutrophils.

Abbreviations LXA4, Lipoxin A4; ALI, Acute lung injury; ARDS, Acute respiratory distress syndrome; LPS, Lipopolysaccharide; TNF-α, Tumor necrosis factor-α; IL-1β, Interleukin-1β; NETs, Neutrophil extracellular traps; ROS, Reactive oxygen species; fMLP, Formyl methionyl leucyl phenylalanine; IL-8, Interleukin-8; PMA, Phorbol ester; Cytochrome C, Cell chromatography C; SOD, Superoxide dismutase; HEPES, Hydroxyethylpiperazine ethylsulfonic acid; FBS, Fetal bovine serum; ELISA, Enzyme-linked immunosorbent assay; CCK8, Cell counting Kit-8; CTX, Cyclophosphamide; MPO, Myeloperoxidase; RA, Rheumatoid arthritis

AUTHOR CONTRIBUTION

The authors contributed in the following manner: study concept and design: all authors; acquisition of data: Wenhao Pan; analysis and interpretation of data: Qichao Xu; and drafting of the manuscript and approval of the final version: Hongxia Mei.

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AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article and are available from the corresponding author upon request.

DECLARATIONS

Ethics Approval All animals received care in compliance with the Principles of Laboratory Animal Care and National standards.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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