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Sodium butyrate relieves lung ischemia-reperfusion injury by inhibiting NF-κB and JAK2/STAT3 signaling pathways

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Abstract. – **OBJECTIVE:** Ischemia-reperfusion (IR) is the main cause of acute lung injury (ALI) in clinical lung transplantation, extracorporeal circulation, lung sleeve resection, trauma and cardiopulmonary resuscitation. The inflammatory response and oxidative stress following IR are factors that cause and aggravate its secondary damage. The purpose of this study was to investigate the efficacy and mechanism of sodium butyrate (NaB) on lung ischemia-reperfusion injury (LIRI).

MATERIALS AND METHODS: We used male C57BL/6 mice to construct the LIRI model and administered the mice with NaB. By examining the expression of inflammatory factors and oxidative stress-related molecules in mouse lung tissue, we investigated the effects of NaB on inflammation and oxidative stress in lung tissue after IR. In addition, the changes in the activity of the NF-κB and JAK2/STAT3 signaling pathways were also examined to determine the mechanism of NaB.

RESULTS: The expression levels of the inflammatory factors (IL-1β, IL-6 and TNF-α) in lung tissue of mice after IR were significantly increased, while NaB reduced the expression of inflammatory factors. In addition, the oxidative stress level of mouse lung tissue after IR increased significantly, showing the decrease of antioxidant molecules SOD1/2, catalase (CAT), and Peroxiredoxin 1 (Prdx1), while the intake of NaB increased the antioxidant level of mouse lung tissue. The activities of NF-κB and JAK2/STAT3 signaling pathways were significantly increased in lung tissue after IR, whereas NaB inhibited the activity of NF-κB and JAK2/STAT3 signaling pathways.

CONCLUSIONS: NaB relieves LIRI by inhibiting NF-κB and JAK2/STAT3 signaling pathways to reduce inflammation and oxidative stress levels in lung tissue of mice after IR.

Key Words:
NaB, Lung ischemia-reperfusion injury, Inflammation, Oxidative stress, NF-κB, JAK2/STAT3.

Introduction

Ischemia reperfusion (IR) is the main cause of acute lung injury caused by clinical lung transplantation, extracorporeal circulation, sleeve resection of the lung, trauma and cardiopulmonary resuscitation[1]. Patients with acute lung injury have a mortality rate of 30-40%, even with intensive support therapy. Lung ischemia reperfusion injury (LIRI) is characterized by severe inflammatory cells infiltration, inflammatory response, lung epithelial and endothelial cell damage, and dysregulation of the blood-gas barrier function[2].

LIRI is a complex pathophysiological process. During lung ischemia, macrophages, endothelial cells and other immune cells produce reactive oxygen species (ROS) and activate calcitonin-dependent nitric oxide synthase (NOS), nicotinamide adenine dinucleotide phosphate, nuclear factor κB (NF-κB) and pro-inflammatory cytokines further upregulate cell surface adhesion molecules. These changes will directly or indirectly lead to physiological changes in the microvasculature, manifested by an increase in pulmonary vascular resistance and an increase in microvascular permeability[3]. After IR, pulmonary vascular resistance can increase up to 3 times as much as normal one due to pre-pulmonary capillary constriction[4]. Pulmonary vascular resistance and increased microvascular permeability together result in varying degrees of pulmonary edema in the ischemic and perfusion phases. After pulmonary edema, due to the imbalance of ventilation/blood flow, gas exchange disorder is caused, and the arterial oxygen partial pressure drop, airway pressure peak and alveolar-arterial oxygen partial pressure difference increase,

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which aggravates lung injury[5]. Therefore, reducing the damage caused by excessive inflammatory response in LIRI is the key to alleviating LIRI.

Sodium butyrate (NaB) is a metabolite of intestinal non-digestible sugars fermented by probiotics (*clostridium perfringens* in sodium caseinate, etc.). It belongs to histone deacetylase inhibitors (HDACi) and can inhibit the inflammatory response in the intestinal tract and promote the regeneration and repair of intestinal epithelial cells[6]. Kovanda et al[7] have shown that NaB has anti-inflammatory and anti-oxidative effects in lung diseases. Aguilar et al[8] found that NaB can increase the concentration of p50 in the cytosol, reduce the concentration of p65 in the cytosol, and inhibit its transfer to the nucleus, resulting in an increase in the amount of homodimer p50/p50, thereby inhibiting the activity of NF-κB pathway. In addition, NaB can also inhibit the activity of the NF-κB pathway by attenuating the LPS response mechanism and inhibiting the phosphorylation of IκB. Stempelj et al[9] have shown that NaB inhibits JAK2 activity and γ-interferon-induced phosphorylation of tyrosine and serine on STAT protein, thereby inhibiting the activity of the JAK/STAT pathway and ultimately reducing the synthesis of pro-inflammatory cytokines. The inhibition of the JAK/STAT pathway prevents the synthesis of inducible NOS and nitric oxide, thereby inhibiting the mucosal inflammatory response. However, there is still no relevant study on the role of NaB in LIRI. Therefore, we hope to provide help for clinical LIRI treatment by studying the effect and mechanism of NaB on LIRI.

Materials and Methods

Animals and Grouping

12-week-old male C57BL/6 mice were used in the study. All mice were purchased from Beijing Charles River Experimental Animal Co., Ltd. (Beijing, China) and housed in Specific Pathogen Free (SPF) class barrier facilities. The animal feeding environment is clean and regularly sterilized. Mice are free to consume clean food and drinking water. We divided mice into sham operation group, IR group, and treatment group. Mice in the sham group were dissociated from the left hilum, but the left hilum was not blocked. Mice in the IR and treatment groups were subjected to LIRI model surgery. The mice in the treatment group were given daily NaB (5 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) for 1 week before modeling. This study was approved by the Animal Ethics Committee of Jinzhou Medical University Animal Center.

Modeling of LIRI

The mice were anesthetized and supported by a small animal ventilator. After disinfecting the mouse's chest with 75% ethanol, we used scissors to cut the left chest of the mouse and expose the left lung. We injected mice with heparin 20 U/kg *via* tail vein injection. Vascular clamp was used to close the left lung hilum when the left lung was filled in mice. After blocking the blood flow for 45 minutes, we loosened the vascular clamp and restored the blood for 180 minutes. We, then, sacrificed the mice and took lung tissues of the mice.

Preparation of Bronchoalveolar Lavage Fluid (BALF)

At the end of the modeling, we used 0.5 mL normal saline to perfusion from the trachea of mice and collected the perfusion fluid. After repeating three times, we collected BALF from each group and examined the total number of cells in BALF. Then, we used the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) to detect the protein concentration in BALF.

Wet/dry Weight Ratio (W/D) of Lung Tissue

W/D reflects the inflammatory exudation and edema of lung tissue after LIRI. We took 1/3 of the left lung tissue and used a clean spot of saline to clear the lung tissue and use filter paper to dry the surface of the lung tissue. At this time, the weight of the lung tissue is wet weight. The lung tissue was then placed in an incubator at 70°C for 24 hours. The weight of the lung tissue after drying is dry weight. The ratio of wet weight to dry weight is W/D.

Detection Myeloperoxidase (MPO) and Malonaldehyde (MDA) Activity

We collect and grind lung tissue. Then, we used MPO activity kit (R&D Systems, Minneapolis, MN, USA) and MDA active kit (R&D Systems, Minneapolis, MN, USA) to detect the content of MPO and MDA in lung tissue according to the manufacturer's instructions.

Western Blot

We extracted proteins from mouse lung tissue using radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China). The BCA

method was used to detect protein concentration. 30 µg of total protein was added to the electrophoresis gel and subjected to gel electrophoresis. We transferred the protein to the polyvinylidene difluoride (PVDF) membrane by transmembrane (Millipore, Billerica, MA, USA). After blocking the non-specific antigen with 5% skim milk, we incubated the PVDF membrane at 4°C overnight with primary antibody dilution (Abcam, Cambridge, MA, USA). We, then, washed the PVDF membrane with Phosphate-Buffered Saline and Tween (PBST) and incubated the PVDF membrane with secondary antibody dilution (Abcam, Cambridge, MA, USA) for 2 hours at room temperature. Finally, we detected the protein bands by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

RNA Isolation and quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract RNA from mouse lung tissue. The lung tip of the left lung of the mouse was used to extract RNA. We took approximately 20 mg of the lung tip of the mouse into 1 mL of TRIzol. After dissolving lung tissue using a homogenizer, we extracted total RNA using the phenol-chloroform-ethanol method. SuperScript Mix (Invitrogen, Carlsbad, CA, USA) was used to reverse mRNA to complementary deoxyribose nucleic acid (cDNA) and SYBR Green Mix (Invitrogen, Carlsbad, CA, USA) was used to amplify cDNA. The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The relative expression level of RNA is represented by $2^{-\Delta\Delta CT}$. All primer sequences were shown in Table I.

Enzyme Linked Immunosorbent Assay (ELISA)

Lung tissue specimens were dissolved in physiological saline at 1:5 and homogenized on ice. The solution was then placed in a centrifuge for centrifugation and the supernatant was collected. We, then, used ELISA kits (R&D Systems, Minneapolis, MN, USA) to determine the concentration of IL-1β, IL-6, and TNF-α in the solution according to the instructions.

Hematoxylin and Eosin (HE) Staining

We collected mouse lung tissue and placed the lung tissue in 4% paraformaldehyde for 24 hours. After dehydrating the tissue with gradient alcohol, we placed the tissue in xylene and paraffin in turn and made paraffin blocks. Then, we used a slicer to make paraffin sections. Before HE staining (Boster, Wuhan, China), we performed tissue dewaxing and dehydration in sequence. Tissue sections were stained in hematoxylin stain for 5 minutes, then, we washed the sections with running water and differentiated with hydrochloric acid alcohol. Then, we used the erythrocyte dyeing solution to stain the cy-

Table I. RT-PCR primers sequences.

Name	Sense/Anti-sense	Sequence (5′-3′)
IL-1β	Sense	GTTGACGTACTACGTACGTGATC
	Anti-sense	AACGAGCATGCGGACTCTGCTAC
IL-6	Sense	ACGACGTTCTACGTACGTACGTCT
	Anti-sense	GCGTATCATGCGATCTCTGGTCA
TNF-α	Sense	ATCGTCGCAGCTTCAGCAGTCTGCA
	Anti-sense	ATCCTGCCGCATATCTCAGTAGCA
SOD1	Sense	GGCATCTATCAGTCCGACTGCATC
	Anti-sense	AGACGCTATCGATCGAGTCGCTAGT
SOD2	Sense	AGGCTCAATCTGACTATGCGCATCA
	Anti-sense	CTATGCAACGCACGTATGCGTACTG
CAT	Sense	AACCTTGGGCATGTTATCTCAGGA
	Anti-sense	GGATCTCTAGAGTCTATCGCGTATCG
Prdx1	Sense	ACTACTCATGCAGGCGATCTTACGC
	Anti-sense	CCGATCTGCTGAGTACGTCATGCAGT
p65	Sense	AAGCTCTCGGCCCGGTAGCGTA
	Anti-sense	CAGCCTTCGCACGATGCAGTCGTG
IKKα	Sense	GGATTTGTGTACCAACTTACGGAA
	Anti-sense	GTTGGCAAATCGAGAGCCAATGT
GAPDH	Sense	GGTTTCGACACCGTGTGGCATCGT
	Anti-sense	AGCGCCCTTTGGGCTATCGACTA

toplasm of the tissue. Finally, we used neutral gum seal to seal and record the staining results with a microscope.

Immunohistochemical (IHC) Staining

After the tissue sections were dewaxed and hydrated, we placed the tissue sections in citrate buffer and heated to 95°C for 10 minutes. After the buffer was naturally cooled, we added 3% hydrogen peroxide to the sectioned tissue and incubate for 30 minutes at room temperature. 10% goat serum was used to block the tissue for 1 hour. We configured the primary antibody dilution (Abcam, Cambridge, MA, USA) and added dropwise to the tissue for overnight incubation at 4°C. After washing the sections with PBS, we incubated the tissue for 1 hour at room temperature using the secondary antibody dilution in the immunohistochemistry kit (Genetech, Shanghai, China). We washed the sections with PBS and used the developer to detect the staining results.

ROS Level Detection

We collected mouse lung tissue and ground the lung tissue using 2% FBS-DSB. Then, we used a mesh to filter the impurities to get the resuspended cells. We added 1 μg/ml dichlorofluorescein diacetate (DCFH-DA; Genechem, Shanghai, China) working solution to 1 ml single cell suspension and incubated for 30 minutes in the dark. Then, we used a flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA) to measure the fluorescence intensity at 488 nm.

Statistical Analysis

All experiments were repeated 3 times. Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM Corp., Armonk, NY, USA) was used to study the analysis of the data. Study data is expressed as mean ± standard deviation. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ indicates that the difference was statistically significant.

Results

NaB Relieves LIRI and Reduces Pulmonary Edema

In order to prove the prevention and treatment of NaB on LIRI, we examined the morphological changes of lung tissue and pulmonary edema. The results of HE staining (Figure 1A) showed that the lung tissue after IR had evident edema and alveolar epithelial cells were disordered, while

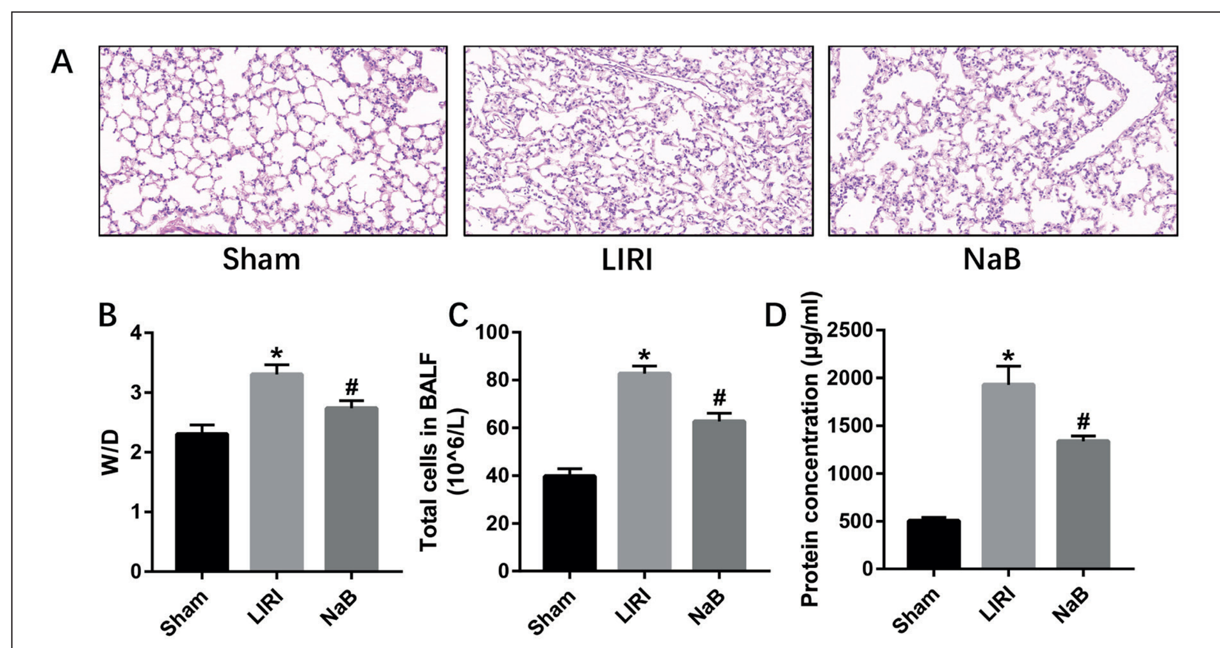

Figure 1. NaB relieves LIRI and reduces pulmonary edema. **A,** HE staining of mice lung tissue (200×). **B,** W/D of mice lung tissue. **C,** Total cells number in BALF. **D,** Protein concentration of BAFL. ("*" means $p<0.05$ *vs.* the Sham group and "#" means $p<0.05$ *vs.* the LIRI group).

the pulmonary edema of the lung tissue of the treatment group was significantly alleviated. The W/D (Figure 1B) of the treatment group was also significantly lower than that of the LIRI group. In addition, the mice in the LIRI group had a large number of cells in the BALF, while the number of cells in the BALF of the mice in the treated group was significantly reduced (Figure 1C). The results of the protein concentration (Figure 1D) in BALF detected by the BCA method showed that a large amount of protein was exuded in the lung tissue of the mouse after IR, indicating an increase in pulmonary capillary permeability. The protein concentration in BALF was significantly reduced after treatment of mice with NaB.

NaB Reduces the Inflammation Level in Lung Tissue After IR

IHC staining (Figure 2A) examined the expression of IL-1β and TNF-α in lung tissue and showed that after IR, the expression of IL-1β and TNF-α in lung tissue was significantly increased while NaB reduced their expression. The results of ELISA (Figure 2B-2D) and RT-PCR (Figure

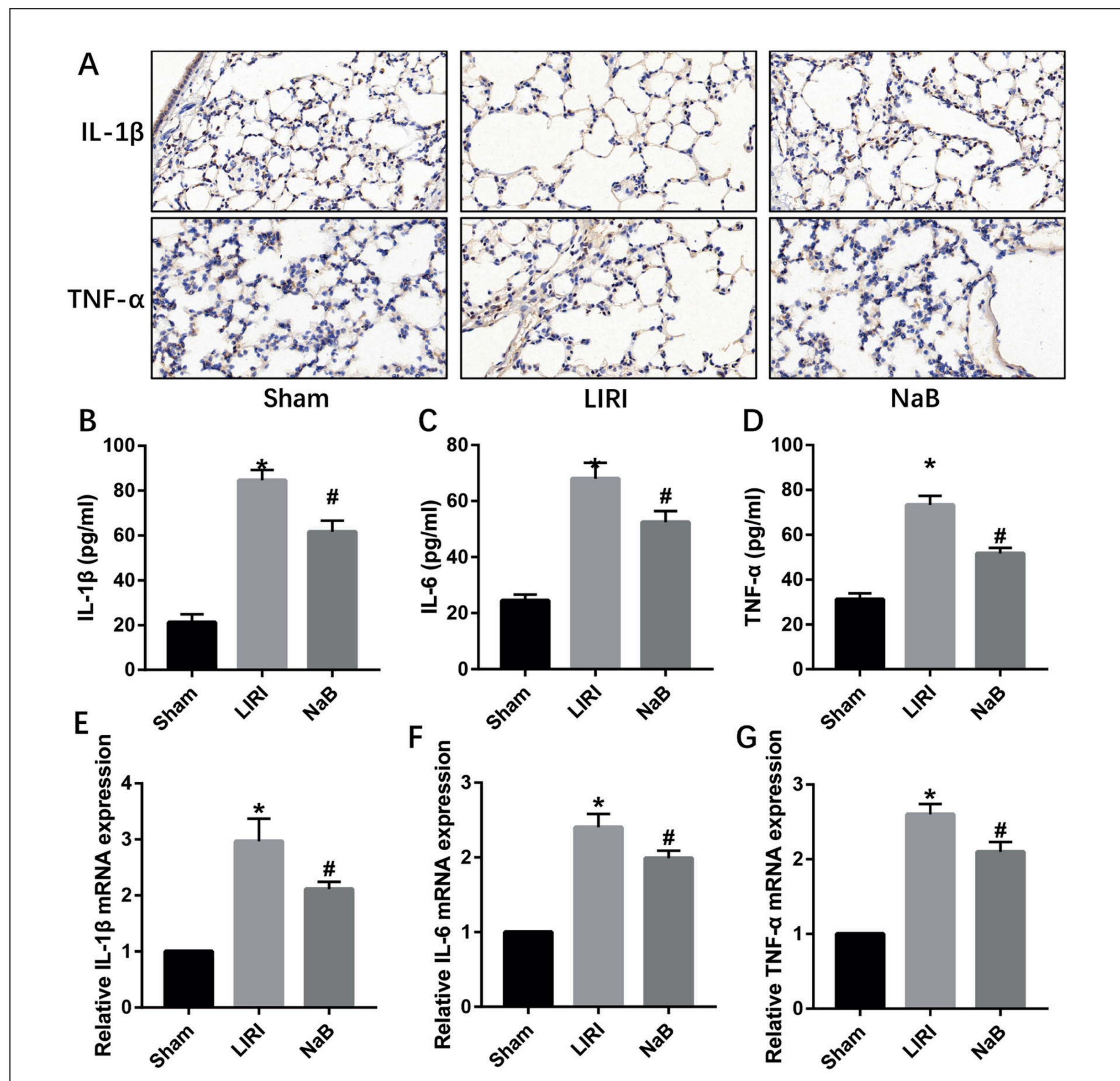

Figure 2. NaB reduces the inflammation level in lung tissue after IR. **A,** IHC staining of IL-1β and TNF-α in mice lung tissue (400×). **B-D,** ELISA of IL-1β, IL-6 and TNF-α. **E-G,** RT-PCR of IL-1β, IL-6 and TNF-α. ("*" means $p<0.05$ *vs.* the Sham group and "#" means $p<0.05$ *vs.* the LIRI group).

2E-2G) also showed that NaB can reduce the expression of IL-1β, IL-6 and TNF-α.

NaB Reduces Oxidative Stress Levels in Lung Tissue After IR

To determine the effect of NaB on oxidative stress in lung tissue, we examined the expression of SOD1 and SOD2 in lung tissue by IHC staining (Figure 3A). The results showed that after IR, the expression of SOD1 and SOD2 in lung tissue decreased and NaB increased their expression. In addition, the results of RT-PCR (Figure 3B-3E) also showed that NaB exerted significant antioxidant effects and increased the expression of SOD1/2, catalase (CAT) and Peroxiredoxin 1 (Prdx1). Flow cytometry (Figure 3F) detected changes of ROS levels in lung tissue and showed that NaB promoted clearance of ROS in lung tissue. In addition, we also examined the levels of MPO (Figure 3G) and MDA (Figure 3H) in mouse lung tissue and the results indicated that NaB can reduce the expression of MPO and MDA.

NaB Inhibits NF-κB and JAK2/STAT3 Signaling Pathways in Mouse Lung Tissues

IHC staining (Figure 4A) detected the expression of p65 and IKKβ in mouse lung tissue and resulted in a significant increase in NF-κB activity in lung tissue of LIRI but NaB reduced the expression of p65 and IKKα. The results of RT-PCR

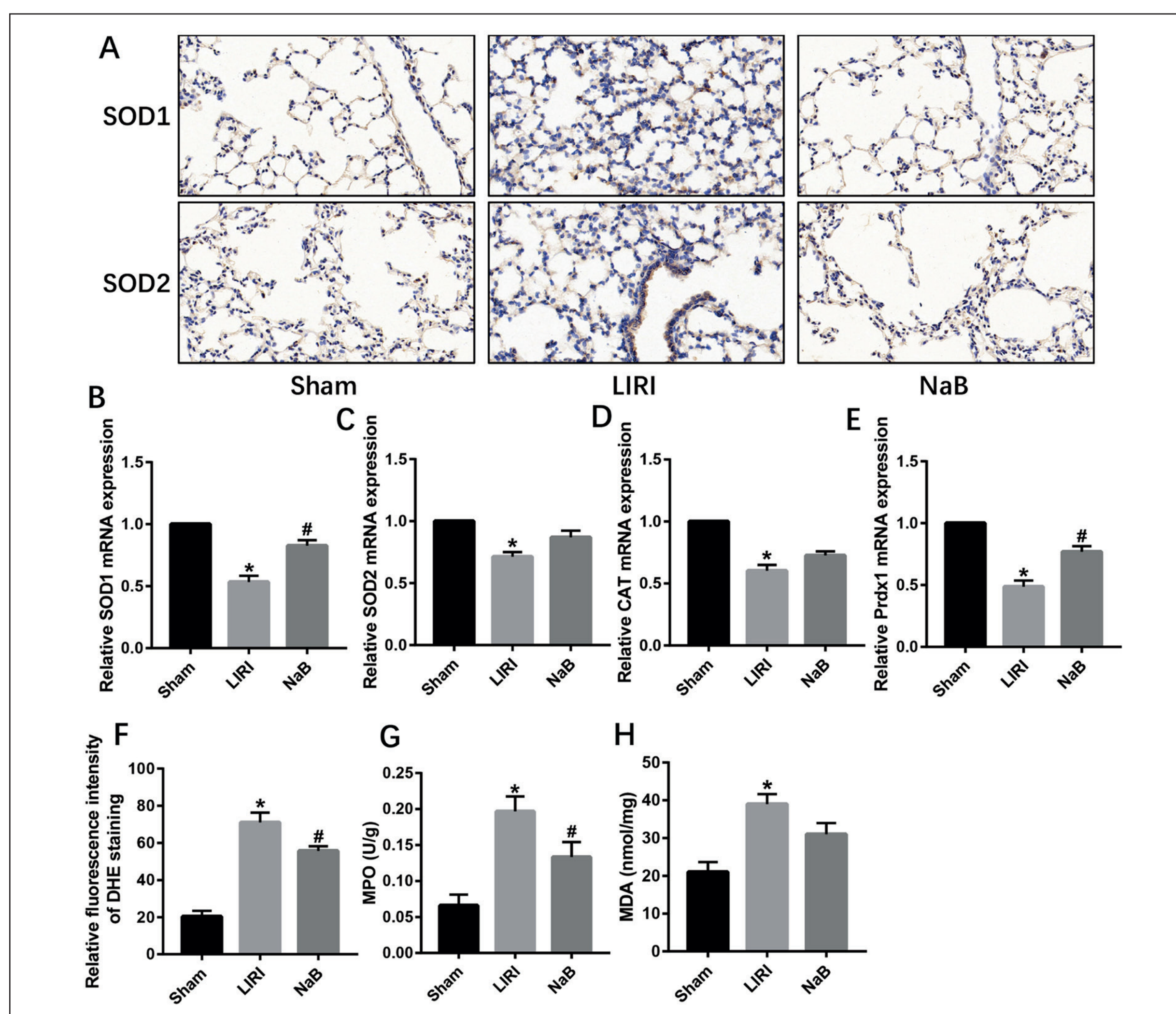

Figure 3. NaB reduces oxidative stress levels in lung tissue after IR. **A,** IHC staining of SOD1 and SOD2 in mice lung tissue (400×). **B-E,** RT-PCR of SOD1, SOD2, CAT and Prdx1. **F,** ROS levels in mice lung tissue. **G, H,** MPO and MDA activity in mice lung tissue. ("*" means $p<0.05$ *vs.* the Sham group and "#" means $p<0.05$ *vs.* the LIRI group).

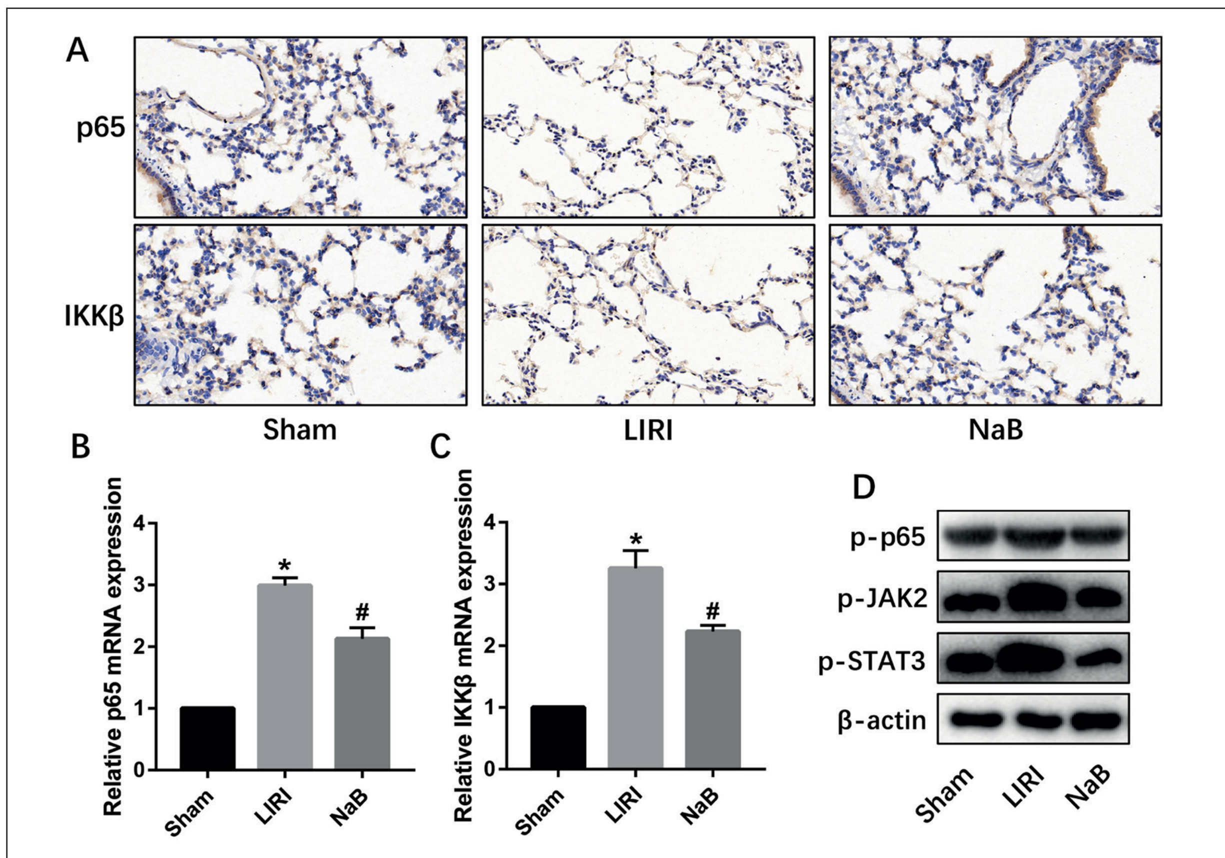

Figure 4. NaB inhibits NF-κB and JAK2/STAT3 signaling pathways in mouse lung tissues. **A,** IHC staining of p65 and IKKβ in mice lung tissue (400×). **B, C,** RT-PCR of p65 and IKKβ. **D,** Western blot of p-p65, p-JAK2 and p-STAT3. ("*" means $p<0.05$ *vs.* the Sham group and "#" means $p<0.05$ *vs.* the LIRI group).

(Figure 4B-4C) were similar to IHC staining. The phosphorylation levels of p65, JAK2, and STAT3 were detected by Western blot (Figure 4D). The results showed that phosphorylation levels of p65, JAK2, and STAT3 were increased in lung tissue after IR, suggesting that nuclear translocation of p65, JAK2, and STAT3 occurred. Phosphorylation levels of p65, JAK2, and STAT3 were significantly reduced after treatment with NaB in mice, suggesting that NaB inhibited the activity of NF-κB and JAK2/STAT3 signaling pathways.

Discussion

LIRI is a common and difficult clinical problem after heart surgery and lung transplantation. Inflammatory response and oxidative stress are important mechanisms of LIRI[10]. At present, it is believed that neutrophils, free radicals, and other inflammatory mediators in IR cause lung injury. Our study found that NaB has excellent anti-inflammatory and antioxidant effects in the prevention and treatment of LIRI.

The inflammatory response after IR is one of the factors that cause and aggravate its secondary injury. In the process of inflammatory response, a variety of inflammatory factors and inflammatory mediators aggregate, causing and aggravating LIRI[11]. The characteristic lesion of LIRI is inflammatory exudation, which is mainly manifested as pulmonary capillary dilatation and rupture of alveolar epithelial cell connection, increasing permeability of endothelial cells, leakage of water and protein in the blood vessels, migration, and aggregation of inflammatory cells, and ultimately leading to pulmonary edema[12]. Even after unilateral pulmonary ischemia, the same physiological changes occur immediately after reperfusion in the contralateral lung, suggesting that injury signals may be secreted through body fluids. The accumulation

of monocytes and neutrophils in the lungs is a key factor in lung inflammation and injury. Both cellular and humoral factors regulate inflammatory responses[13]. However, recent studies have shown that inflammatory reflexes may also modulate innate immune responses. During the IR process, peripheral blood leukocytes and activated endogenous microglia increase the formation of oxygen free radicals in the early stage of ischemia and cause necrosis of cell tissues, which leads to a cascade of inflammatory responses and promotes oversecretion of inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α. In addition, the expression of adhesion molecules on the wall of the tissue is upregulated, causing leukocytes to migrate to the vascular endothelium, infiltrating and accumulating in the ischemic area, and inducing a secondary inflammatory response, eventually forming a vicious circle. In the early stage, TNF-α is mainly secreted by mononuclear macrophages. When the blood flow is recanalized, the macrophage system of the body can be changed from stationary state to activated state and promotes the release of TNF-α or other interleukins[14]. When LIRI occurs, the circulating TNF-α levels increased and TNF-α can stimulate the release of monocyte chemotactic protein, keratinocyte chemokines, etc. and change the alveolar vascular exudation rate leading to pulmonary edema. Our study found that the inhibitory effect of NaB on the inflammatory response after LIRI is mainly reflected in the inhibition of the expression of IL-1β, IL-6, and TNF-α. The IR model of mice contained large number of inflammatory factors in the serum and lung tissues, while NaB significantly reduced their expression.

In this study, NaB effectively increased the expression of anti-oxidant stress molecules SOD1/2, CAT, and Prdx1 and promoted ROS clearance. Oxygen free radicals refer to a class of highly chemically reactive oxygen-containing groups, mainly including superoxide anion oxygen radicals (O^{2-}), nitric oxide radicals (NO) and hydroxyl radicals (OH-). Among them, O^{2-} widely exists in living organisms and is the initial link of radical chain reaction. Its properties are extremely unstable and can continuously generate new ROS. OH- is the most harmful free radical. For a long time, we have recognized the importance of oxygen free radicals in LIRI and vascular injury. Activated neutrophils or lung tissue may produce oxygen free radicals after LIRI[15]. Ischemia can cause hypoxia in lung tissues and reintroducing molecular oxygen during reperfusion can produce toxic oxygen metabolites through xanthine oxidase and mitochondrial cytochrome P450 systems. Pak et al[16] have found that hypoxia activates p38 kinase, which causes xanthine oxidase to be activated by phosphorylation, resulting in the production of ROS. In the canine model, ventilation of 100% oxygen or room air during lung ischemia resulted in more severe pulmonary edema than 100% nitrogen ventilation or SOD pretreatment[17]. Oxidative stress markers, such as lipid peroxidation products have been found to be closely related to lung injury in animal lung IR models. Free radical lipid peroxidation can produce aldehydes MDA in the IR process, which has a strong destructive effect on the cell membrane[18]. It can reflect the degree of free radical production and lipid peroxidation, and indirectly assess the degree of cell damage. Therefore, the inhibitory effect of NaB on oxidative stress is very beneficial for the prevention and treatment of LIRI.

The NF-κB nuclear transcription factor family is present in most vertebrate cells and is an important inflammatory regulator that is central to immune regulation and inflammation[14]. In general, NF-κB in the body is in a state of inhibition. When the body develops inflammatory response, inhibitory NF-κB kinase (IKK) is activated by oxidized low-density lipoprotein (oxLDL) and inflammatory cytokines, resulting in phosphorylation and ubiquitination of IκB, thereby promoting the activation and migration of NF-κB to the nucleus and ultimately stimulating the production of the chemokine CCL2 and vascular cell adhesion molecule-1 (VCAM-1). The NF-κB signaling pathway is closely related to histone acetyltransferase[17]. NaB, as HDACi, inhibits the acetylation of various proteins in the NF-κB signaling pathway family, thereby inhibiting the activity of the NF-κB signaling pathway[19]. The JAK-STAT pathway is a specific component of many cytokine receptor systems involved in cell growth, survival, proliferation, and hematopoiesis and immune regulation. This pathway is mainly composed of three parts, namely tyrosine kinase JAK, tyrosine kinase-related receptors (such as epidermal growth factor, γ-interferon, etc.) and transcription factor STAT. When the cytokine binds to the corresponding receptor, it activates the JAK kinase coupled to the receptor, which leads to phosphorylation of the tyrosine residue at the receptor, thereby activating the STAT protein, and finally the

STAT protein enters the nucleus in the form of a dimer[20]. The NF-κB and JAK/STAT signaling pathways play an important role in IR-induced lung injury, with significant pro-inflammatory and pro-oxidative effects. The inhibitory effect of NaB on NF-κB and JAK/STAT signaling pathways also explains the protective effect of NaB on the lungs and the anti-inflammatory and anti-oxidative effects in the IR process. Liang et al[21] also studied the effect of NaB on lung injury. They made a burn model of rats and gave rats oral ringer's solution containing NaB. They found that severely burned rats were accompanied by acute lung injury and that rats treated with NaB had less lung injury, with reduced levels of inflammation and oxidative stress. This suggests that NaB can reduce burn-induced acute lung injury. Therefore, NaB has a good therapeutic effect on lung injury induced by various factors. We believe that the study of the lung protection of NaB can provide a new direction for the clinical treatment of LIRI.

Conclusions

NaB effectively alleviates IR-induced lung injury and can inhibit IR-induced inflammatory responses and oxidative stress. In addition, NaB can inhibit NF-κB and JAK2/STAT3 signaling pathways in lung tissue, which is beneficial to the prevention and treatment of lung injury during IR.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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