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Original Article

Role of transcriptional factor Nrf2 in the acute lung injury of mice

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Abstract: Objective: This study aimed to investigate the expression and role of Nrf2 in the acute lung injury (ALI) of mice. Methods: A total of 60 BABL/c mice were randomly divided into 2 groups: ALI group and control group. In ALI group, ALI was introduced by injection of LPS. Immunohistochemistry was performed to detect Nrf2 expression in the lung; Western blot assay was employed to detect the expression of Nrf2 in the lung homogenate; ELISA was conducted to detect the expression of Nrf2 in the lung homogenate and BALF. Results: As compared to control group, ALI mice had a high Nrf2 expression in the lung as shown in immunohistochemistry, and the Nrf2 expression in the lung homogenate and BALF also increased markedly ($P<0.05$). Conclusion: The Nrf2 expression increases in the lung and BALF of ALI mice, suggesting that Nrf2 is involved in the inflammation during ALI and may serve as a new target in the therapy of ALI.

Keywords: Transcription factor, Nrf2, acute lung injury, mouse

Introduction

Acute lung injury (ALI) is a syndrome consisting of acute hypoxemic respiratory failure with bilateral pulmonary infiltrates that is associated with both pulmonary and nonpulmonary risk factors and that is not primarily due to left atrial hypertension [1]. Common causes of ALI include sepsis, trauma, aspiration, multiple blood transfusion, acute pancreatitis, inhalation injury, and certain types of drug toxicity. There is evidence showing that approximately 190,000 cases per year of ALI in the United States each year, with an associated 74,500 deaths per year, and the in-hospital mortality was 38.5% for ALI [2]. Inflammation and oxidative stress are two major mechanisms underlying the pathogenesis of ALI [3-5]. Thus, increasing studies have been conducted to develop strategies targeting the inflammation and/or oxidative stress for the therapy of ALI.

Nuclear factor, erythroid 2 related factor 2 (Nrf2) is a member of the Cap'n'collar/basic region leucine zipper (CNC-bZIP) transcription factor family, and can be activated by diverse oxidants, pro-oxidants, antioxidants, and chemopreventive agents. After phosphorylation and dissociation from the cytoplasmic inhibitor, Kelch-like ECH-associated protein 1 (Keap1), Nrf2 translocates to the nucleus and binds to an antioxidant response element (ARE). Through transcriptional induction of ARE-bearing genes that encode antioxidant-detoxifying proteins (such as heme oxygenase), Nrf2 activates cellular rescue pathways against oxidative injury, inflammation/immunity, apoptosis, and carcinogenesis [6]. Nrf2 is ubiquitous and relatively abundant in tissues such as liver, kidney, and lung, where routine detoxification processes occur. The airways are particularly vulnerable to oxidant injury because they are continuously exposed to environmental airborne toxicants, and thus redox balance needs to be tightly controlled. Studies have confirmed that Nrf2 is closely related to the pathogenesis of pulmonary disorders including ALI and Nrf2 may protect the lung against a variety of various oxidative environmental toxicants and pollutants, medicinal agents, allergens, and pathogens [7, 8].

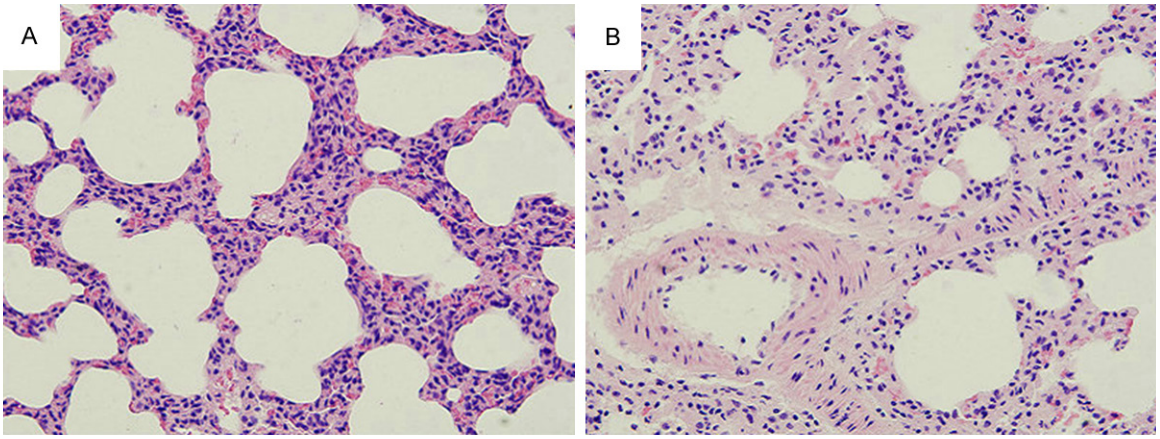

Figure 1. HE staining of mouse lung. A. Control group; B. ALI group. (×200).

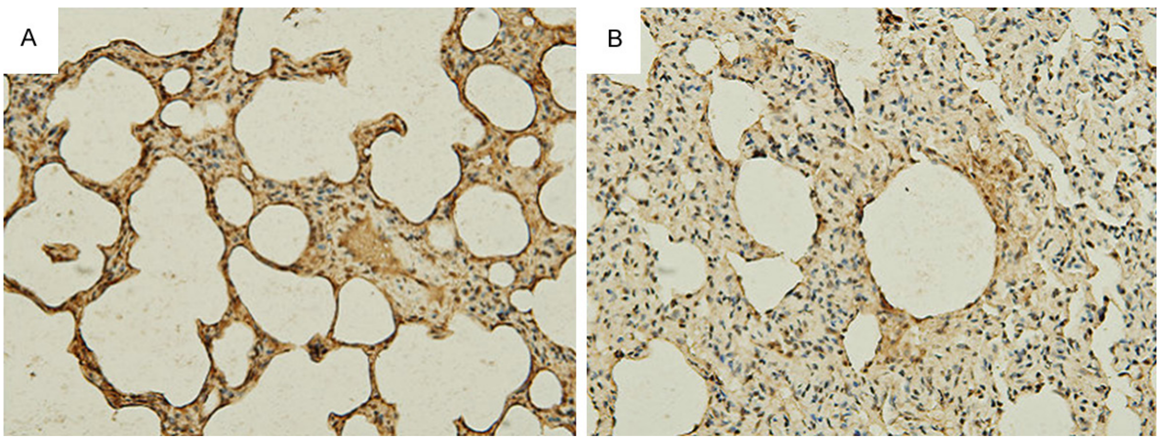

Figure 2. Immunohistochemistry for Nrf2 in the mouse lung. A. Control group; B. ALI group. (×200).

In this study, we investigated the Nrf2 expression in the lung and BALF of mice undergoing LPS induced ALI, aiming to explore the role of Nrf2 in this disease.

Materials and methods

Main reagents

Lipopolysaccharide (LPS, E.coil 0127 B8, Sigma-Aldrich) was dissolved in sterilized tube (12 mg: 10 ml) followed by sonification for 15 min until suspended substances were absent. The LPS solution was stored at -20°C [2]. Mouse Nrf2 ELISA kit (R&D), mouse Nrf2 antibody (ab89443, Abcam), RIPA lysis buffer (P0013B, Beyotime Institute of Biotechnology) were used in this study.

Grouping and ALI animal model

A total of 60 BALB/c mice aged 8-10 weeks and weighing 17-23 g were purchased from the Experimental Center of Chinese Academy of Sciences in Shanghai and given ad libitum access to water and food. Mice were randomly assigned into control group and ALI group (n=30 per group). ALI was induced by injection of LPS according to previously reported [3]. In brief, mice were intraperitoneally anesthetized with 20% pentobarbital sodium (1 g/kg) and then fixed on a table. The hair in the neck was removed and an incision was made at the center (1-2 cm in length). The subcutaneous tissues were separated and blood vessels were completely exposed. The trachea was carefully pulled up with a forceps, and threads were placed under the trachea. A hole was made at the trachea besides the cartilagines peltata, and a microsyringe (0.1 ml) was inserted through this hole. Then, LPS (3 mg/kg) was injected at the bronchial bifurcation. The microsyringe was retracted and wound closed. Finally, mice fixed on the table were rotated for

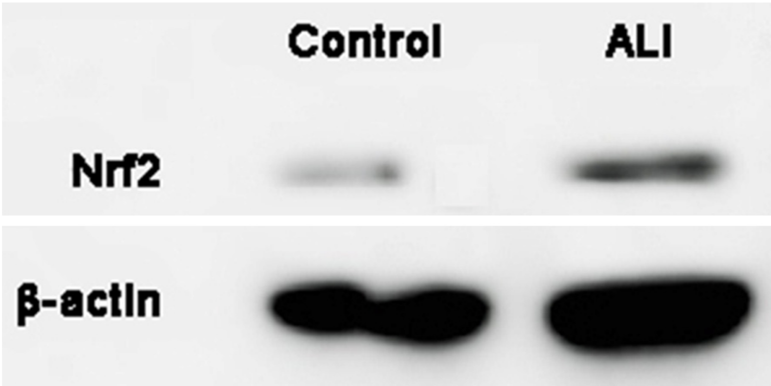

Figure 3. Detection of Nrf2 expression in the lung homogenate by Western Blot assay.

Table 1. Nrf2 expression normalized to β-actin expression ($\bar{x}\pm s$) (n=10)

Group	Control group	ALI group
Relative expression	0.125±0.037	0.198±0.021*

Note: q^{*}=3.9669, P<0.05 vs. control group.

Table 2. Nrf2 expression in the lung homogenate and BALF of mice (pg/mg pro, $\bar{x}\pm s$)

Group	n	Lung homogenate	BALF
ALI group	30	10.03±0.23**	7.16±0.21*
Control group	30	2.92±0.81	1.21±0.59
t		8.163	9.032
P		0.014	0.019

Note: $^{*}P$<0.01, $^{**}P$<0.01 vs. control group.

1-2 min to assure that LPS was evenly distributed in the lung. After treatment, mice were placed into a box and transferred back to cages when they woke.

Collection of bronchoalveolar lavage fluid

After experiment, mice were sacrificed by cervical dislocation, and thoracotomy was performed, followed by ligation of right main bronchus. The lung was lavaged with 1 ml of normal saline at 4°C (about 60-80% of saline was retrieved). The lavage fluid was centrifuged at 3000 rpm for 10 min, and the supernatant was harvested and stored at -20°C.

Detection of Nrf2 protein expression by Western Blot assay

The right lung was collected and weighed, and then mixed with RIPA lysis buffer (10 mg: 0.1 ml), followed by homogenization on ice. The homogenate was then transferred into a pre-cold EP tube (4°C) and centrifuged at 10000 rpm for 20 min at 4°C. The supernatant was harvested and protein concentration was determined. Then, 5 μg of proteins were loaded, subjected to 12% SDS-PAGE and transferred onto PVDF membrane at 60 V for 3 h. The PVDF membrane was blocked in 3% non-fat milk in PBS-T for 2 h and then treated with rabbit anti-mouse Nrf2 (1:500) at 4°C over night. After washing thrice, the membrane was treated with secondary antibody (1:500) at room temperature for 60 min. Following washing, visualization was done with DAB, and protein bands were photographed. The integrated optical densities (IOD) of Nrf2 and β-actin were determined with IPP image analysis software and compared between groups.

Pathological examination

The upper lobe of the left lung was collected and fixed in 4% formaldehyde, embedded in paraffin and cut into 4-μm sections, followed by HE staining and immunohistochemistry. Immunohistochemistry was performed with SP method, and the primary antibody was rabbit anti-mouse Nrf2 monoclonal antibody (1:100). Five fields were randomly selected, and images were captured at a magnification of (200×; a total of 150 images in each group). No cytoplasmic staining was scored 0, light brown cytoplasm was scored 1, brown cytoplasm was scored 2 and dark brown cytoplasm was scored 3. In addition, the Nrf2 expression was also scored according to the proportion of positive cells: <30%, 1; 30-70%, 2; >70%, 3; 0%, 0. The product of both scores was the final score in pathological examination: negative (-), 0; weakly positive (+), 2-3; positive (++), 4; strong positive (+++), 5-6.

Lung homogenization

The lower lobe of the left lung was collected, ground and homogenized in normal saline (v:v 1:9), followed by centrifugation at 3000 rpm for 10 min. The supernatant was harvested and stored at -20°C.

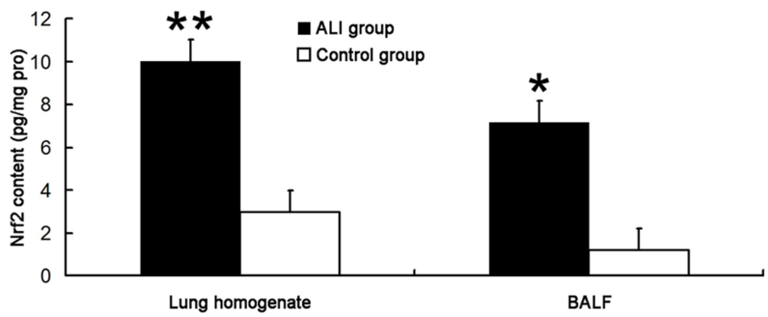

Figure 4. Nrf2 expression in the lung homogenate and BALF of mice. Note: *P<0.01, **P<0.01 vs. control group.

Detection of Nrf2 expression by ELISA

The lung was homogenized as above mentioned. BALF was also collected for the detection of Nrf2 expression. The lung homogenate and BALF were independently centrifuged and the supernatant was harvested. Nrf2 expression was measured by ELISA according to manufacturer's instructions. Optical density (OD) was measured at 450 nm. The OD of samples was normalized to that of blank control. The standard curve was delineated according to the standard samples at 500, 250, 125, 62.5, 31.25, 15.625 and 0 ng/ml, and the Nrf2 expression was calculated according to the standard curve. Nrf2 expression was expressed as pg/mg pro.

Statistical analysis

All the data are expressed as mean ± standard deviation (SD) from three independent experiments and compared with t test for quantitative data and chi square test for qualitative data. A value of P<0.05 was considered statistically significant. Statistical analysis was conducted with SPSS version 15.0.

Results

Lung pathology

In control group, HE staining showed the structure of bronchus and lung was intact, the alveolar space was clear, alveolar septum had no edema and inflammatory infiltration (**Figure 1A**). In ALI group, the lung injury was obvious: the alveolar wall had edema, the lung interstitium was thickened, there were a lot of inflammatory cells in the alveolar space (such as eosinophils, lymphocytes), and the alveolar structure was significantly disrupted (**Figure 1B**).

Immunohistochemistry for Nrf2

Under a light microscope, Nrf2 protein was mainly found to express in the cytoplasm and nucleus of epithelial cells of the bronchus and lung (**Figure 2A** and **2B**). In the control group, weak expression of Nrf2 was observed in the lung; in the ALI group, strong Nrf2 expression was found in the lung.

Nrf2 expression in the lung homogenate by Western blot assay

Western blot assay showed Nrf2 expression in the lung homogenate increased significantly in ALI mice (**Figure 3**). As shown in **Table 1**, the protein expression of Nrf2 was normalized to that of β-actin as the relative expression of Nrf2. Significant difference was observed in the relative expression of Nrf2 between two groups (P<0.05) (**Table 1**).

NNrf2 expression in the lung homogenate and BALF

In ALI group, the Nrf2 expression increased markedly in the lung homogenate and BALF as compared to control group (P<0.01) (**Table 2** and **Figure 4**).

Discussion

ALI refers to the early stage of acute respiratory distress syndrome and is a disorder of acute inflammation that causes disruption of the lung endothelial and epithelial barriers. The alveolar-capillary membrane is comprised of the microvascular endothelium, interstitium, and alveolar epithelium. Cellular characteristics of ALI include loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration, and release of pro-inflammatory, cytotoxic mediators. Following infection or trauma, up-regulation of pro-inflammatory cytokines occurs as a direct response and/or as a marker of ongoing cellular injury [9]. In addition, it is well known that oxidant production within lung is also related to the pathogenesis of ALI

[4, 10]. The most important source of damaging oxidants is phagocytic cells (residential macrophages and recruited neutrophils [PMNs]) that can generate toxic oxygen metabolites. Defenses against toxic oxygen metabolites in the lung include superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione, which is present in mM concentrations in lung lining fluids. These antioxidant enzymes are inducible in a variety of situations such as during hyperoxia, in the presence of LPS, etc. [11]. The presence of these antioxidant enzymes and reducing factors in lung illustrates how the lung adapts to maintain a redox balance and can respond to oxidizing conditions that may threaten the structural and functional integrity of the lung. The lung is susceptible to such threats, because it is the single organ in the body that is exposed to the highest concentrations of O_2.

Nrf2 is a "master regulator" in response to oxidative/electrophilic stresses and chemical insults through the coordinated induction of a wide array of cytoprotective genes. Therefore, activation of Nrf2 is considered to be an important approach for preventing diseases triggered by stresses and toxins [12]. Available studies have confirmed that Nrf2 is protective against inflammation and oxidative stress in a variety of diseases including ALI [5, 13, 14]. Cho et al investigated the association of Nrf2 polymorphism haplotypes with ALI phenotypes and they found Nrf2 as a genetic determinant in ALI pathogenesis [15]. As compared to wild-type mice, the lung hyperpermeability, inflammation, and epithelial cell injury were enhanced in $Nrf2^{-/-}$ mice with hyperoxia induced acute lung injury [16], and enhanced pro-inflammatory cytokines, diminished ARE-responsive glutathione biosynthesis enzymes, disturbed redox balance were also found in $Nrf2^{-/-}$ mice relative to wild types in a ventilation induced lung injury (VILI) model [17], and supplementation of $Nrf2^{-/-}$ mice with the antioxidant N-acetyl cysteine significantly attenuated VILI [17]. Thus, a variety of investigators attempt to protect the lung against via up-regulating Nrf2 expression or activity. Shan et al found that ATF3 could protect the lung against acute and ventilator-induced lung injury by preventing Nrf2 degradation [18]. Yao et al also found propofol could activate Nrf2 pathway to ameliorate ALI in a rat liver transplantation model [19].

In the present study, ALI was induced by intratracheal injection of LPS, and Nrf2 expression was detected in the lung and BALF with different methods. Our results showed the Nrf2 expression increased significantly in the lung (immunohistochemistry, Western blot and ELISA) and BALF, suggesting that Nrf2 is associated with the pathogenesis of LPS induced ALI. However, how Nrf2 is activated during ALI is still unclear.

Taken together, our findings indicate that Nrf2 expression increases in the lung and BALF following ALI, suggesting that Nrf2 is involved in the pathogenesis of ALI. Our findings provide evidence for the further investigations on the pathogenesis of ALI and Nrf2 may become a promising target in the therapy of ALI.

Disclosure of conflict of interest

None.

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