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Expression of Nrf2-Keap1-ARE signal pathway in traumatic lung injury and functional study

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Abstract. – OBJECTIVE: Traumatic lung injury (TLI) can cause inflammation and oxidative stress, or even leads to acute respiratory distress syndrome (ARDS) and death. Nuclear factor erythroid-2 related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1)-antioxidant response element (ARE) signal pathway participates in disease occurrence and progression via regulating inflammatory and oxidative stress response, but with its expression and functional roles in TLI largely unknown.

MATERIALS AND METHODS: Wistar rats were randomly divided into control group, TLI group by crushing method, and Nrf2 activation group which received Nrf2 specific agonist sulforaphane 30 min before TLI treatment. Artery blood gas (ABG), wet/dry mass ratio (W/D) of lung tissues, myeloid peroxidase (MPO) and superoxide dismutase (SOD) activity of lung tissue were analyzed. Keap1 and ARE mRNA levels were tested by Real-time PCR, while Nrf2 protein was measured by Western blot. Inflammatory factors including tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2) were quantified by enzyme-linked immunosorbent assay (ELISA).

RESULTS: TLI model had lower ABG or SOD, higher W/D ratio, MPO value, elevated expressions of TNF-α, IL-2, and Keap1, plus decreased Nrf2 and ARE expression ($p<0.05$). Nrf2 activation significantly improved ABG, decreased W/D ratio and MPO value, enhanced SOD activity, decreased TNF-α and IL-2 secretion, suppressed Keap1 expression, and facilitated Nrf2 and ARE expressions ($p<0.05$).

CONCLUSIONS: Nrf2-Keap1-ARE signal pathway can improve TLI-related pathology via modulating oxidative stress response and suppressing inflammation.

Key Words:
Nrf2-Keap1-ARE, Traumatic lung injury, Cytokines, Oxidative stress, Inflammation.

Introduction

With the development of transportation, industry, and mineralization, plus rapid progression of civil engineering, the incidence of traumatic lung injury (TLI) as caused by car accidences or falling is also increased by years[1,2]. TLI frequently causes acute respiratory distress syndrome (ARDS), which elevates mortality rate[3]. Due to high incidence, morbidity, and medical cost of TLI, it causes both severe physical and mental injury on patients and major economic burden for the health system[4,5]. TLI has a complicated pathogenesis mechanism that is not fully illustrated yet, thus causing difficulty for effective treatment[6].

Nuclear factor erythroid-2 related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1)-antioxidant response element (ARE) signal pathway is one major anti-oxidant signal pathway inside the body, and is responsible for maintaining the balance between peroxidation and anti-oxidation[7,8]. In this pathway, Nrf2 plays a critical role in body's anti-oxidative process[9]. Keap1 belongs to cytoplasmic protein chaperone molecule and is one specific inhibitor receptor of Nrf2 with structural domain for regulating Nrf2. Keap1 negatively regulates Nrf2[10]. Nuclear ARE is one DNA promoter binding sequence, and can be activated by Nrf2 to further regulate expression of anti-oxidation enzyme gene for participating in the regulation of body oxidative stress level[11,12]. Under normal physiological conditions, Nrf2 locates in the cytoplasm via binding with specific inhibitory receptor Keap1. As Keap1 is one negative regulator, Nrf2 stays at inactive status with binding. Keap1 also facilitates ubiquitin degradation of Nrf2, making Nrf2 expression at

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lower level[13]. During oxidative stress, stimuli by oxidants dissociate Nrf2 and Keap1, forming dimers with c-Jun N-terminal kinase (JNK) in mitogen-activating protein kinase (MAPK) family, plus the binding onto ARE sites for initiating downstream promoter of protein transcription[14,15]. The previous study[16] has found the correlation between Nrf2-Keap1-ARE signal pathway with various refractory diseases in respiratory system including pulmonary fibrosis, lung cancer, bronchial asthma, and chronic obstructive pulmonary disease (COPD). The expression and function of Nrf2-Keap1-ARE signal pathway in TLI, however, has not been fully illustrated.

Materials and Methods

Experimental Animal

Healthy male Wistar rats (2 month old, specific pathogenic free (SPF) grade, body weight 250 ± 20 g) were purchased from Laboratory Animal Center of Shandong University (Shandong, China), and were kept in an SPF grade facility with fixed temperature (21 ± 1°C) and relative humidity (50%-70%) in 12 h light/dark cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Eighty-ninth Hospital of the Chinese People's Liberation Army.

Major Materials and Instruments

Pentobarbital sodium and lidocaine were purchased from Zhaohui Pharm (Shanghai, China). IL-2 and TNF-α ELISA kit were purchased from RD Systems (Minneapolis, MN, USA). MPO and SOD activity assay kit was purchased from Cell Signaling Technology (Beverly, MA, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences Inc. (Pensacola, FL, USA). Western blotting reagents were purchased from Beyotime Bio. Ltd. Co. (Shanghai, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-mouse Nrf2 monoclonal antibody and goat anti-rabbit horseradish peroxidase (HRP)-labelled IgG secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). RNA extraction kit and reverse transcription kit were purchased from ABI (Foster City, CA, USA). Microscopic surgical instruments were purchased from Suzhou Medical Instrument (Suzhou, China). Amp PCR System 2400DAN amplifier was purchased from PE Gene Applied Biosystems (Foster City, CA, USA). Imark microplate reader was purchased from RD Systems (Minneapolis, MN, USA). Other common reagents were purchased from Sangon Bio. Ltd. Co. (Shanghai, China).

Animal Grouping and Treatment

Wistar rats were randomly divided into control group, TLI group, which was generated TLI model using crush method, and Nrf2 activating group, which received 5 mg/kg Nrf2 specific agonist sulforaphane 30 min before TLI model generation via intraperitoneal injection.

TLI Model Generation

Crush method was used to generate rat TLI model as previously recorded[17]. Rats were fasted 12 h before surgery with water provided *ad libitum*. 30 mg/kg pentobarbital sodium was used to anesthetize rats, which were later fixed on the table. A 200 g weight was made to freely fall from 1 m height to knock onto bilateral pulmonary of rats.

Sample Collection and Blood-gas Analysis

About 24 h after treatment, 0.5 ml blood was collected from right common carotid artery for artery blood gas analysis. 5 ml blood samples were further collected from the tail vein. Blood samples were also collected from control group. Blood was centrifuged at 3000 r/min for 15 min. The supernatant was placed in Eppendorf tube and was kept in -80°C fridge for further use. Rats were also sacrificed to collect right pulmonary tissues, which were kept in -80°C fridge for further use.

W/D Ratio

About 24 h after treatment, right pulmonary tissues were collected for weighting wet mass on an electronic balance. Tissues were then dried at 80°C and, then, weighted for dry weight for calculating W/D ratio.

Enzyme-linked Immunosorbent Assay (ELISA) for Serum TNF-α and IL-2

Serum samples were collected from all groups of rats to detect the level of TNF-α and IL-2 following the manual instruction of test kits. In brief, 96-well plate was added with 50 µl serially diluted samples, which were used to plot standard curves. Total of 50 µl test samples was, then, added to test wells in triplicates. After washing for 5 times, liquids were discarded to fill with washing buffer for 30 s vortex. The rinsing procedure was repea-

ted for 5 times. Total of 50 µl enzyme labeling reagent was then added into each well except blank control. After gentle mixture, the well was incubated for 30 min at 37°C. Chromogenic substrates A and B were sequentially added (50 µl each), followed by 37°C dark incubation for 10 min. The test plate was then mixed with 50 µl quenching buffer as the blue color turned into yellow. Using blank control well as the reference, absorbance (A) values at 450 nm wavelength were measured by a microplate reader within 15 min after adding quenching buffer. A linear regression model was then plotted based on the concentration of standard samples and respective OD values. Sample concentration was further deduced based on OD values and regression function.

MPO Activity and SOD Activity Assay

Using SOD activity assay kit, SOD activity was tested in intestinal tissues following manual instruction. In brief, tissue were denatured at 95°C for 40 min, and were centrifuged at 4000 r/min for 10 min. Ethanol-chloroform mixture (5:3, v/v) was used to extract ethanol phase in the homogenate for total SOD activity assay. MPO activity was measured following manual instruction of test kit. Tissue homogenate was prepared by vortex homogenizer. After 95°C heating for 40 min, tissue lysate was washed with cold water, and was centrifuged at 4000 r/min for 10 min. Samples were added into phosphate buffer containing 30 mM H_2O_2 (pH 7.0). The mixture was incubated for 10 min. Enzymatic activity was measured by detecting the decrease of absorbance light density at 240 nm wavelength.

Real-time PCR for Nrf2, Keap1 and ARE mRNA Expression in Pulmonary Tissues

Pulmonary tissues were rinsed in phosphate buffered-saline (PBS) under sterile condition. Tissues were homogenized in liquid nitrogen and were extracted for mRNA using TRIzol reagents. cDNA was synthesized using specific primers (Table I). Real-time PCR was used to detect the expression of target genes under the following conditions: 52°C for 1 min, followed by 35 cycles each containing 90°C denature for 30 s, 58°C annealing for 50 s, and 72°C elongation for 35 s. Fluorescent quantitative PCR was used to collect data and analyzed for CT value concerning GAPDH. Relative expression level was determined by 2^{-Ct} method.

Western Blot for Nrf2 Protein Expression

Total proteins were extracted from pulmonary tissues after homogenization on liquid nitrogen and were mixed with lysis buffer for 15-30 min iced incubation. Using ultrasonic rupture (5 s, 4 times) and centrifugation (10000 ×g, 15 min), proteins were collected and kept at -20°C for western blotting. Proteins were separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and were transferred to PVDF membrane by semi-dry method (160 mA, 1.5 h). Non-specific binding sites were blocked by 5% defatted milk powders for 2 h. Anti-Nrf2 monoclonal antibody (1:1000) was applied for 4°C overnight incubation. Goat anti-rabbit IgG (1:2000) was then added for 30 min incubation. After PBS-tween 20 (PBST) washing and ECL development for 1 min, the membrane was exposed under X-ray. An imaging analyzing system and Quantity one software were used to scan X-ray films and to detect the density of bands with repeated measures (n=4).

Statistical Analysis

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to collect all data. The measurement data were expressed as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between two groups. Tukey's post hoc test was used to validate the ANOVA for comparing measurement data between groups. A statistical significance was defined when $p<0.05$.

Table I. Primer synthesis sequence.

Target gene	Forward primer 5′-3′	Reverse primer 5′-3′
GAPDH	AGTGCCAGCCTCGTCTCATAG	ACTTGCAACTTGCCGTGGGTAG
Nrf2	CTCATCGTAAGCGAACAATGG	GCACCGTTCTTAGCG
Keap1	TAAGAGGAACGGAATG	ACATCATCTATTCTCT
ARE	TCATCATCTAGCCTC	ACTTGCTTGCACGGG

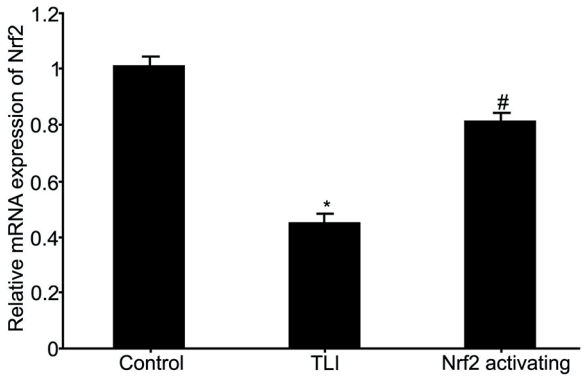

Figure 1. Nrf2 mRNA expression in rat pulmonary tissues. *, $p<0.05$ compared to control group; #, $p<0.05$ compared to TLI group.

Results

Blood-gas Analysis

TLI rats had decreased pH and PaO_2 values, and elevated $PaCO_2$ ($p<0.05$ compared to control group). The Nrf2 activating group had remarkably elevated pH and PaO_2, plus lower $PaCO_2$ ($p<0.05$ compared to TLI group, Table II).

W/D Ratio and Oxidative Stress of Rats

TLI rats had higher W/D ratio and MPO activity, and lower SOD activity ($p<0.05$ compared to control group). Nrf2 activating significantly decreased W/D ratio and MPO activity, plus higher SOD activity ($p<0.05$ compared to TLI group, Table III).

Nrf2 mRNA and Protein Expression in Rat Pulmonary Tissues

Real-time PCR and Western blot were used to test mRNA and protein expressions of Nrf2 in rat pulmonary tissues. Results showed a decreased Nrf2 mRNA and protein expression in TLI group ($p<0.05$ compared to control group). Nrf2 activating group significantly elevated Nrf2 mRNA and protein expression ($p<0.05$ compared to TLI group, Figures 1 and 2).

Keap1 and ARE mRNA Expression in Rat Pulmonary Tissues

Real-time PCR was used to test Keap1 and ARE mRNA expression levels in rat pulmonary tissues. Results showed elevated Keap1 mRNA expression in TLI group, and decreased ARE mRNA expression ($p<0.05$ compared to control group). Nrf2 activating significantly inhibited Keap1 mRNA and facilitated ARE mRNA expression ($p<0.05$ compared to TLI group, Figure 3).

Serum Levels of Inflammatory Factors TNF-α and IL-2

ELISA was used to test serum levels of inflammatory factors TNF-α and IL-2 in rats. Results showed elevated serum levels of inflammatory factors TNF-α and IL-2 in TLI group ($p<0.05$ compared to control group). Nrf2 activating significantly inhibited serum levels of inflammatory factors TNF-α and IL-2 ($p<0.05$ compared to TLI group, Figure 4).

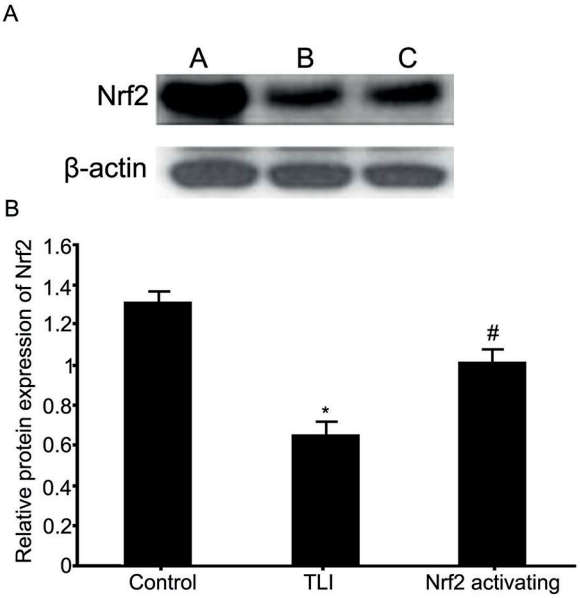

Figure 2. Nrf2 protein expression in pulmonary rats. ***(A) A,*** Control group; ***B,*** TLI group; ***C,*** Nrf2 activating group. ***(B)*** *, $p<0.05$ compared to control group; #, $p<0.05$ compared to TLI group.

Table II. Blood-gas analysis of rats.

Group	pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)
Control group	7.38±0.02	102.5±7.6	37.7±4.5
TLI group	7.22±0.05*	66.3±8.5*	52.6±5.6*
Nrf2 activating group	7.35±0.06#	89.2±9.1#	42.5±4.1#

Note: *, $p<0.05$ compared to control group; #, $p<0.05$ compared to TLI group.

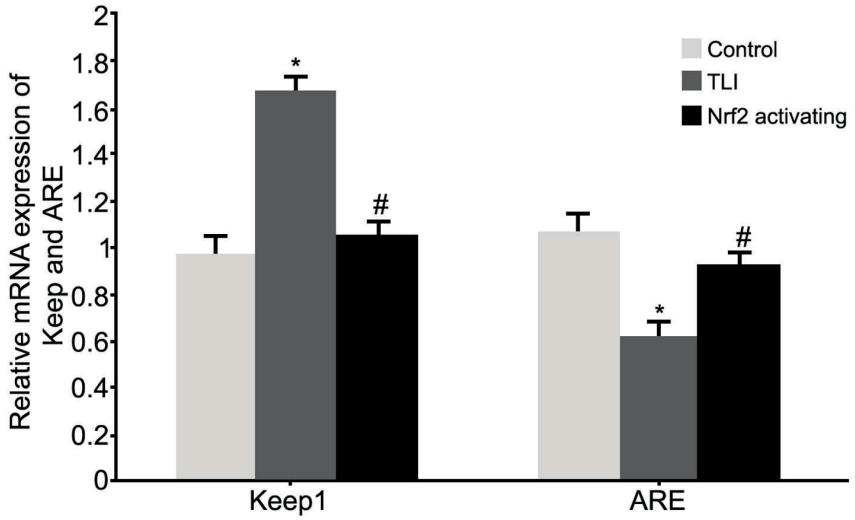

Figure 3. Keap1 and ARE expression in rat pulmonary tissues. *, $p<0.05$ compared to control group; #, $p<0.05$ compared to TLI group.

Table III. W/D ratio and oxidative stress of rats.

Group	W/D	MPO (U/g)	SOD (U/g)
Control group	4.21±0.22	0.45±0.06	137.2±13.1
TLI group	5.47±0.35*	1.12±0.09*	82.2±15.16*
Nrf2 activating group	4.55±0.56#	0.61±0.07#	121.1±14.2#

Note: *, $p<0.05$ compared to control group; #, p<0.05 compared to TLI group.

Discussion

Under normal physiological conditions, Nrf2 tightly binds with Keap1 to inactivate Nrf2. With electrophilic stress, oxidative stress, toxic substances, and metabolites can cause dissociation of Nrf2 with Keap1, further causing the binding between Nrf2 and ARE[18]. The study has shown the participation of Nrf2-Keap1-ARE signal pathway in regulating normal physiological status of human body, antagonizing tumor, detoxifying and maintaining body ho-

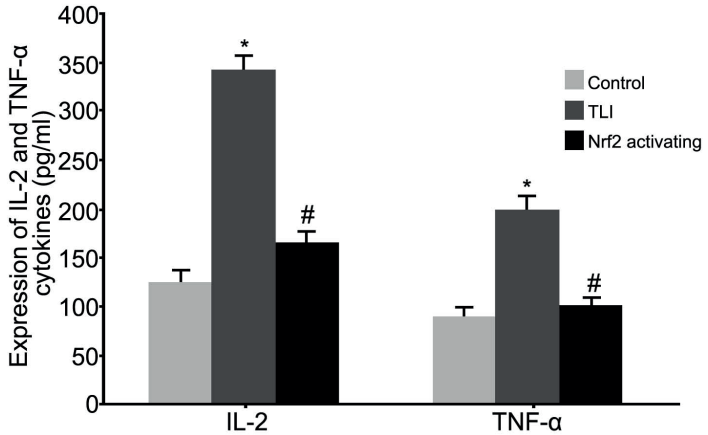

Figure 4. Serum levels of inflammatory factors TNF-α and IL-2. *, $p<0.05$ compared to control group; #, $p<0.05$ compared to TLI group.

meostasis. Nrf2 is one transcriptional factor with cell protective roles[19]. When body is under oxidative stress status, over-production of free radicals such as reactive oxygen species may exceed clearance ability of body anti-oxidation system, making the imbalance of oxidation/anti-oxidation system, further causing tissue inflammation and injury. Therefore, oxidation-reduction status of the body is closely correlated with disease occurrence[20]. Nrf2-Keap1-ARE signal pathway can resist both internal and external oxidation and chemical substances via regulating oxidation-reduction balance, thus inhibiting oxidative stress response for defense reaction, making it one signal pathway critical for endogenous anti-oxidation[21].

As one critical condition in clinics, TLI can cause systemic inflammatory syndrome. TLI has multiple inducing factors and mechanisms, with major pathological changes attributed to increased vascular penetrability, leading to abundant release of inflammatory factors, as presented by pneumonia syndrome. On the other hand, TLI can lead to oxidative stress, further aggravating inflammatory body injury[22]. Although the previous study[23] showed that activation of Nrf2-Keap1-ARE signal pathway could inhibit pulmonary fibrosis, impeding COPG progression, or even regulating occurrence and progression of lung cancer, its expression and function in TLI, however, have not been illustrated. This study generated TLI model using crush method, with addition of Nrf2 specific agonist sulforaphane. Results demonstrated elevated Keap1 mRNA and lower Nrf2 mRNA/protein expressions, plus decreased ARE mRNA expression in TLI model, whilst Nrf2 activation significantly inhibited Keap1 expression, and facilitated Nrf2 and ARE expressions, indicating abnormal expression of Nrf2-Keap1-ARE signal pathway in TLI patients. Further functional study showed aggravated blood-gas of artery in TLI patients, which also had increased W/D ratio. Nrf2 agonist, on the other hand, improved artery blood-gas indexes and lower W/D ratio, indicating that the activation of Nrf2-Keap1-ARE signal pathway could improve TLI. Further mechanism study showed that TLI model could induce oxidative stress, lower SOD activity, and higher MPO activity, thus facilitating secretion of inflammatory factors and aggravating inflammation. The Nrf2 activating group had significantly lowered MPO activity, enhanced SOD activity, and decreased secretion of TNF-α and IL-2, for further improvement of inflammation. These results collectively suggested that Nrf2-Keap1-ARE signal pathway could inhibit inflammation and regulate TLI via modulating oxidation/anti-oxidation balance.

Conclusions

We showed that Nrf2-Keap1-ARE signal pathway could alleviate inflammation and further improve pathological change of TLI via modulating oxidative stress response.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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