Nrf2-Keap1-ARE-NQO1 signaling attenuates hyperoxia-induced lung cell injury by inhibiting apoptosis

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Abstract. Bronchopulmonary dysplasia (BPD) is one of the main causes of chronic lung disease in premature infants. Acute lung injury following exposure to hyperoxia contributes to the development of BPD in preterm infants. The nuclear factor-erythroid 2-related factor 2 (Nrf2) signaling pathway is an endogenous antioxidant defense mechanism that is involved in the pathogenesis of numerous hyperoxia-induced diseases. In the present study, the expression of Nrf2, Kelch-like ECH-associated protein 1 (Keap1) and NAD(P)H quinone oxidoreductase 1 enzyme (NQO1) was detected in A549 cells exposed to hyperoxia and transfection with small interfering RNA (siRNA) using reverse transcription-quantitative polymerase chain reaction and western blotting, and cellular apoptosis was detected using flow cytometry. The results demonstrated that apoptosis increased significantly following exposure of the cells to hyperoxia, and Nrf2, Keap1 and NQO1 expression levels were significantly upregulated under hyperoxic conditions. Furthermore, following transfection with Nrf2 siRNA, the expression levels of these genes were significantly downregulated and apoptosis was significantly increased compared with the respective values in untransfected cells. These findings suggest that the Nrf2-Keap1-antioxidant

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Abbreviations: BPD, bronchopulmonary dysplasia; ALI, acute lung injury; Nrf2, nuclear factor-erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1; ARE, antioxidant response element; NQO1, NAD(P)H quinone oxidoreductase 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ROS, reactive oxygen species; PBS, phosphate-buffered saline; AECII, alveolar epithelial cell type II; siRNA, small interfering RNA; sMAF, small musculo-aponeurotic fibrosarcoma

Key words: hyperoxia-induced lung injury, nuclear factor-erythroid 2-related factor 2, NAD(P)H quinone oxidoreductase 1 enzyme, premature

response element-NQO1 signaling pathway may play a protective role in hyperoxia-induced lung injury via the inhibition of apoptosis.

Introduction

Acute lung injury (ALI) is a condition in which progressive hypoxemia and respiratory distress are caused by non-cardiogenic factors, including hyperoxia and infection (1). Exposure to hyperoxia can induce ALI, which is a key risk factor for the occurrence and development of bronchopulmonary dysplasia (BPD). Hyperoxia-induced lung injury may result in atelectasis, poor lung compliance and susceptibility to infection as a consequence of surfactant deficiency, mucociliary dysfunction and histological damage (2). Experimental models have demonstrated that hyperoxia can disrupt alveolar and microvascular development, and thereby cause alveolar simplification (3). Similarly, exposure to hyperoxia at birth is known to increase the risk of BPD (4). Markers of oxidative stress have been shown to be associated with the development of lung disease (5). Between the years 2007-2011 and 2012-2015, the incidence of BPD among preterm infants in 11 high-income countries exhibited a significant increase, from 23.3 to 27.5% (6).

The oxidative stress triggered by reactive oxygen species (ROS) contributes to ALI by causing pulmonary parenchymal damage (4). Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a member of the cap 'n' collar family of transcription factors, and the Nrf2-kelch-like ECH-associated protein 1 (Keap1)/antioxidant response element (ARE) signaling pathway has been shown to regulate antioxidant proteases, scavenge ROS, maintain intracellular redox homeostasis, and regulate apoptosis and anti-inflammatory responses (7). NAD(P)H quinone oxidoreductase 1 (NQO1) is a phase II stress response protein that regulates the production of ROS and is able to alleviate oxidative stress injury induced by the exposure of respiratory epithelial cells to hyperoxia (8). However, the relationships among Nrf2, NQO1 and hyperoxia-induced lung injury remain unclear.

Reparative responses to lung epithelial lesions in infants with BPD are dependent on type II alveolar epithelial cells (AECIIs) (9); however, AECIIs tend to degenerate in primary culture. The A549 cell line is derived from human alveolar basal epithelium adenocarcinoma, is suitable for gene

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transfection, and has characteristics similar to those of AECIIs; therefore, A549 cells are often used in the study of pulmonary antioxidation mechanisms (10). Since previous studies have used A549 cells to investigate the pathogenesis of BPD in premature infants (11,12), A549 cells exposed to hyperoxia were used in the present study as a model to investigate the molecular processes that contribute toward BPD in premature infants.

In the present study, the expression of Nrf2, Keap1 and NQO1 in A549 cells was investigated under exposure to hyperoxia and with small interfering RNA (siRNA) transfection, and their associations with cellular apoptosis were elucidated. Thus, the aim of the study was to provide insights into the pathogenesis of BPD in premature infants.

Materials and methods

Cell line. A549 cells were obtained from The Chinese Academy of Sciences (Shanghai, China) and cultured at the Cell Laboratory Center, Shanghai Ssmdata Medical Information Technology Company (Shanghai, China).

Reagents. Three pairs of 21-base siRNAs were designed by Suzhou GenePharma Co., Ltd. based on the human *Nrf2* gene sequence in GenBank (National Institutes of Health) using standard design principles (Table I). Lipofectamine[®] 2000, TRIzol[®], fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Thermo Fisher Scientific, Inc., and the PrimeScript RT reagent kit was obtained from Takara Bio, Inc.

Cell culture and grouping of A549 cells. A549 cells (5x10⁴/well) were inoculated into a 24-well culture plate with 10% FBS, DMEM, supplemented with 100 U/ml penicillin at 37°C the day before transfection, and grown to 40-70% confluence within 24 h. The siRNA (100 nM) was mixed gently with 50 μ l serum-free DMEM, and 1 μ l Lipofectamine 2000 was mixed with 50 µl DMEM at 25°C for 5 min. The siRNA and Lipofectamine reagents were then mixed and added to the culture plate containing the cells at 37°C for 12 h. The cells were divided into the following four groups: Normoxic without transfection (group I), hyperoxia-exposed without transfection (group II), normoxic with transfection (group III) and hyperoxia-exposed with transfection (group IV). After transfection for 24 h, the hyperoxia-exposed groups (II and IV) were exposed to 95% O2 and 5% CO2 for 24 h while the normoxic groups (I and IV) were incubated with 5% CO₂ in air for 24 h.

Nrf2 siRNA screening. Cells were in inoculated into a 6-well culture plate at a density of $4x10^5$ cells/well and transfected with one of three siRNAs (100 nM) targeting Nrf2 expression (siRNA-1, siRNA-2 or siRNA-3) or a negative control siRNA (100 nM) using Lipofectamine 2000. The cells were cultured in an incubator at 37°C with 5% CO₂ for 12 h and collected to determine the transfection efficiency. The siRNA with the highest efficiency for the repression of Nrf2 expression (siRNA-1) was used for subsequent experiments.

Immunofluorescence and confocal laser scanning microscopy. The A549 cells transfected with siRNA-1 were fixed with 4% paraformaldehyde at -20°C for 20 min and then treated with 0.3% Triton-X100 for membrane permeabilization. Subsequently, 3% bovine serum albumin (Thermo Fisher Scientific, Inc.) was added, and the cells were blocked in an incubator at 37°C for 1 h. Then, the cells were washed with phosphate-buffered saline (PBS) and incubated overnight at 4°C with Nrf2 (cat. no. ab137550; 1:1,000 Abcam) according to the manufacturer's instructions. After incubation for 1 h at 37°C, the cells were washed in PBS and then incubated in the dark with diluted goat-anti-rabbit IgG (cat. no. ab150077; 1:10,000; Abcam) secondary antibody at 37°C for 1 h. The cells were then mounted on slides and the nuclei were stained with 4',6-diamidino-2-phenylindole at 25°C for 5 min. All samples were analyzed using confocal laser scanning microscopy.

Reverse transcription-qPCR (RT-qPCR). A549 cells from each group were collected and total RNA was extracted from them using TRIzol. The total RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc.) at 42°C for 15 min and 85°C for 5 sec. The cDNA was then subjected to fluorogenic qPCR. Differences in gene expression between groups were compared using a relative quantitation method with *GAPDH* as the internal reference gene. The samples were pre-amplified at 95°C for 15 min, followed by 40 cycles of qPCR at 95°C for 20 sec and 60°C for 45 sec. Relative target gene mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (13). The sequences of the primers used are shown in Table I.

Western blotting. A549 cells from each group were lysed and total protein was extracted using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). The bicinchoninic acid method was used for total protein quantification. The proteins (30 μ g) were separated using SDS-PAGE on 7.5% gels (Beyotime Institute of Biotechnology), transferred to polyvinylidene fluoride membranes and blocked with PBS containing 5% (w/v) skimmed milk powder for 2 h at 25°C. The membranes were then incubated with Nrf2 (cat no. ab137550; Abcam), Keap1 (cat. no. ab139729; Abcam), NQO1 (cat no. ab2346; Abcam) and GAPDH (cat. no. ab9485; Abcam) primary antibodies diluted 1:1,000 overnight at 4°C followed by horseradish peroxidase-conjugated goat-anti-rabbit (cat no. ab6721; Abcam) secondary antibodies diluted 1:5,000 for 1 h at 25°C. Next, the membranes were washed with 150 mM NaCl and 50 mM Tris-Cl at 25°C three times. Finally, the bands were visualized using Pierce enhanced chemiluminescence western blotting substrate (Thermo Fisher Scientific, Inc.) was added, and the blots were scanned using a Bio-Rad Gel Doc XR+ gel documentation system (Bio-Rad Laboratories, Inc.). Bio-Rad Image Lab Software (version 5.1; Bio-Rad Laboratories, Inc.) was used for densitometric analysis.

Flow cytometry. Cell apoptosis in groups I, II and IV was detected using flow cytometry. Briefly, A549 cells $(3x10^5 \text{ cells/well})$ from each group were inoculated into 6-well culture plates and cultured at 37°C for 48 h, then collected for the detection of apoptosis. After removal of the culture medium, the cells were digested with trypsin, centrifuged at 1,000 x g for 5 min at 25°C, then resuspended in PBS. The cells (1x10⁵) were centrifuged again at 1,000 x g for 5 min at

Table I. Primer and siRNA sequences.

siRNA or gene Primer		Sequence (5'-3')		
siRNA-1	Sense	AUACUUCUCGACUUACUCCAA		
	Antisense	GGAGUAAGUCGAGAAGUAUUU		
siRNA-2	Sense	AAACGUAGCCGAAGAAACCUC		
	Antisense	GGUUUCUUCGGCUACGUUUCA		
siRNA-3	Sense	AAUAUUAAGACACUGUAACUC		
	Antisense	GUUACAGUGUCUUAAUAUUGA		
NC	Sense	UUCUCCGAACGUGUCACGUTT		
	Antisense	ACGUGACACGUUCGGAGAATT		
Nrf2	Sense	ATGGATTTGATTGACATACTTT		
	Antisense	ACTGAGCCTGATTAGTAGCAAT		
Keapl	Sense	TGCGCTGCGAGTCCGAGGTCTTC		
	Antisense	TCGAAGATCTTGACCAGGTAGT		
NQO1	Sense	ACATATAGCATTGGGCACACTC		
	Antisense	TCATTAAGAATCCTGCCTGGAAGT		
GAPDH	Sense	CATCACTGCCACCCAGAAGACTG		
	Antisense	ATGCCAGTGAGCTTCCCGTTCAG		

siRNA, small interfering RNA; NC, negative control; Nrf2, nuclear factor-erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1; NQO1, NAD(P)H quinone oxidoreductase 1 enzyme.

Table	II.	Nrf2	mRNA	suppression	following	siRNA	transfec-
tion.							

siRNA	Nrf2 expressio		
siRNA-1	0.1871±0.0592		
siRNA-2	0.3135±0.1262		
siRNA-3	0.3703±0.0182		
NC	0.9634±0.0574		

Data are relative mRNA expression ratios of Nrf2 to GAPDH and are presented as the mean \pm SD (n=3). Nrf2, nuclear factorerythroid 2-related factor 2; siRNA, small interfering RNA; NC, negative control.

25°C. After resuspension, the cells were incubated in the dark with 5 μ l Annexin V-FITC at 4°C for 15 min and then with 5 μ l propidium iodide staining solution in the dark at 4°C for 5 min. Unstained cells were used as the negative control. The flow cytometry data were acquired using an Attune NxT flow cytometer (Thermo Fisher Scientific, Inc.) and analyzed using FlowJo (version 10; FlowJo LLC).

Statistical analysis. All statistical analyses were conducted using SPSS 20.0 software (IBM Corp.) and the results are presented as the mean \pm standard deviation. Results were analyzed by one-way ANOVA. Pairwise comparisons using Bonferroni correction were performed if one-way ANOVA indicated a significant difference. P<0.05 was considered to indicate a statistically significant difference.

Results

Nrf2 siRNA efficiency. The extent by which Nrf2 was downregulated following transfection with three different siRNAs was investigated using RT-qPCR and western blotting. Nrf2 expression was significantly downregulated by Nrf2 siRNA-1, -2 and -3, with siRNA-1 displaying the highest inhibition efficiency (80.57% for *Nrf2* siRNA; Table II, Fig. 1). Therefore, siRNA-1 was used in subsequent experiments.

Nrf2 protein expression and distribution in A549 cells. The expression and distribution of Nrf2 in A549 cells incubated under two different conditions were examined using immuno-fluorescence. Nrf2 was preferentially distributed throughout the cytoplasm of A549 cells under normoxic conditions (group I). However, the expression of Nrf2 protein was down-regulated following transfection with Nrf2 siRNA (group III; Fig. 2).

Hyperoxia upregulates Nrf2, Keap1 and NQO1 in A549 cells. To determine the effects of hyperoxia on Nrf2, Keap1 and NQO1, their expression levels were measured in A549 cells incubated under hyperoxic and normoxic conditions. Relative Nrf2, Keap1 and NQO1 mRNA expression levels in the cells exposed to hyperoxia without siRNA transfection (group II; 4.553 ± 0.498 , 3.299 ± 0.483 and 5.866 ± 0.582 , respectively) were significantly higher compared with those in untransfected cells under normoxic conditions (group I; F=65.310-209.249, P<0.01; Fig. 3). Similarly, relative Nrf2, Keap1 and NQO1 protein expression levels were significantly higher in cells exposed to hyperoxia without transfection (group II; 1.118 ± 0.143 , 1.217 ± 0.070 and 1.064 ± 0.053 ,



Figure 1. Suppression of Nrf2 by siRNA transfection in A549 cells. siRNA-1, siRNA-2 and siRNA-3 are three different Nrf2 siRNAs that were compared with NC siRNA. Data are presented as the mean \pm SD. **P<0.01 vs. NC. siRNA, small interfering RNA; Nrf2, nuclear factor-erythroid 2-related factor 2; NC, negative control.



Figure 2. Nrf2 protein expression and distribution in A549 cells. The localization of Nrf2 was visualized in (A1-C1) group III and (A2-C2) group I using immunofluorescence. Nrf2 was preferentially distributed in the cytoplasm of the A549 cells under normoxic conditions. Nrf2, nuclear factor-erythroid 2-related factor 2.

respectively) compared with those in untransfected cells under normoxic conditions (group I; F=49.103-96.875, P<0.01; Fig. 4). Therefore, it appears that hyperoxia upregulates Nrf2, Keap1 and NQO1 expression.

Nrf2 siRNA downregulates Nrf2, Keap1 and NQO1 in A549 cells. The effects of Nrf2 siRNA on Nrf2, Keap1 and NOO1 expression were examined in A549 cells exposed to hyperoxia. Relative Nrf2, Keap1 and NOO1 mRNA expression levels in cells exposed to hyperoxia after transfection (group IV; 0.937±0.057, 0.854±0.067 and 0.789±0.058, respectively) were significantly lower compared with those in untransfected cells exposed to hyperoxia (group II; F=75.337-226.208, P<0.01; Fig. 3). Similarly, relative Nrf2, Keap1 and NQO1 protein expression levels were significantly lower in hyperoxia-exposed cells after Nrf2 siRNA transfection (group IV; 0.703±0.036, 0.996±0.036 and 0.701±0.037, respectively) compared with those in untransfected cells exposed to hyperoxia (group II; F=23.600-93.816, P<0.01; Fig. 4). These results indicate that Nrf2 siRNA downregulates Nrf2, Keap1 and NQO1 in cells exposed to hyperoxia.



Figure 3. Effects of hyperoxia and Nrf2 siRNA on *Nrf2*, *Keap1* and *NQO1* mRNA expression in A549 cells. Reverse transcription-quantitative polymerase chain reaction demonstrated that *Nrf2*, *Keap1* and *NQO1* mRNA expression levels were significantly higher in the hyperoxia without Nrf2 siRNA group than in the normoxia without Nrf2 siRNA group, but significantly lower in the hyperoxia after Nrf2 siRNA transfection group than in the hyperoxia without Nrf2 siRNA group. II, hyperoxia without SiRNA; group II, normoxia without siRNA; group II, hyperoxia after Nrf2 siRNA transfection; group IV, hyperoxia after Nrf2 siRNA, group II, normoxia after Nrf2, nuclear factor-erythroid 2-related factor 2; siRNA, small interfering RNA; Keap1, kelch-like ECH-associated protein 1; NQO1, NAD(P)H quinone oxidoreductase 1 enzyme.



Figure 4. Effects of hyperoxia and Nrf2 siRNA on Nrf2, Keap1 and NQO1 protein expression. Western blotting demonstrated that Nrf2, Keap1 and NQO1 protein expression levels were significantly higher in the hyperoxia without Nrf2 siRNA group than in the normoxia without Nrf2 siRNA group, but were significantly lower in the hyperoxia after Nrf2 siRNA transfection group than in the hyperoxia without Nrf2 siRNA group. Data are presented as the mean ± SD. **P<0.01. Group I, normoxia without siRNA; group II, hyperoxia without siRNA; group IV, hyperoxia after Nrf2 siRNA transfection; Nrf2, nuclear factor-ery-throid 2-related factor 2; siRNA, small interfering RNA; Keap1, kelch-like ECH-associated protein 1; NQO1, NAD(P)H quinone oxidoreductase 1 enzyme.



Figure 5. Effects of hyperoxia and Nrf2 siRNA on A549 cell apoptosis. Flow cytometry demonstrated that the rate of apoptosis was significantly higher in the hyperoxia without Nrf2 siRNA group than in the normoxia without Nrf2 siRNA group, and was significantly higher again in the hyperoxia after Nrf2 siRNA transfection group. Data are presented as the mean \pm SD. **P<0.01. Group I, normoxia without siRNA; group II, hyperoxia without siRNA; group IV, hyperoxia after Nrf2 siRNA transfection; Nrf2, nuclear factor-erythroid 2-related factor 2; siRNA, small interfering RNA.

Effects of hyperoxia and Nrf2 siRNA on cell apoptosis. Finally, the effects of hyperoxia and Nrf2 siRNA on apoptosis in A549 cells were investigated. In untransfected cells, apoptosis following exposure to hyperoxia (group II; $20.15\pm1.08\%$) was significantly higher compared with that without hyperoxia exposure (group I; $7.59\pm0.39\%$; F=357.466, P<0.01). Furthermore, the rate of apoptosis was significantly higher in cells exposed to hyperoxia after transfection with Nrf2 siRNA (group IV; $35.64\pm0.49\%$) than in untransfected cells exposed to hyperoxia (group II; F=510.221, P<0.01; Fig. 5). Together, these findings indicate that Nrf2, Keap1 and NQO1 may protect against hyperoxia-induced lung injury via the inhibition of apoptosis.

Discussion

Improvements in perinatal care have increased the survival rates of premature infants and thus have also increased the incidence of BPD (14). Multiple factors serve roles in the etiology of BPD, including hyperoxia, postnatal infection and ventilator-induced lung injury. However, the degree of prematurity and exposure to hyperoxia are the most important predisposing factors for BPD in neonates (15,16). The specific pathogenesis of ALI following hyperoxia exposure has not yet been fully defined. In the present study, transfection with Nrf2 siRNA was used to advance our understanding of the pathogenesis of ALI and help to improve the prevention and management of BPD.

The results of the present study demonstrated that relative Nrf2, Keap1 and NQO1 expression levels were significantly higher in untransfected cells exposed to hyperoxia than in untransfected cells under normoxic conditions, as was the rate of apoptosis. These findings indicate that the oxidative stress caused by hyperoxia leads to cell injury and apoptosis, and suggest that the Nrf2-Keap1-ARE-NQO1 signaling pathway may play an essential role in hyperoxia-induced ALI in addition to serving as a key endogenous antioxidant defense mechanism. Hyperoxia is known to result in oxidative stress and cell apoptosis, and to serve an important role in the development of BPD in premature infants (11). Therefore, the upregulation of genes in antioxidative signaling pathways, such as the Nrf2-Keap1-ARE-NQO1 pathway, may reduce hyperoxia-induced lung injury in preterm infants.

siRNAs are double-stranded RNAs, 20-25 base pairs in length, that provide RNA interference (17), and can be used to explore the mechanism of BPD (18). In the present study, siRNA was used to interfere with Nrf2 expression, and it was found that Nrf2 siRNA significantly decreased Keap1 and NQO1 expression under hyperoxic conditions, and increased the susceptibility of A549 cells to hyperoxia-induced damage, as demonstrated by the aggravation of apoptosis. These findings suggest that Nrf2 exerts regulatory effects on Keap1 and NQO1, which may be involved in cellular apoptosis during the occurrence of hyperoxia-induced ALI.

Nrf2 contains a highly conserved basic region leucine zipper and induces the transcription of numerous cytoprotective genes via signal transduction (19). In the present study, hyperoxia significantly increased Nrf2 expression and the rate of apoptosis, whereas Nrf2 siRNA significantly decreased Nrf2 expression and downregulated the expression of its downstream mediators Keap1 and NQO1. Furthermore, when the A549 cells were transfected with Nrf2 siRNA, they were more susceptible to hyperoxia-induced damage and exhibited an increased rate of apoptosis. These findings indicate that Nrf2 plays a key role in oxidative stress reactions and suggest that the self-protective mechanisms of lung cells exposed to hyperoxia are associated with Nrf2-Keap1-ARE-NQO1 signaling.

Nrf2 is a key genetic determinant of ALI pathogenesis (20), with Nrf2-knockout mice displaying increased susceptibility to hyperoxia-induced damage and exacerbated ALI compared with wild-type mice (21,22). Previous studies have shown that Nrf2 and its downstream effectors are significantly upregulated in the lung tissues of premature mice exposed to hyperoxia, and confer protection against BPD (23,24). Moreover, hyperoxia induces a BPD-like phenotype for which mortality rates, arrested lung development, apoptosis, inflammation, and structural protein and membrane lipid oxidation are more severe in Nrf2-/- neonatal mice compared with Nrf2+/+ neonatal mice (25). In addition to its ability to provide enhanced antioxidative effects, Nrf2 also displays strong anti-inflammatory activity (26,27). Therefore, Nrf2 appears to be a promising focus for the prevention and treatment of BPD owing to its ability to alleviate oxidative stress reactions through multiple mechanisms.

Keap1 is a cysteine-rich protein that acts as a redox damage sensor, whereas ARE is a cis-acting enhancer in a Nrf2 target gene cluster (28). The ARE consensus sequence has been identified in the promoter region of numerous genes that encode phase II detoxification enzymes (29). In the present study, it was found that Nrf2 was primarily localized in the cytoplasm of untransfected normoxic A549 cells. Moreover, relative Keap1 expression was significantly increased under hyperoxic conditions, and decreased following transfection with Nrf2 siRNA. Under physiological conditions, Keap1 traps and ubiquitinates Nrf2 in the cytoplasm, leading to its rapid degradation by the ubiquitin-proteasome system (30,31). In addition, broad complex-tramtrack-bric-a-brac and cap 'n' collar homology1 forms a heterodimer with small musculo-aponeurotic fibrosarcoma (sMAF) protein and prevents Nrf2 from binding to the ARE (32). However, hyperoxia modifies the reactive cysteine residues of Keap1, preventing it from targeting Nrf2 for ubiquitination and degradation. Consequently, Nrf2 is translocated into the nucleus and forms a heterodimer with sMAF (33), which recognizes and binds to the ARE, and activates a series of antioxidant enzymes such as NQO1 (34). Therefore, it appears that Keap1 and ARE may serve critical roles in redox homeostasis during hyperoxia-induced lung injury.

NQO1 is an antioxidant enzyme activated by cytoprotective Nrf2-Keap1-ARE target gene products (35). This enzyme catalyzes the two-electron reduction of quinone compounds to generate less reactive hydroquinones. In the present study, NQO1 expression was significantly upregulated in untransfected cells exposed to hyperoxia. This upregulation was accompanied by apoptosis, indicating the involvement of NQO1 in hyperoxia-induced ALI. Moreover, transfection with Nrf2 siRNA significantly decreased the expression of NQO1 under hyperoxic conditions and further increased the rate of apoptosis, suggesting that downregulation of the Nrf2-Keap1-ARE-NQO1 pathway exacerbates hyperoxia-induced ALI. In addition, the hyperoxia-induced cellular apoptosis exhibited a negative association with Nrf2 and NQO1 expression, suggesting that high Nrf2 and NQO1 expression in the lung tissue may strengthen its antioxidant defenses and reduce the injury induced by oxidative stress and apoptosis.

A previous study demonstrated the significant upregulation of NQO1 expression in transgenic mice carrying the human CYP1A1-Luc promoter upon exposure to hyperoxia, and suggested that these mice are less susceptible than wild-type mice to hyperoxia-induced ALI and alveolar simplification (36). In another study, miR-494 was shown to negatively regulate NQO1 and block the Nrf2 signaling pathway, resulting in the acceleration of ALI in rats with sepsis-associated acute respiratory distress syndrome (37). Furthermore, in cells exposed to hyperoxia, oxidative stress has been shown to increase the expression of NQO1 and regulate ROS generation, thereby preventing cells and tissues from undergoing hyperoxia-induced lung injury (38). The findings of the present study indicate that hyperoxia-induced activation of the Nrf2-Keap1-ARE-NQO1 signaling pathway is Nrf2-dependent and protects against hyperoxia-induced lung injury via the inhibition of apoptosis. However, further studies are required to fully elucidate the relationship between the Nrf2-Keap1-ARE-NQO1 signaling pathway

and the duration of hyperoxia exposure, as well as the threshold of its protective effect. In addition, the effect of upregulation of the Nrf2-Keap1-ARE-NQO1 pathway on apoptosis should be explored to confirm the conclusions of the present study.

In summary, the present study demonstrated that the Nrf2-Keap1-ARE-NQO1 signaling pathway protects against the hyperoxia-induced injury of lung cells by inhibiting apoptosis. The protective effects of the Nrf2-Keap1-ARE-NQO1 signaling observed in the *in vitro* model provide insights into the pathogenesis of hyperoxia-induced ALI and indicate that drugs that induce Nrf2 and NQO1 expression may be promising agents for the prevention and treatment of BPD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BWW, CC and XHG designed the study. BWW and CC managed the experiments, analyzed the data and drafted the manuscript. CC, XHG, XYZ and XYC interpreted the results and revised the manuscript. The authors agree to be accountable for the version published. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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