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Assessment of Protection Offered by the NRF2 Pathway Against Hyperoxia-Induced Acute Lung Injury in NRF2 Knockout Rats

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Abstract

Nuclear factor erythroid 2-related factor (Nrf2) is a redox-sensitive transcription factor that responds to oxidative stress by activating expressions of key antioxidant and cytoprotective enzymes via the Nrf2-antioxidant response element (ARE) signaling pathway. Our objective was to characterize

hyperoxia-induced acute lung injury (HALI) in Nrf2 knock-out (KO) rats to elucidate the role of this pathway in HALI. Adult Nrf2 wildtype (WT), and KO rats were exposed to room air (normoxia) or >95% O₂ (hyperoxia) for 48 h, after which selected injury and functional endpoints were measured *in vivo* and *ex vivo*. Results demonstrate that the Nrf2-ARE signaling pathway provides some protection against HALI, as reflected by greater hyperoxia-induced histological injury and higher pulmonary endothelial filtration coefficient in KO versus WT rats. We observed larger hyperoxia-induced increases in lung expression of glutathione (GSH) synthetase, 3-nitrotyrosine (index of oxidative stress), and interleukin-1 β , and *in vivo* lung uptake of the GSH-sensitive SPECT biomarker ^{99m}Tc-HMPAO in WT compared to KO rats. Hyperoxia also induced increases in lung expression of myeloperoxidase in both WT and KO rats, but with no difference between WT and KO. Hyperoxia had no effect on expression of Bcl-2 (anti-apoptotic protein) or peroxiredoxin-1. These results suggest that the protection offered by the Nrf2-ARE pathway against HALI is in part via its regulation of the GSH redox pathway. To the best of our knowledge, this is the first study to assess the role of the Nrf2-ARE signaling pathway in protection against HALI using a rat Nrf2 knockout model.

INTRODUCTION

Acute lung injury (ALI) is one of the most frequent causes of admission to medical intensive care units^{1, 2}. ALI and its most serious form Acute Respiratory Distress Syndrome (ARDS) are characterized by rapidly progressing hypoxic lung failure and carry high morbidity. ALI is often triggered by overwhelming infections associated with sepsis or pneumonia, including that caused by COVID-19³. Even prior to COVID-19, ARDS occurred in ~250,000 patients/year in the United States, carried a mortality rate that exceeded 40%, lacked early detection tools, and had limited therapies^{1, 2}. Ventilation with high concentrations of oxygen (hyperoxia) is required to maintain adequate oxygenation to systemic organs and tissues⁴. However, sustained exposure to high fractions of oxygen causes or exacerbates ALI⁵.

Oxidative stress, inflammation, and mitochondrial dysfunction are key pathways in the pathogenesis of ALI, with the pulmonary capillary endothelium a primary and early target⁶⁻¹². Nuclear factor erythroid 2-related factor (Nrf2) is a redox-sensitive transcription factor that responds to oxidative stress by activating the expression of key antioxidant and cytoprotective enzymes via the Nrf2-antioxidant response element (ARE) signaling pathway^{13, 14}. As such, Nrf2 has been shown to regulate glutathione (GSH) biosynthesis, protect mitochondrial function, and inhibit apoptosis, all of which are pertinent to the pathogenesis of ALI¹³⁻¹⁷.

Previous studies in mice have suggested that the Nrf2-ARE signaling pathway provides protection against acute lung injuries, including ARDS¹⁸⁻²². Using Nrf2 knockout mouse strains, Cho et al. suggested that the Nfe2l3 gene, which encodes the Nrf2 transcription factor, diminishes susceptibility to hyperoxic lung injury²². In another study, Cho et al. showed that Nrf2 knockout mice were more sensitive to hyperoxia (>95% O₂ for 72 h) than wild-type mice¹⁸. Additionally, Reddy et al. demonstrated a role for Nrf2-regulated GSH synthesis not only in protection against hyperoxia-induced injury, but also in the resolution of lung injury following exposure to hyperoxia²⁰. These results suggest a protective role for Nrf2 against hyperoxic lung injury.

Priestley et al. developed a Nrf2 knock-out (KO) rat model²³. They reported that mRNA expression for catalase, HO-1, superoxide dismutase 1 and 2, and glutathione reductase in livers of KO rats were 35% to 55% lower than those in wild-type (WT) rats. The objective of the present study is to use the rat Nrf2 KO model to assess specific lung injury endpoints and the role of this pathway in protection against hyperoxia-induced ALI in rats. Nrf2 WT and KO rats were compared under normoxic and hyperoxic conditions using both *in vivo* molecular imaging to demonstrate alterations in the pulmonary oxidant status and *ex vivo* assays to determine changes in selected functional and structural endpoints. It is important to perform studies on the role of the Nrf2 pathway in hyperoxia-induced ALI (HALI) in this rat Nrf2 KO model since rats undergo a unique acclimation process during exposure to hyperoxia and develop tolerance to otherwise lethal hyperoxia that mice do not^{24, 25}. Moreover, the sequence of events leading to O₂ toxicity is best described in rats⁵.

MATERIALS AND METHODS

Materials

HMPAO (Ceretek®) was purchased in kit form from GE Healthcare (Arlington Heights, IL), and technetium-labeled macroaggregated albumin (^{99m}Tc-MAA, particle sizes 20–40 μm) was purchased from Cardinal Health (Wauwatosa, WI). Antibodies were purchased from Abcam for GSH synthetase (cat # 124811), Bcl-2 (cat # ab59348), myeloperoxidase (MPO) (cat # ab188211), and 3-nitrotyrosine (3-NT) (cat # ab52309). Antibodies for interleukin-1β (IL-1β) and peroxiredoxin-1 (Prdx-1) were purchased from RD systems (cat # MAB5011) and Cell Signaling (cat # 8499), respectively.

Rat model of human ALI

All treatment protocols were approved by the Institutional Animal Care and Use Committees of the Zablocki Veterans Affairs Medical Center, the Medical College of Wisconsin, and Marquette University.

Male littermate Sprague Dawley Nrf2 KO (*SD-Nfe212^{em1M^{cwi}}*) wildtype (WT) and homozygous (KO) rats were bred by and obtained from the Medical College of Wisconsin Rat Research Model Service Center as described by Priestley et al.²³. For normoxia (control) studies, adult (68–77 days old) WT and KO rats (321 ± 7 (SE) g, n = 25) were housed in chambers with room air. For hyperoxia studies, age- and weight-matched WT (331 ± 8 g, n = 18) and KO rats (332 ± 14 g, n = 17) were housed adjacent to the room-air chambers in exposure chambers with > 95% O₂ for 48 h, as previously described²⁶. This exposure period was chosen since it is relatively early in the pathogenesis of hyperoxia-induced ALI, prior to clinical evidence of respiratory distress^{5, 26, 27}. Four groups of rats were studied: WT normoxia, WT hyperoxia, KO normoxia, and KO hyperoxia.

Lung wet-to-dry weight ratio, weight of pleural effusion

Heart and lungs from a randomly selected subset of each group of rats were isolated as previously described²⁶. The lungs were dissected free of the heart, trachea and mainstem bronchi and total lung wet weight was obtained. The left lung lobe was weighed and dried at 60°C for 72 h for wet-to-dry weight ratio and the remaining lung lobes were used for histological studies described below²⁶. For a subset of rats exposed to hyperoxia, cotton gauze was inserted into the chest cavity to absorb any pleural effusion²⁸. The gauze was weighed before and after use and the difference in weights was determined.

Histology

In a randomly selected subset of rats from each group, excised lungs were fixed after inflation in 10% neutral buffered formalin (Fisher Scientific, Pittsburg, PA) and embedded in paraffin. Whole-mount sections of lung were cut (4 μm thick), processed and stained with Hematoxylin & Eosin (H&E, Richard Allan, Kalamazoo, MI). Using high resolution jpeg images of the slides, an investigator masked to the treatment groups obtained 3 to 5 representative images from preselected areas of the lung on each slide, avoiding large vessels or airways at 100X (for neutrophils and edema) and 400X (for alveolar septum thickness). These images were then scored for injury independently and values for each rat averaged for a single "n." We used a 0 to 2 scoring system suggested by Matute-Bello et al.²⁹ for neutrophil influx, edema, and thickness of the diffusion barrier (Table 1).

Table 1 - Endpoints for histological injury grading on a scale of 0–2 for each injury

Histology injury endpoint/score	0	1	2
Neutrophilic influx	None to very rare	Perivascular or peribronchiolar only	Intra-alveolar and widely distributed
Edema	None to very rare	Proteinacious material in <5% and <20% field	proteinacious material in >20% field
Thickness of alveolar septum	$\leq 1.8 \times$ control thickness	>1.8 but $<2.5 \times$ control thickness	$>2.5 \times$ control thickness

Western blots

Western blot analysis was carried out as previously described³⁰ on whole lung tissue homogenate to quantify the expressions of glutathione (GSH) synthetase as an indicator of the GSH redox pathway, the anti-apoptotic protein Bcl-2, and pro-inflammatory cytokine interleukin-1 β (IL-1 β) (for electrophoresis, protein used was 40 $\mu\text{g}/\text{lane}$). For myeloperoxidase (MPO), as an indicator of inflammation, and the anti-oxidant peroxiredoxin 1 (Prdx-1), 45 $\mu\text{g}/\text{lane}$ protein was loaded. Western blot analysis was also carried out on whole lung tissue homogenate (protein concentration 30 $\mu\text{g}/\mu\text{L}$) to quantify the expression of 3-nitrotyrosine (3-NT) as an indicator of oxidative stress²⁷.

Pulmonary vascular endothelial filtration coefficient (K_f)

Randomly selected rats from normoxia and 48-h hyperoxia WT and KO groups were anesthetized with pentobarbital sodium (50–100 mg/kg i.p.). The lungs were then removed and suspended from a calibrated force displacement transducer (ModelFT03; Grass Instruments), attached to a rat lung ventilation-perfusion system, and lung weight was monitored continuously^{26, 31}. The value of K_f , a measure of vascular permeability, was then determined using the approach described by Bongard et al.³¹. Briefly, after a 10 min stabilization period with the venous pressure (P_V) set at atmospheric pressure, P_V was raised to 3.7 mm Hg and the lung perfused for 10 min. Then, P_V was raised to 10 mmHg and perfusion continued for an additional 10 min. K_f was determined by dividing the difference in the rate of lung weight gain measured 10 min after increasing P_V from 3.7 to 10 mm Hg and after increasing P_V from 0 to 3.7 mm Hg by the difference in pulmonary capillary pressure at these P_V values. For each P_V , the capillary pressure was estimated as the average of arterial and venous pressures. K_f was normalized to gram of dry lung weight.

Imaging studies

In vivo imaging studies described below were conducted on randomly selected subsets of rats from each group. Sample sizes were chosen to achieve a power $\geq 85\%$ using power analysis (ANOVA power) based on previously published means and standard deviations of the lung uptake of ^{99m}Tc -HMPAO^{26, 28, 32}.

^{99m}Tc -HMPAO was constituted and labeled according to kit directions as previously described^{26, 28}. Rats were anesthetized with sodium pentobarbital (40–50 mg/kg, i.p.) and a tail vein was cannulated. The rat was then placed supine on a plexiglass plate (4 mm) positioned directly on the face of a parallel-hole collimator (hole diameter = 2 mm, depth = 25 mm) attached to a modular gamma camera (*Radiation Sensors, LLC*) for planar imaging^{26, 28}. An injection (37–74 MBq) of ^{99m}Tc -HMPAO was administered via the tail vein catheter. ^{99m}Tc -HMPAO reaches steady-state in the lung by 20 min postinjection, at which time three thirty-second planar images were acquired^{26, 28}.

A subsequent injection of ^{99m}Tc -MAA (37 MBq) was made via the same tail-vein cannula and the rat re-imaged. The ^{99m}Tc -MAA injection provided a planar image in which the lung boundaries were clearly identified, since $>95\%$ of ^{99m}Tc -MAA lodged in the lungs. After imaging, the rats were euthanized with an overdose of pentobarbital.

Image analysis

Images were analyzed using MATLAB-based software developed in-house. The boundaries of the upper portion of the lungs were identified in the high-sensitivity ^{99m}Tc -MAA images and manually outlined to construct a lung region of interest (ROI) free of liver contribution²⁶. The ^{99m}Tc -MAA lung ROI mask was then superimposed on the ^{99m}Tc -HMPAO image yielding a lung ^{99m}Tc -HMPAO ROI. No registration was required since the animal was maintained in the same location throughout the imaging study. Background regions in the upper forelimbs were also identified in the ^{99m}Tc -HMPAO image to normalize lung activity for injected ^{99m}Tc -HMPAO specific activity, dose, and decay^{26, 28}. Mean counts/sec/pixel/injected dose within both the lung and forelimb-background ROIs were then determined and decay corrected. The ratio of the lung and background ROI signals averaged over the 3x30 second time interval, when the ^{99m}Tc -HMPAO signal within the ROIs had reached steady state, was used as the measure of lung ^{99m}Tc -HMPAO uptake^{26, 28}.

Statistical analysis

Statistical evaluation of data was carried out using SigmaPlot version 12.0 (Systat Software Inc., San Jose, CA). Results are expressed as means \pm SE unless stated otherwise. To evaluate differences between two groups of rats, an unpaired two-tailed *t* test was used with the level of statistical significance set at 0.05.

RESULTS

Lung wet/dry weight ratios, pleural effusion, and K_f

Rat exposure to hyperoxia for 48 h increased lung wet/dry weight ratio in both WT and KO groups and the increase was not different between WT and KO groups (Table 2). No pleural effusion was observed in either group of normoxic rats. Pleural effusions were identified in both of the hyperoxia cohorts, but no differences between the WT and KO groups were observed (Table 2).

Table 2 - Injury endpoints

Group	Lung wet/dry wt	Pleural effusion (g)	3-NT	MPO	IL-1 β
WT normoxia	5.28 \pm 0.21 (n = 12)	ND	1.0 \pm 0.26 (n = 6)	\pm 0.27 (n = 6)	\pm 0.17 (n = 6)
WT hyperoxia	6.07 \pm 0.16* (n = 15)	3.53 \pm 0.79 (n = 16)	2.45 \pm 0.35* (n = 6)	3.41 \pm 0.36* (n = 6)	1.92 \pm 0.28* (n = 6)
KO normoxia	5.35 \pm 0.23 (n = 8)	ND	1.0 \pm 0.36 (n = 4)	\pm 0.28 (n = 3)	\pm 0.32 (n = 4)
KO hyperoxia	5.96 \pm 0.12* (n = 17)	3.82 \pm 1.02 (n = 14)	6.45 \pm 1.67* , [†] (n = 6)	3.13 \pm 0.37* (n = 6)	4.95 \pm 1.05* , [†] (n = 6)

3-NT indicates 3-nitrotyrosine; MPO, myeloperoxidase; IL-1 β , interleukin-1 β . 3-NT, MPO, and IL-1 β band density normalized to β actin and resulting values normalized to corresponding normoxia values. n = number of rats. ND, not detectable. Values are mean \pm SE.

*Different from normoxia.

[†]Different from WT hyperoxia ($P < 0.05$).

The lung vascular endothelial filtration coefficient (K_f) was elevated in both the WT and KO rats after 48 h of hyperoxia (Fig. 1) compared to normoxia. However, the hyperoxia-induced increase in K_f was ~50% greater in the KO group than the WT group.

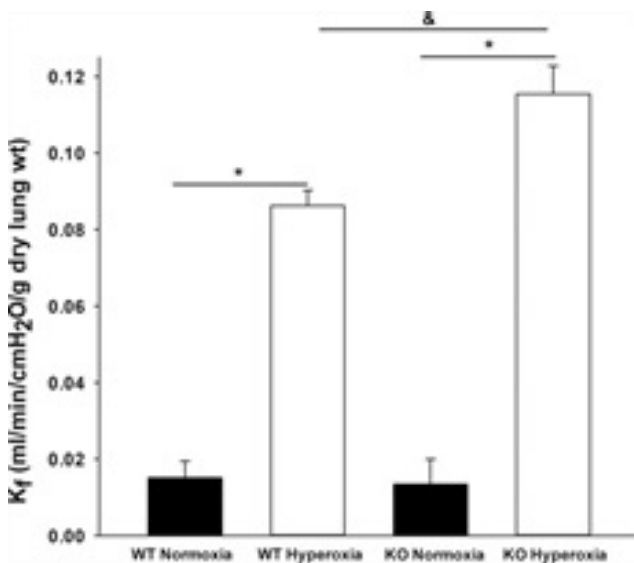


Fig. 1: Pulmonary vascular endothelial filtration coefficient, K_f , in lungs of wild-type (WT) and knockout (KO) rats exposed to normoxia or hyperoxia. * Different between normoxia and hyperoxia. & Different between WT hyperoxia and KO hyperoxia. n = number of rats: WT normoxia⁴, WT hyperoxia⁴, KO normoxia³, and KO hyperoxia⁴.

Histology

Images of representative lung sections stained with H&E appear in Figure 2. Images from WT normoxia (A) and KO normoxia (B) rats show the expected lacy architecture of the lungs. The results (Table 3) show a higher overall histology score for the hyperoxic KO lungs than for hyperoxic WT lungs. This

reflects the higher edema score in the KO group, with no difference in the neutrophilic influx or alveolar septum thickness scores between the hyperoxic WT and KO groups.

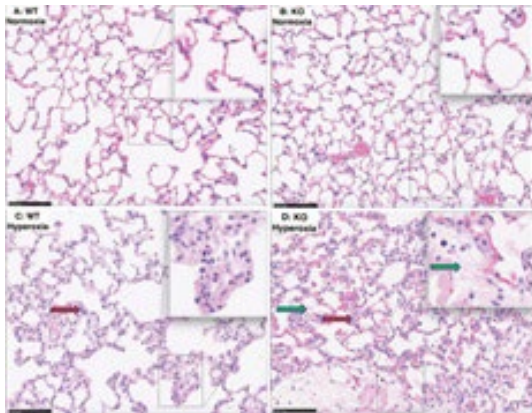


Fig. 2: Representative images of WT normoxia (A), KO normoxia (B), WT hyperoxia (C), and KO hyperoxia (D) H&E stained lungs slices. Scale is 100 μm.

Table 3 - Histological injury grading

Group	Total score	Neutrophilic influx	Edema	Thickness of alveolar septum
WT normoxia (n = 3)	0.05 ± 0.05	0.05 ± 0.05	0	0
WT hyperoxia (n = 6)	1.86 ± 0.26	1.17 ± 0.11	0.25 ± 0.14	0.44 ± 0.08
KO normoxia (n = 3)	0.11 ± 0.06	0	0	0.11 ± 0.06
KO hyperoxia (n = 6)	2.81 ± 0.09*	1.28 ± 0.06	0.86 ± 0.10*	0.67 ± 0.09

n, number of rats. Values are mean ± SE.

*Different from WT hyperoxia ($P < 0.05$).

Western blots

Expression of MPO increased with hyperoxia in both WT and KO rats (Table 2), however the increase was not different between WT and KO rats. The measured hyperoxia-induced increases in MPO lung expression in both WT and KO rats (Table 2) are consistent with the increases in the neutrophilic influx histological scores (Table 3). Table 2 also shows that the hyperoxia-induced increases in both 3-NT and cytokine IL-1β expressions, relative to normoxic values, were larger in KO rats than in WT rats.

Table 4 shows that expression of GSH synthetase was elevated following 48 h of exposure to hyperoxia in WT rats, but not in KO rats. Expressions of Bcl-2 and Prdx-1 were not elevated following exposure to hyperoxia in either the WT or KO groups (Table 4).

Table 4 - Western blots of anti-oxidants and anti-apoptotic proteins

Group	Bcl-2	Prdx-1	GSH synthetase
WT normoxia	1.00 ± 0.13 (n = 6)	1.00 ± 0.04 (n = 6)	1.00 ± 0.18 (n = 6)
WT hyperoxia	1.14 ± 0.16 (n = 6)	1.03 ± 0.09 (n = 6)	1.76 ± 0.22* (n = 6)
KO normoxia	1.00 ± 0.05 (n = 4)	1.00 ± 0.07 (n = 4)	1.00 ± 0.18 (n = 4)
KO hyperoxia	1.07 ± 0.14 (n = 6)	1.13 ± 0.09 (n = 6)	1.04 ± 0.09 (n = 6)

GSH indicates glutathione; Prdx-1, peroxiredoxin-1. Bcl-2, Prdx 1 and GSH synthetase band density normalized to β actin and resulting values normalized to corresponding normoxia values. n = number of rats. Values are mean \pm SE.

*Hyperoxia different from normoxia ($P < 0.05$).

Imaging results

Lung uptake of ^{99m}Tc -HMPAO was quantified from the biomarker images in groups of WT and KO rats exposed to either normoxia or hyperoxia. Figure 3 shows lung uptake (the ratio of lung-to-background signal at steady-state) of ^{99m}Tc -HMPAO in all four groups of rats studied. One-way ANOVA using the Holm-Sidak method was used to evaluate differences between means of the four groups. ^{99m}Tc -HMPAO uptake was $\sim 120\%$ greater in WT hyperoxia rats compared to WT normoxia rats, consistent with our previously published results in Sprague Dawley rats^{26, 27}. ^{99m}Tc -HMPAO uptake was modestly increased in KO hyperoxia rats compared to KO normoxia; this increase was not significant and was 72% smaller than the increase with hyperoxia in the WT group.

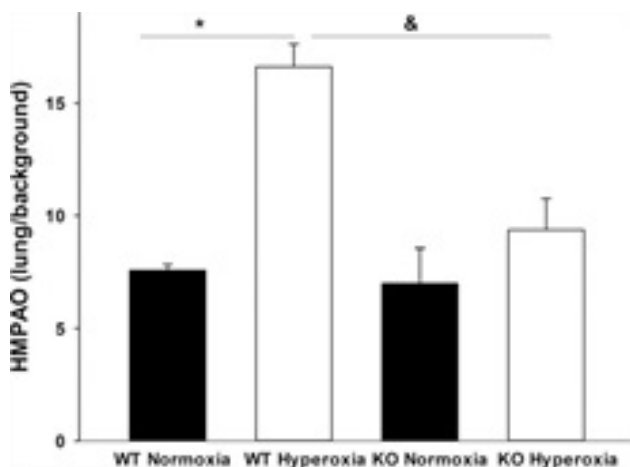


Fig. 3: Lung uptake of ^{99m}Tc -HMPAO in wild-type (WT) and knockout (KO) rats exposed to normoxia or hyperoxia. * Different between WT normoxia and WT hyperoxia. & Different between WT hyperoxia and KO hyperoxia. n = number of rats: WT normoxia³, WT hyperoxia⁵, KO normoxia⁵, and KO hyperoxia⁷.

DISCUSSION

The transcription factor Nrf2, which is constitutively localized mainly in the cytosol, translocates to the nucleus in response to oxidative stress, where it up-regulates a wide range of cytoprotective genes via the anti-oxidant response element (ARE) signaling pathway. As such, the Nrf2-ARE signaling pathway is a positive regulator of many cytoprotective genes, including those that encode for antioxidant enzymes (e.g., GSH system), anti-inflammatory enzymes (e.g., hemeoxygenase1), anti-apoptotic proteins (e.g., Bcl-2), and phase II detoxifying enzymes (e.g., NQO1), and is a key regulator of redox homeostasis^{13-15, 33}. The objective of this study was to assess the role of the Nrf2-ARE signaling pathway in protection against hyperoxia-induced acute lung injury (HALI) using Nrf2 KO rats. The results are the first to demonstrate that the Nrf2-ARE signaling pathway provides some protection against lung injury in rats exposed to hyperoxia for 48 h, as reflected by greater hyperoxia-induced histological injury, higher filtration coefficient, and higher hyperoxia-induced increases in lung expressions of 3-NT and IL-1 β in KO rats compared to WT rats. Other novel aspects of this study include the use of SPECT imaging to

track the protection offered by the Nrf2 pathway against HALI *in vivo*, and the assessment of the protective role of this pathway against any hyperoxia-induced increase in pulmonary vascular endothelial filtration coefficient, a measure of vascular permeability, in isolated perfused lungs.

The SPECT biomarker ^{99m}Tc -HMPAO was originally developed as a brain perfusion agent but its uptake and retention in several tissues serves as a marker of tissue redox state^{26, 34}. We have previously shown that in the lung, ^{99m}Tc -HMPAO reduction and thus its cellular retention, is strongly dependent on the oxidoreductive state of the tissue including intracellular GSH content. This is evidenced by a strong correlation between ^{99m}Tc -HMPAO lung uptake and lung tissue GSH content under a range of experimental conditions including duration of exposure to hyperoxia, mild and severe ALI resulting from intratracheal instillation of lipopolysaccharide, and treatment of HALI with hydrogen therapy^{26, 27}. One of our long-term goals is to use SPECT imaging for early detection of clinical ALI and for stratifying the risk of ARDS development in hosts with risk factors for ARDS. Knockout models such as Nrf2 provide a platform for assessing differences in key cellular processes that make a host more or less likely to develop ARDS. The measured differences in lung uptake of HMPAO *in vivo* between Nrf2 KO and WT rats following exposure to hyperoxia could be reflective of the protection offered by the Nrf2 pathway against hyperoxia-induced ALI. These results may have prognostic value in terms of early detection of ALI and/or for stratifying the risk of ARDS development in patients with risk factors for ARDS.

This study also reported higher lung expression of GSH synthetase and higher *in vivo* lung uptake of the GSH-sensitive SPECT biomarker HMPAO in WT compared to KO rats. These results suggest a larger hyperoxia-induced increase in the lung GSH redox pathway in Nrf2 WT rats compared to KO rats, suggesting that protection offered by the Nrf2-ARE signaling pathway against HALI is via the Nrf2-regulated GSH redox pathway²⁰. Further evidence of protection of the capillary-alveolar barrier is suggested by the lower values of the edema histological scores (Table 3) and K_f (Fig. 1) in hyperoxic WT relative to hyperoxic KO lungs.

We and others have measured reduced (GSH) and oxidized (GSSG) forms of glutathione content of lung tissue homogenates^{24, 35–37}. Results showed that ~99% of lung tissue glutathione content is in the GSH form, with a GSH:GSSG ratio of >100 under various experimental conditions^{24, 35–37}. In fact, the lung tissue GSSG content is low enough that in some conditions it was lower than the detection limit of the assay²⁴. As such, GSH/GSSG is not a sensitive or accurate measure of pulmonary oxidative stress. Instead, for the present study we measured lung tissue expression of 3-NT as an index of oxidative stress. Results (Table 2) show higher hyperoxia-induced increases in lung expressions of 3-NT in KO rats compared to WT rats. Curiously, normoxic Nrf2 WT lung tissue had higher expressions of 3-NT and IL-1 β than normoxic Nrf2 KO lung tissue (Supplemental Digital Content, <https://links.lww.com/SHK/B378>). Some sort of compensatory responses to shifts in pro- and anti-oxidant defense stress proteins in the KO relative to WT rats is possible, but is beyond the scope of the present work.

Previous studies using genetically-modified mice have suggested that the Nrf2-ARE signaling pathway provides protection against HALI^{18–22}. Reddy et al. showed that after 48 h of hyperoxia, the expressions of Gclc glutamate-cysteine catalytic subunit (a key enzyme for GSH biosynthesis) and GSH peroxidase were increased significantly in WT compared to normoxia mice, but not in KO mice²⁰. Consistent with

these results, they reported that total lung GSH levels increased with hyperoxia in WT mice but decreased in KO mice²⁰. Cho et al. reported that the lung expression of GSH S transferases (GST-Ya) increased more in Nrf2 WT mice than KO mice following exposure to hyperoxia for 72 h as compared to normoxia¹⁸. Furthermore, they showed that lung expression of GSH peroxidase increased more in Nrf2 WT mice than in KO mice following exposure to hyperoxia¹⁸. Those results are consistent with results of the present study in rats which show a hyperoxia-induced increase in the lung expression of GSH synthetase (Table 4) and in the lung uptake of the GSH-sensitive SPECT biomarker HMPAO (Fig. 3) in Nrf2 WT, but not KO, rats. Furthermore, the results of the present study suggest less HALI in WT rats than Nrf2 KO rats, consistent with results from a previous study regarding a role for the Nrf2-regulated GSH redox pathway in the protection offered by the Nrf2-ARE signaling pathway against HALI²⁰.

Reddy et al. showed histological evidence of hemorrhage and alveolar damage in lungs of both Nrf2 WT and KO mice following exposure to hyperoxia for 48 h, but the injury endpoints and lung wet-to-dry weight ratios were not different between Nrf2 WT and KO mice²⁰. TUNEL staining supported an increase in cell death in both WT and KO mice with hyperoxia, but the difference between WT and KO was not significant²⁰. On the other hand, the lung expression levels of the pro-inflammatory cytokine interleukin 6 (IL-6) increased significantly in WT mice with hyperoxia, compared to a smaller increase in KO mice. These results are generally consistent with results in the present study in rats (Tables 2–4).

Although there is evidence that Nrf2-regulated GSH synthesis offers protection against hyperoxia-induced injury and plays a role in the resolution of lung injury following exposure to hyperoxia, the mechanism of this protection is not well understood. Oxidative-stress induced mitochondrial dysfunction plays a key role in the pathogenesis of HALI^{31, 38–45}. The Nrf2-ARE pathway could protect mitochondria from oxidative stress associated with HALI via its effect on GSH synthesis^{13, 14}. As a regulator of cellular redox homeostasis, Nrf2 has an important effect on mitochondrial function^{14, 17, 46}. Previous studies reported a decrease in basal cellular respiration and mitochondrial ATP production as well as mitochondrial membrane potential depolarization in cells from Nrf2 knockout mice and an increase in cells from Kelch-like ECH-associated protein 1 (Keap1) knockout mice, where Keap1 is a cytoplasmic protein that acts as an inhibitor of Nrf2 transcriptional function by binding at its N-terminal regulatory domain^{13, 14, 17, 46}. Holmstrom et al. suggested that these alterations are due to the role of Nrf2 in regulating substrate availability for mitochondrial respiration via its effect on genes that encode for glucose-6-phosphate dehydrogenase (G6PD), malic enzyme 1, isocitrate dehydrogenase 1, and enzymes of the pentose phosphate pathway^{14, 17}.

One limitation of the current study is that the model used is a global Nrf2 KO model. Thus, the observed increase in susceptibility of KO rats to hyperoxia as compared to WT rats could be due to injury to organs other than the lungs. However, high O₂ levels due to hyperoxia exposure are mostly limited to the lungs because of the small effect of plasma O₂ partial pressure (pO₂) on blood O₂ content above physiological pO₂^{47, 48}. Nevertheless, the global effect could be tested by adding Nrf2 back to the lung by viral-mediated gene transfer. Such studies are outside the scope of the present study.

The lung consists of 40 different resident cell types⁴⁹. In addition, other cell types (e.g., leukocytes) are recruited to the lungs following injury^{5, 26, 27}. For the present study, the results provide no direct information regarding the contributions of the different cell types to the measured increase in susceptibility to hyperoxia in KO rats. However, endothelial cells would be expected to dominate

because of their large surface area and high fraction (~50%) of total cells in normal lungs⁵. These cells are also a primary and early target of HALI^{5, 7, 11, 28, 45, 50}. Hyperoxia-induced cellular infiltration and edema could confound the interpretation of measured hyperoxia-induced changes in the expressions of various proteins (Tables 2 and 4) and other measurements (e.g., SPECT imaging) (Fig. 3).

To the best of our knowledge, this is the first study to assess the role of the Nrf2-ARE signaling pathway in protection against ALI using this rat Nrf2 knockout model. We demonstrated that the Nrf2-ARE signaling pathway provides some protection against lung injury in rats exposed to hyperoxia. Our *in vivo* and *in vitro* results suggest a larger hyperoxia-induced increase in the lung GSH redox pathway in Nrf2 WT rats compared to KO rats, supporting protection offered by the Nrf2-ARE signaling pathway against HALI is via the Nrf2-regulated GSH redox pathway.

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