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Transcriptional responses of neonatal mouse lung to hyperoxia by Nrf2 status ☆

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ABSTRACT

Hyperoxia exposure can inhibit alveolar growth in the neonatal lung through induction of p21/p53 pathways and is a risk factor for the development of bronchopulmonary dysplasia (BPD) in preterm infants. We previously found that activation of nuclear factor erythroid 2 p45-related factor (Nrf2) improved survival in neonatal mice exposed to hyperoxia likely due to increased expression of anti-oxidant response genes. It is not known however, whether hyperoxic induced Nrf2 activation attenuates the growth impairment caused by hyperoxia in neonatal lung. To determine if Nrf2 activation modulates cell cycle regulatory pathway genes associated with growth arrest we examined the gene expression in the lungs of $Nrf2^{-/-}$ and $Nrf2^{+/+}$ neonatal mice at one and 3 days of hyperoxia exposure.

Methods: Microarray analysis was performed in neonatal $Nrf2^{+/+}$ and $Nrf2^{-/-}$ lungs exposed to one and 3 days of hyperoxia. Sulforaphane, an inducer of Nrf2 was given to timed pregnant mice to determine if *in utero* exposure attenuated p21 and IL-6 gene expression in wildtype neonatal mice exposed to hyperoxia.

Results: Cell cycle regulatory genes were induced in $Nrf2^{-/-}$ lung at 1 day of hyperoxia. At 3 days of hyperoxia, induction of cell cycle regulatory genes was similar in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ lungs, despite higher inflammatory gene expression in $Nrf2^{-/-}$ lung.

Conclusion: p21/p53 pathways gene expression was not attenuated by Nrf2 activation in neonatal lung. *In utero* SUL did not attenuate p21 expression in wildtype neonatal lung exposed to hyperoxia. These findings suggest that although Nrf2 activation induces expression of anti-oxidant genes, it does not attenuate alveolar growth arrest caused by exposure to hyperoxia.

1. Introduction

Hyperoxia is commonly used to improve oxygenation in neonates with acute lung injury. However, exposure to hyperoxia can impair alveolar growth during a critical period of postnatal lung development and lead to structural and functional changes in the mature lung [1–3]. Exposure to hyperoxia has also been shown to be major risk factor for the development of chronic lung disease of prematurity also known as bronchopulmonary dysplasia (BPD) [4].

Recent multicenter analyses have found that lower target oxygen saturations can reduce the incidence of retinopathy of prematurity and BPD. However lower target oxygen saturations have been shown to be associated with higher mortality, in very low birth weight infants [5]. This study and others, suggest that supplemental oxygen will continue to be used as an intervention for premature infants, despite it being a major risk factor for the development of chronic lung disease in this population [6].

Neonatal mice are commonly used to model BPD. Similar to the human infant, neonatal mice undergo significant postnatal alveolar growth following delivery [7]. Hyperoxic-exposure in the neonatal mouse can cause inhibition of alveolar growth similar to that found with BPD, through induction of p21 and p53 cell cycle regulatory

Abbreviations: CLDP, chronic lung disease of prematurity; Nrf2, nuclear factor erythroid 2 p45-related factor.

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genes [2,3,8–11]. We previously have shown that neonatal mice with Nrf2 null mutations have increased lung inflammation and decreased survival when exposed to hyperoxia [12]. Since alveolar growth inhibition and airway inflammation are characteristic features of BPD, we were also interested in understanding the temporal impact of Nrf2 activation on cell cycle regulatory gene expression. Recently, Cho and colleagues, used microarray gene profiling and computerized algorithms in Nrf2 mutant null mice to identify Nrf2-mediated mechanisms that may influence alveolar development and hyperoxia-induced lung injury in neonatal mice [13]. Along those lines, strategies to induce activation of Nrf2 have been proposed as potential adjuvant therapies for the treatment of chronic obstructive pulmonary disease (COPD) and possibly BPD. Indeed it has been shown that sulforaphane (SUL), an inducer of Nrf2, can reduce inflammation in wildtype $Nrf2^{+/+}$ mice exposed to chronic cigarette smoke [14]. However, the utility of Nrf2 inducers in BPD is less clear particularly since it is unknown whether hyperoxic growth inhibition can be attenuated by Nrf2 activation in the neonatal lung, independent of its potential modulatory effect on lung inflammation.

In this study we hypothesized that Nrf2 activation will not attenuate hyperoxic induced alveolar growth arrest in the lungs of neonatal mice and will not attenuate the induction of cell cycle regulatory genes despite an increased expression of Nrf2 inducible anti-oxidant genes. To test this hypothesis we examined gene expression in lungs of neonatal $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice exposed to one or 3 days of 85–90% hyperoxia and analyzed results using ingenuity pathway software. We also measured the expression of p21 and IL-6 in $Nrf2^{+/+}$ neonatal mice exposed to prenatal SUL and 3 days of postnatal hyperoxia using RT-PCR to assess the impact of prenatal SUL on markers of lung inflammation and growth arrest.

2. Methods

$Nrf2^{-/-}$ transgenic mice were generated as previously described [15] and bred into a CD-1 background (in the laboratory of S. Biswal). Timed pregnant wildtype CD-1 mice for the SUL experiments were obtained from Charles River Laboratories International, Inc. (251 Ballardvale Street, Wilmington, MA). All experiments were conducted in accordance with the standards established by the United States Animal Welfare Acts, set forth in NIH guidelines, and the Policy and Procedures Manual of the Johns Hopkins University Animal Care and Use Committee.

2.1. Hyperoxia

Newborn mice were kept in room air until 24 h of age and then placed in a hyperoxia chamber (85–90%). Nursing mothers were rotated every 24 h to prevent injury from acute oxygen toxicity. Excess CO_2 was absorbed using anhydrous calcium sulfate (Drierite no. 23001). Newborn mice were exposed to one or 3 days of hyperoxia and then sacrificed immediately on removal from the hyperoxia chamber.

2.2. Sulforaphane

Pregnant $Nrf2^{+/+}$ mice were treated with Sulforaphane (LKT Laboratories, Inc. 545 Phalen Blvd., St. Paul, MN) (SUL), based on a previous published protocol studying the effects of Nrf2 activation in neonatal mice with epidermolysis bullosa simplex [16]. SUL was administered by intra-peritoneal injection to pregnant-timed mice at gestational days of 13, 15, and 17. Mice received 5 micromoles/dose of SUL diluted in 300 μl of sterile PBS as previously described [16]. Control mice were given 300 μl of sterile PBS.

2.3. Lung fixation and morphometry

Lungs were infused through the trachea with 0.5% low-melting agarose, fixed overnight in 4% paraformaldehyde, paraffin-embedded and cut into 5 μm sections. Lung sections were stained with hematoxylin and eosin. Ten randomly chosen areas from each lung section were photographed with the 10x objective of a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) as previously described [3]. Mean airspace chord length (MCL) was measured from each image using NIS-Elements AR (Nikon Instruments Inc., Melville, NY). The software allowed for manual identification and exclusion of large airways and vessels prior to MCL calculations.

2.4. Western

25 μg of lung protein was loaded, transferred and blocked in 5% milk in TBS-T. Primary antibodies were incubated overnight (p21, BD Pharmingen, catalog no. 556431, 1:2000 dilution, Xedar, Sigma, catalog no. WH0060401M2, 1:3000 dilution, β-actin, Abcam, catalog no.: ab8227 1:10,000 dilution). Secondary antibody (horse anti-mouse IgG, HRP-linked antibody, Cell signaling, catalog no. 7076, 1:10,000 concentration) was incubated for 1 h in 5% milk in TBS-T, washed and developed.

2.5. Microarray data analysis

Lung was isolated from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice and used for gene expression profiling. Total RNA was isolated from lung tissue using the RNeasy kit (Qiagen). Total lung RNA ($n = 3$ for each group, for a total of 24 specimens) was applied to mouse gene 1.0 ST arrays from Affymetrix. Microarray data was imported as CEL files into Genomic Suite Software (Partek, St. Louis, MO), Robust Multi-chip Analysis (RMA) background was corrected for GC content and quantile normalization and median polish were used for probe summarization. Principal component analysis was performed on the analyzed gene expression data. Array data was analyzed using Ingenuity Pathway analysis (IPA; Ingenuity Systems Inc.) Differentially expressed genes were considered significant if p-value (P) $\leqslant 0.05$ at a fold change (FC) $\geqslant 1.5$ with a FDR < 0.05.

2.6. Quantitative RT–PCR (QRT–PCR) analysis

Reverse transcription was performed using total RNA and processed with the SuperScript first-strand synthesis system for RT–PCR according to the manufacturer's protocol (Invitrogen). QRT–PCR was performed using the Applied Biosystems (Foster City, CA) TaqMan assay system, as previously described [3]. Probes and primers were designed and synthesized by Applied Biosystems (cyclin-dependent kinase inhibitor 1A and interleukin 6). The GADPH gene was used an internal endogenous control.

Differences in measured variables between treated and control groups were determined using Student's t test (two-tailed, equal variance). Statistical significance was accepted at $p < 0.05$, error bars reflect standard error of the mean.

3. Results

3.1. Nrf2. status and cell cycle regulatory and inflammatory pathway gene expression in neonatal lung after 3 days of hyperoxia

Since alveolar growth inhibition and lung inflammation are characteristic of BPD [17,18] we were interested in determining if Nrf2 status influenced the expression of cell cycle regulatory and inflammatory pathway genes in neonatal mice exposed

Table 1
Fold change of Nrf2 inducible genes after 3 days of hyperoxia.

Nrf2 inducible genes	Gene symbol	FC of $Nrf2^{+/+}$ ($3dO_2$) above $Nrf2^{-/-}$ ($3dO_2$)
Glutathione S-transferase, alpha 3	Gsta3	5.33
Glutathione peroxidase 2	Gpx2	5.16
Aldo–keto reductase family 1, member B1	AKR1B1	3.61
NAD(P)H dehydrogenase, quinone 1	Nqo1	3.04
Aldehyde oxidase 1	Aox1	2.80
Aldehyde dehydrogenase family 1, subfamily A1	Aldh1a1	2.65
Aldehyde oxidase 3	Aox3	2.59
Glutathione S-transferase, mu 5	Gstm5	2.49
Pirin	Pir	2.39
Carboxylesterase 1g	Ces1g	2.15
Carboxylesterase 1e	Ces1e	2.10
Carbonyl Reductase 2	Cbr2	2.07
Microsomal glutathione S-transferase 1	Mgst1	1.88
Aldo–keto reductase family 1, member B10	AKR1B10	1.75
Glutathione S-transferase, alpha 4	Gsta4	1.62

Numbers indicate fold changes (FC) found in lungs of $Nrf2^{+/+}$ neonatal mice exposed to 3 days (3 d) of hyperoxia (O_2) above that of lungs of $Nrf2^{-/-}$ mice exposed to $3dO_2$.

to hyperoxia. To address this question we exposed neonatal $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice to one or 3 days of hyperoxia starting at 24 h of age. All mice were immediately sacrificed following removal from hyperoxia to assess differential gene expression by microarray. Assessment was done immediately after removal from hyperoxia, based on our previous finding that neonatal CD-1 $Nrf2^{-/-}$ mice had a high mortality during the first 24 h of room air recovery after 3 days of hyperoxia [12]. Control mice were kept in room air and were matched for age, background and genotype.

As expected, hyperoxia caused a marked induction of Nrf2 inducible genes in the lungs of wildtype mice, in contrast to that of $Nrf2^{-/-}$ mice. After 3 days of hyperoxia, expression of glutathione S-transferase alpha was 5.3-fold greater, glutathione peroxidase 2 was 5.1-fold greater and NAD(P)H dehydrogenase, quinine 1 was 3.0-fold greater in the $Nrf2^{+/+}$ lung above that of $Nrf2^{-/-}$ lung (Table 1). Glutathione peroxidase 3, a gene not regulated by Nrf2 [19] was found to be equally induced in the lungs of $Nrf2^{-/-}$ and $Nrf2^{+/+}$ hyperoxia exposed mice (2.16 and 2.26-fold respectively above room air controls).

After 1 day of hyperoxia, X-linked ectodermal dysplasia receptor (Xedar) and p21, were the most highly induced genes in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice (Supplemental Table 1). These genes have been shown to regulate cell cycle progression and are p53 mediated. Interestingly, 3 days of hyperoxia caused similar induction of cell cycle regulatory genes in both $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice, despite higher induction of inflammatory genes in the $Nrf2^{-/-}$ mice (Table 2 and Supplemental Table 2). We then examined protein expression of p21 and Xedar in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. In the $Nrf2^{+/+}$ mice exposed to hyperoxia, expression of lung p21 and Xedar protein was significantly increased above room air controls. In the lungs of $Nrf2^{-/-}$ mice exposed to hyperoxia, p21 but not Xedar was significantly increased above room air controls. There was no significant difference in lung p21 and Xedar protein expression between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice exposed to hyperoxia (Fig. 1).

Since induction of cell cycle regulatory genes, including p21 have been associated with alveolar growth inhibition in neonatal mice [11], we then measured mean chord lengths (MCLs) in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice exposed to hyperoxia and room air to assess the effect of hyperoxia on alveolar growth with regard to Nrf2 status. We found that $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice exposed to 3 days of hyperoxia had larger and more simplified appearing alveoli compared to room air controls and there was no difference in MCL measurements between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice exposed to 3 days of hyperoxia, (Fig. 2).

Table 2
Fold change of cell cycle regulatory genes mediated by p53 after 3 days of hyperoxia.

Cell cycle regulatory and apoptosis genes	Gene symbol	FC of $Nrf2^{-/-}$ $3dO_2$ lung above $Nrf2^{-/-}$ 3dRA	FC of $Nrf2^{+/+}$ $3dO_2$ lung above $Nrf2^{+/+}$ 3dRA	Description
X-linked ectodermal dysplasia receptor	Xedar/ TNFRSF27	19.03	19.52	Inhibits cell growth/apoptosis
Cyclin-dependent kinase inhibitor 1A	p21	8.67	10.11	G1 checkpoint regulator
Cyclin G1	Ccng1	4.64	4.73	Cell cycle inhibitor
Zinc finger matrin type 3	Zmat3/Wig1	4.48	4.86	Inhibits tumor growth
Proline/serine-rich coiled-coil 1	Psrc1/Dda3	5.13	4.67	Promotes cell growth
Pleckstrin homology-like domain, family A, member 3	PHLDA3	3.66	3.05	Regulates AKT
Adenylate kinase 1	AK1	3.79	3.57	Growth regulation
Sestrin 2	Sesn2	3.35	2.99	Inhibits mTOR
Ribosomal protein S27-like	Rps27L	2.71	2.31	Positively regulates p21
Growth differentiation factor 15	Gdf15	3.48	2.79	Induced in response to stress
Glycoprotein (transmembrane) nmb	GPNMB	3.64	2.23	Growth delay
G2 S phase expressed protein 1	Gtse1	2.49	2.08	Regulates p21 stability
Transformation related protein 53 inducible nuclear protein 1	Trp53inp1	2.42	2.04	Regulates cell progression
B-cell translocation gene 2, anti-proliferative	BTG2	2.31	1.57	Inhibits cyclin D1
Tumor necrosis factor receptor superfamily, member 10b	Tnfrsf10b	3.05	3.22	Apoptosis
Apoptosis enhancing nuclease	Aen	3.84	3.16	Exonuclease involved in apoptosis induction
Nuclear protein 1	Nupr1/p8	2.30	2.63	Regulates cell cycle/ apoptosis
BCL2-associated X protein	Bax	2.80	2.69	Apoptosis
Prostaglandin-endoperoxide synthase 2	Ptgs2	4.71	2.20	Apoptosis
Solute carrier family 19, member 2	SLC19A2	4.81	3.77	Thiamin transporter protein
Sulfatase 2	SULF2	4.56	2.30	Regulates signaling pathways

Numbers indicate fold changes in lungs of $Nrf2^{-/-}$ mice and $Nrf2^{+/+}$ neonatal mice exposed to $3dO_2$ above that of $Nrf2^{-/-}$ and $Nrf2^{+/+}$ RA controls. Fold changes of 1.5 and above/below are significant, $p < 0.05$.

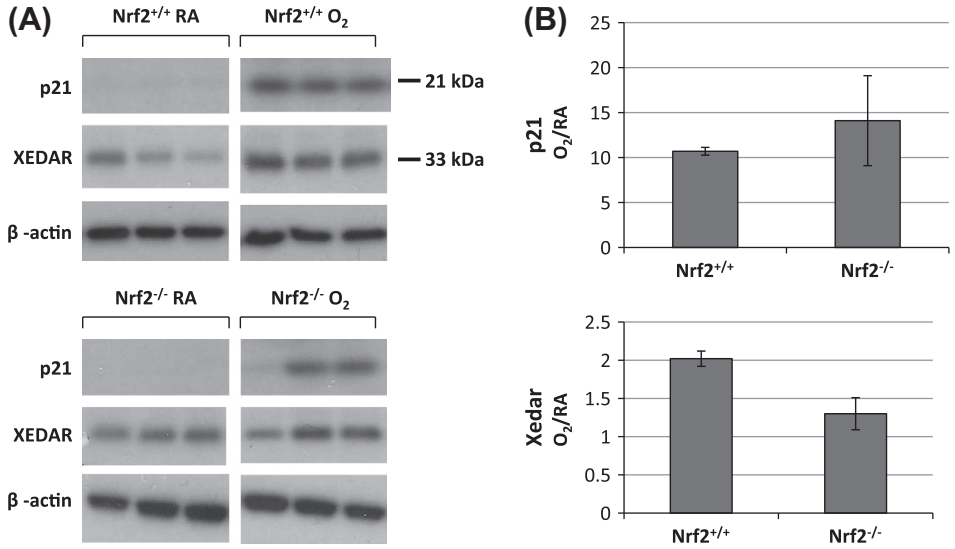

Fig. 1. Induction of p21 and Xedar in the lungs of neonatal $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice exposed to 3 days of hyperoxia. (A) Representative examples of western blots using lung homogenate from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice. Increased protein expression of p21 and Xedar found in lung from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice exposed to 3 days of hyperoxia. (B) Quantification of protein expression normalized to room air controls by densitometry. Significant differences were found between $Nrf2^{+/+}$ O_2 and $Nrf2^{+/+}$ RA for p21 and Xedar respectively ($p < 0.0001$ and $p < 0.0001$) and $Nrf2^{-/-}$ O_2 and $Nrf2^{-/-}$ RA for p21 ($p < 0.03$) but not Xedar. No differences in p21 or Xedar expression were found between $Nrf2^{+/+}$ O_2 and $Nrf2^{-/-}$ O_2, $n = 5–6$.

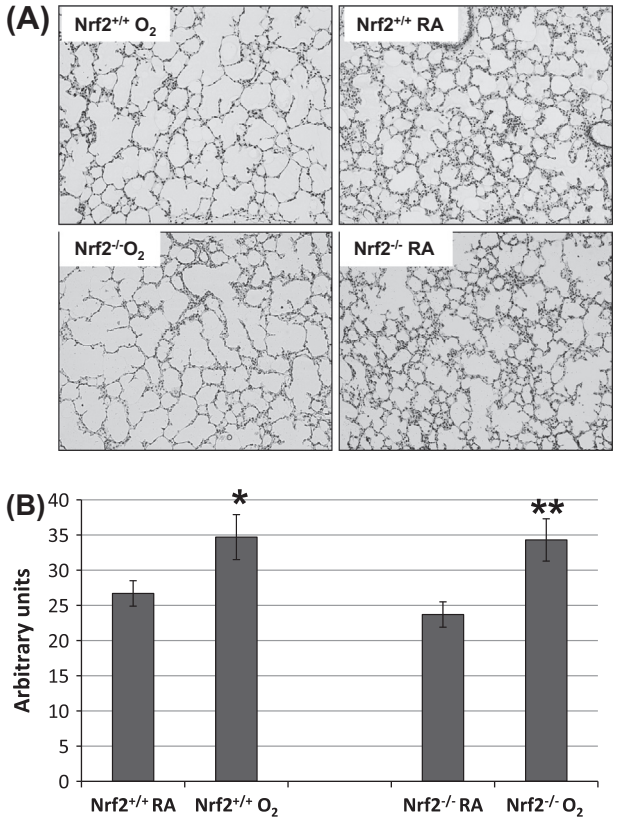

Fig. 2. Enlarged and fewer alveoli in 4 day old $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice exposed 3 days of hyperoxia. (A) Representative examples of lung histology from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice exposed to 3 days of hyperoxia or room air. The $Nrf2^{+/+}$ and $Nrf2^{-/-}$ exposed to hyperoxia had fewer and more simplified appearing alveoli compared to room air controls (20X magnification). (B) Mean chord lengths (MCLs) of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ neonatal mice in room air and after 3 days of hyperoxia. $Nrf2^{+/+}$ O_2 mice had significantly larger MCLs compared to room air $Nrf2^{+/+}$controls ($^{*}p < 0.046$) and $Nrf2^{-/-}$ O_2 mice had significantly larger MCLs compared to room air $Nrf2^{-/-}$ controls ($^{**}p < 0.028$). There was no difference between $Nrf2^{+/+}$ O_2 and $Nrf2^{-/-}$ O_2 mice, $n = 3–6$.

3.2. In utero exposure to SUL in $Nrf2^{+/+}$ neonatal mice after 3 days of hyperoxia

Induction of pro-inflammatory cytokine, IL6 has been shown to be associated with increased levels of reactive oxygen species [20]. Although IL6 was only minimally induced by microarray in $Nrf2^{+/+}$ lung exposed to 3 days of hyperoxia, we previously found a modest but significant increase in IL6 expression in neonatal wildtype lung exposed to 3 days of hyperoxia using RT–PCR [12]. To this end we treated pregnant wildtype mice with SUL, an inducer of Nrf2, to determine if *in utero* exposure to SUL would attenuate lung IL-6 expression in offspring exposed to hyperoxia (O_2). By real-time PCR, we found significantly greater expression of lung IL6 in PBS-treated $Nrf2^{+/+}$ mice exposed to postnatal O_2 compared to SUL-treated $Nrf2^{+/+}$ mice exposed to postnatal O_2 ($p < 0.01$). SUL treatment however, had no effect in attenuating the expression of p21 in the lungs of either PBS or SUL neonatal mice exposed to hyperoxia. Lung p21 was significantly induced in both PBS and SUL-treated O_2 exposed mice with no significant difference between the two groups ($p < 0.15$) (Fig. 3). Although total cell counts in the BAL of the PBS-treated O_2 mice trended towards higher numbers, we found no significant difference in BAL cell counts between the PBS and SUL-treated O_2 exposed mice. Also we did not find significant differences between PBS and SUL treated O_2 exposed neonatal mice with regard to IL6 protein expression in the BAL or lung homogenate (data not shown).

4. Discussion

Alveolar growth inhibition and lung inflammation are common features of BPD. Interventions that minimize the impact of hyperoxia on growth inhibition and inflammation in neonatal lung may prevent long-term respiratory sequelae. Gene profiling was performed on $Nrf2^{-/-}$ and $Nrf2^{+/+}$ lung to examine the influence of Nrf2 status on cell cycle regulatory and pro-flammatory gene expression in neonatal mice exposed to hyperoxia. At 1 day of hyperoxia, greater expression of the cell cycle regulatory genes Xedar [21] and p21 [10] were found in the lungs of neonatal $Nrf2^{-/-}$ mice. However, at 3 days of hyperoxia, expression of cell cycle reg-

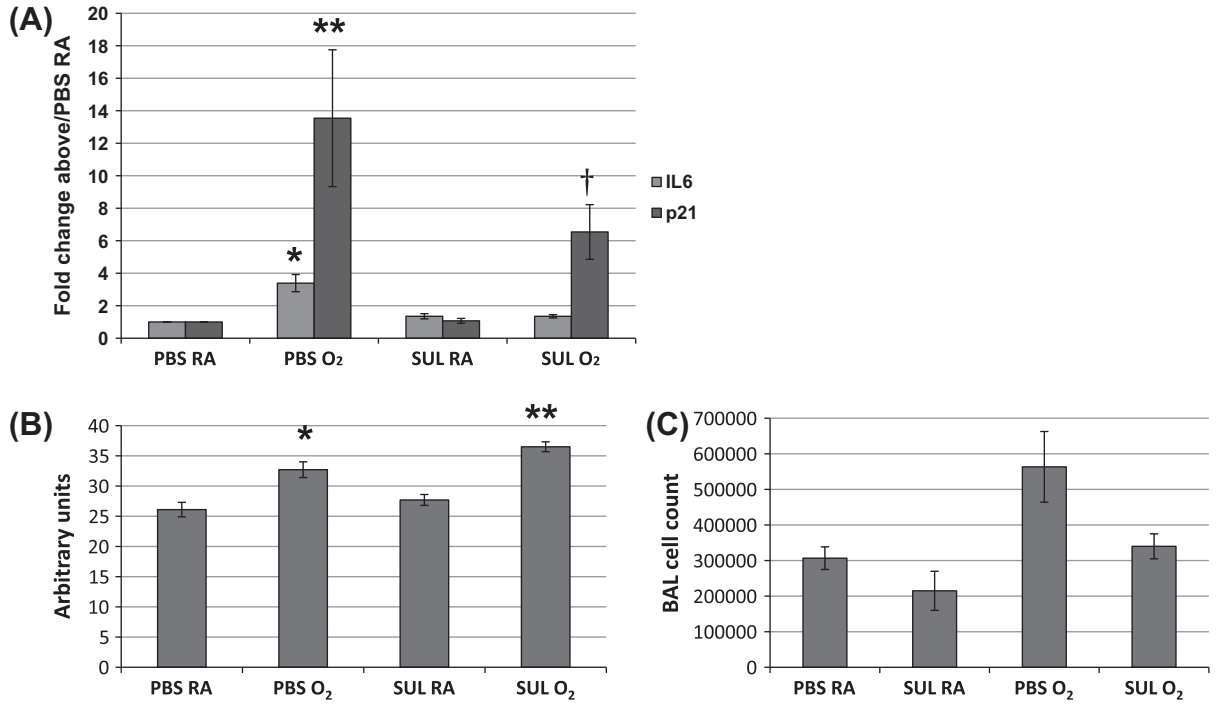

Fig. 3. *In utero* SUL exposure attenuated induction of lung IL6 but not p21 expression in neonatal wildtype ($Nrf2^{+/+}$) mice exposed to 3 days of postnatal hyperoxia. (A) Lung IL6 expression by real-time PCR was significantly greater in neonatal wildtype mice treated with *in utero* PBS and 3 days of postnatal O_2 (PBS O_2) compared to neonatal wildtype mice treated with *in utero* SUL and 3 days of postnatal O_2 (SUL O_2) ($^{*}p < 0.01$). Expression of p21 was significantly higher in PBS O_2 and SUL O_2 mice compared to PBS RA and SUL RA controls ($^{**,\dagger}p < 0.03$). No significant difference, in p21 expression was found between PBS O_2 and SUL O_2 mice, $n = 5–6$. (B) Mean chord lengths (MCLs) of PBS RA, PBS O_2, SUL RA and SUL O_2 were measured in neonatal mice. MCLs of PBS O_2 and SUL O_2 mice were significantly larger compared to PBS RA and SUL RA mice ($p < 0.02$ and $p < 0.001$, respectively). There were no significant differences between PBS RA and SUL RA ($p < 0.37$) or PBS O_2 and SUL O_2 mice, ($p < 0.06$), $n = 3$. (C). There was a trend towards higher number of total cell counts in the bronchoalveolar lavage of PBS-treated O_2 compared to SUL-treated O_2 mice ($p < 0.10$), $n = 3$.

ulatory genes, including p21 and Xedar were equally induced in both $Nrf2^{-/-}$ and $Nrf2^{+/+}$ lung despite higher expression of inflammatory pathway genes in $Nrf2^{-/-}$ lung. These findings suggest that Nrf2 induction can attenuate hyperoxia-induced lung inflammation but may be less effective in attenuating alveolar growth inhibition, particularly with longer exposures to hyperoxia.

In response to an oxidative stress such as hyperoxia, the induction of p21 and other p53- mediated cell cycle regulatory genes can help preserve the integrity of the genome by limiting progression of the cell into S phase and mitosis [11,22]. The p21senescence pathway may also be activated by unrepaired double stranded DNA breaks from reactive oxygen species and p53 accumulation [23]. Therefore, growth inhibition and senescence through induction of cell cycle regulatory genes may help preserve genomic integrity in neonatal lung exposed to hyperoxia but may also impair alveolar growth during a critical period of development. Indeed, although we found that genes involved in cell cycle regulation were induced at 1 day of hyperoxia in the lungs of $Nrf2^{-/-}$ mice, by 3 days of hyperoxia the lungs of $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice had similar induction of these genes. Supporting this was an earlier study in which we found that p21 was similarly induced in neonatal $Nrf2^{+/+}$ and $Nrf2^{-/-}$ lung exposed to 3 days of hyperoxia [12].

We previously reported that neonatal O_2 exposed $Nrf2^{+/+}$ mice had better survival compared to neonatal O_2 exposed $Nrf2^{-/-}$ mice. In our present study we found that lung IL-6 mRNA expression was significantly higher in neonatal O_2 exposed $Nrf2^{-/-}$ mice compared to neonatal O_2 exposed $Nrf2^{+/+}$ mice, but we were unable to find differences at the protein level. Nevertheless Choo-Wing and colleagues reported that neonatal IL6 transgenic mice had markedly worse survival in hyperoxia [24] similar to what we found in neonatal $Nrf2^{-/-}$ O_2 exposed mice. In their study they also reported that preterm infants with respiratory distress syndrome had higher levels of tracheal IL6 levels compared to controls. Another study reported that the Nrf2 activator CDDO-IM decreased IL-6 levels in LPS-treated peritoneal neutrophils [25]. We speculate that the induction of Nrf2 responsive anti-oxidant genes attenuates the induction of inflammatory pathway genes such as IL6 in the $Nrf2^{+/+}$ mice exposed to hyperoxia and that this in turn may help to improve survival. Similarly Cho and colleagues noted augmented lung injury and decreased survival of $Nrf2^{-/-}$ mice with prolonged hyperoxia [13]. Taken together these studies suggest that Nrf2 activation may limit injury by attenuating hyperoxia- induced inflammation in the lungs of neonates exposed to hyperoxia.

A recent study in neonatal mice found that hyperoxia caused epigenetic changes which induced p21 expression, alveolar growth inhibition and cell senescence [26]. Interestingly in this study azithromycin a drug that has both anti-inflammatory and antimicrobial properties was not effective in attenuating the alveolar hypoplasia caused by hyperoxia. In addition, clinical studies in which preterm infants at risk for BPD were treated with the antioxidant n-acetylcysteine failed to show a decrease in incidence of BPD in those treated with n-acetylcysteine [27,28]. It is possible that these studies [26–28] were not beneficial because n-acetylcysteine and/or azithromycin does not attenuate the induction of regulatory genes associated with impaired alveolar growth in BPD caused by hyperoxia. Indeed the lack of efficacy of these animal and human studies suggests that anti-oxidant and/or anti-inflammatory interventions alone cannot attenuate alveolar growth inhibition in the BPD lung. When we gave SUL to pregnant mice we found that *in utero* exposure attenuated IL-6 expression in the lungs of neonatal mice exposed to hyperoxia, but SUL did not decrease p21 expression or mitigate the effect of hyperoxia on alveolar growth inhibition. Our findings indicate that SUL has other

complex biological effects that may have influenced the results of our study. For example SUL has been reported to have growth inhibitory effects [29]. While this may be potentially beneficial as an anti-carcinogenic therapy, in the neonate SUL may potentially interfere with critical postnatal alveolar growth in the developing lung. Nevertheless, SUL or other selective Nrf2 inducers could potentially be useful as adjuvant therapies in preventing or modulating the severity of BPD by decreasing lung inflammation. Studies examining the long term effects of *in utero* Nrf2 inducers on alveolar growth, lung inflammation, lung function and structure and overall survival would be helpful in determining the utility of these agents.

Our study is similar to Cho and colleagues and our microarray analysis revealed similar results with regard to the hyperoxia experiments. Some differences however exist in our methodology and focus. We used a different background of mice and we exposed mice to 85–92% for one or 3 days of exposure rather than 100% hyperoxia for up to 3 days. We limited our interpretation of the data to pathways known to be dysregulated in BPD, specifically pathways involved in cell cycle regulation and inflammation. Cho and colleagues, in turn reported on many cellular pathways that were differentially expressed in lungs of neonatal $Nrf2^{-/-}$ mice exposed to 100% hyperoxia compared to wildtype lung. Redox homeostasis and ARE-anti-oxidant pathways were among some of the pathways they found to be suppressed, while endocytosis, transport and developmental pathways were noted to be induced in $Nrf2^{-/-}$ lung. Although informative, further interpretation of the relationships and clinical significance of this data with regard to the multiple pathways found to be differentially regulated is required to determine if these pathways are relevant with regard to BPD development or severity.

In summary using transgenic mice, we found that Nrf2 activation appears to attenuate the induction of inflammatory gene expression, but not cell cycle regulatory gene expression in a model of BPD in neonatal mice. We also found that *in utero* exposure to SUL helped attenuate lung inflammation in neonatal mice exposed to hyperoxia but not alveolar growth arrest. These findings suggest that caution should be exercised in using Nrf2 inducers such as SUL as a therapy for chronic lung disease in the neonate. For instance, although inflammation in the neonatal lung may be attenuated, growth inhibition during a period of rapid postnatal alveolar growth may be exacerbated or not improved by induction of Nrf2. Further study evaluating the efficacy of Nrf2 inducers is warranted in infants with chronic lung disease before clinical use of this strategy should be recommended.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.09.021.

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