

# S-Nitrosoglutathione Attenuates Airway Hyperresponsiveness in Murine Bronchopulmonary Dysplasia

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## ABSTRACT

Bronchopulmonary dysplasia (BPD) is characterized by lifelong obstructive lung disease and profound, refractory bronchospasm. It is observed among survivors of premature birth who have been treated with prolonged supplemental oxygen. Therapeutic options are limited. Using a neonatal mouse model of BPD, we show that hyperoxia increases activity and expression of a mediator of endogenous bronchoconstriction, S-nitrosoglutathione (GSNO) reductase. MicroRNA-342-3p, predicted in silico and shown in this study in vitro to suppress expression of GSNO reductase, was decreased in hyperoxia-exposed pups. Both pretreatment with aerosolized GSNO and inhibition of GSNO reductase attenuated

airway hyperresponsiveness in vivo among juvenile and adult mice exposed to neonatal hyperoxia. Our data suggest that neonatal hyperoxia exposure causes detrimental effects on airway hyperreactivity through microRNA-342-3p-mediated upregulation of GSNO reductase expression. Furthermore, our data demonstrate that this adverse effect can be overcome by supplementing its substrate, GSNO, or by inhibiting the enzyme itself. Rates of BPD have not improved over the past two decades; nor have new therapies been developed. GSNO-based therapies are a novel treatment of the respiratory problems that patients with BPD experience.

## Introduction

Globally, more than 11% of babies are born before 37 weeks of gestation (premature), and the number of premature births is increasing worldwide (Blencowe et al., 2012). Bronchopulmonary dysplasia (BPD) is the major pulmonary morbidity of extreme prematurity, with an estimated 14,000 diagnoses made annually in the United States (Lemons et al., 2001; Van Marter, 2009) and annual healthcare expenditures in excess of \$4.5 billion (Maitre et al., 2015). After their initial care, half of

extremely premature patients will be rehospitalized in early childhood for respiratory causes (Furman et al., 1996). Follow-up studies of children and young adults born prematurely show evidence of impaired pulmonary function, manifesting signs of bronchospastic airway hyperresponsiveness (Kim et al., 2006; Lum et al., 2011) and obstructive pulmonary disease with decreased predicted forced expiratory volume in 1 second (Vrijlandt et al., 2006; Fawke et al., 2010; Vollsaeter et al., 2013), decreased predicted forced expiratory flow (Fawke et al., 2010; Vollsaeter et al., 2013), and reduced exercise capacity (Vrijlandt et al., 2006). Indeed, airway hyperreactivity and asthma-like symptoms are common long-term pulmonary consequences of both premature birth and BPD (Greenough, 2006, 2013; Jaakkola et al., 2006; Baraldi et al., 2009).

S-nitrosothiols (SNOs) are molecules in which nitric oxide is bound to a cysteine thiol. They regulate the biologic activity of many target proteins (Foster et al., 2009). One such SNO is S-nitrosoglutathione (GSNO), an endogenous bronchodilator, which is 100-fold more potent than the asthma medication theophylline (Gaston et al., 1994). GSNO is capable of relaxing smooth muscle in both a guanylate cyclase-dependent (Mayer et al., 1998) and -independent manner (Perkins et al., 1998) in part through decreasing calcium sensitivity (Pabelick et al.,

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**ABBREVIATIONS:** BCA, bicinchoninic acid; BPD, bronchopulmonary dysplasia; BSA, bovine serum albumin; eNOS, endothelial NOS; GSNO, S-nitrosoglutathione; GSNOR, GSNO reductase; iNOS, inducible NOS; miR, micro-RNA; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; Rrs, respiratory system resistance; SNO, S-nitrosothiol; 2C/NOA, copper-cysteine reagent and nitric oxide analysis.

2000). GSNO reductase (GSNOR; also known as alcohol-dehydrogenase 5) is extensively expressed in lung tissue and regulates endogenous SNO levels through the enzymatic breakdown of GSNO to oxidized glutathione and ammonia (Liu et al., 2001). In a homeostatic manner, GSNOR catabolic activity can increase under conditions of elevated nitric oxide synthase (NOS) activity, specifically inducible NOS (iNOS) and endothelial NOS (eNOS) (Bhandari et al., 2006; Brown-Steinke et al., 2010). Airway levels of GSNO are decreased in pediatric cases of severe asthmatic respiratory failure (Gaston et al., 1998), and airway expression of GSNOR is elevated in asthma patients (Que et al., 2009; Marozkina et al., 2015). Consistent with these findings, GSNOR is a critical modulator of airway hyperreactivity in asthmatic animal models (Fang et al., 2000; Que et al., 2005; Ferrini et al., 2013; Blonder et al., 2014).

In the perinatal period, infants with evolving BPD are frequently treated for airway hyperreactivity (Mhanna et al., 2009; Slaughter et al., 2015), but first-line therapies such as  $\beta$ 2-adrenergic agonists lack consistent bronchodilator efficacy (Motoyama et al., 1987; Kovacs et al., 1990; Denjean et al., 1992) and have not been shown to prevent BPD nor facilitate weaning of respiratory support (Denjean et al., 1998). Of note, older children with BPD also have airway hyperreactivity that is distinct from allergic T-helper-2-high asthma (Halvorsen et al., 2005; Siltanen et al., 2011; Filippone et al., 2013) and may have minimal or even a paradoxical response to traditional asthma medications (Yuksel and Greenough, 1993; De Boeck et al., 1998; Baraldi et al., 2005). Novel treatments are needed in this high-risk patient population. We hypothesized that increased GSNOR activity could underlie the perinatal airway hyperreactivity observed in BPD and thus GSNO repletion would be therapeutic.

We tested this hypothesis in a hyperoxic murine model of BPD and airway hyperreactivity (Raffay et al., 2014). Murine lung development continues postnatally and is similar to the premature human lung (Amy et al., 1977; Berger and Bhandari, 2014). Hyperoxia exposure in neonatal mice creates a lesion very similar to human BPD (Warner et al., 1998) with characteristic long-term alveolar and parenchymal remodeling (Nold et al., 2013; O'Reilly et al., 2014), manifesting increased airway reactivity (Takeda et al., 2009; Raffay et al., 2014; Regal et al., 2014; Wang et al., 2014). We used this model to investigate the role of GSNOR in BPD airway hyperreactivity. We demonstrate that neonatal hyperoxia increases GSNOR expression and activity, in part through a microRNA (miR), and GSNO-based treatments can abolish BPD airway hyperreactivity. This novel mechanism underlying the pathophysiology of bronchospasm in murine BPD is particularly encouraging because there have been no effective new treatments for BPD in decades, and GSNO-mimetic compounds are already in clinical trials for other lung diseases.

## Materials and Methods

**Animal Hyperoxic Exposure.** Animal protocols were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University (Cleveland, OH). Timed pregnant C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were maintained on 12-hour light-dark cycles with ad libitum standard food and water. Within 24 hours of birth, litters were pooled and randomized into exposure groups. Paired with a nursing dam, pups were raised in 60% oxygen or room air (21%) for 21 days. Hyperoxia-exposed animals were housed in standard cages placed in a 38-L Plexiglas chamber with a

continuous flow of blended oxygen (2 L/min). Oxygen concentrations were monitored twice daily via an oxygen analyzer (miniOX I; MSA Medical, Gurnee, IL). To control for oxygen exposures, nursing dams were rotated between paired litters during weekly cage changes. Ventilator studies were conducted and/or tissue harvested within 24 hours of removal from hyperoxia at 3 weeks. A subgroup of animals was returned to room air following 3 weeks of initial hyperoxia exposure and subsequently recovered to 6 weeks of age for adult lung mechanic and GSNOR activity studies.

**GSNOR Activity by Copper Cysteine Reagent and Nitric Oxide Analysis.** Enzyme activity in lung homogenates from 3-week-old and 6-week-old mice was assessed by timed GSNO catabolism (Brown-Steinke et al., 2010) and quantification by copper-cysteine reagent and nitric oxide analysis (2C/NOA). After terminal anesthesia with i.p. ketamine/xylazine (Pfizer, St. Joseph, MO; Lloyd Laboratories, Shenandoah, IA), lungs from mice were harvested and rinsed in ice-cold phosphate-buffered saline (PBS, pH 7.4), placed in centrifuge tubes, snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Tissue in ice-cold radioimmunoprecipitation assay lysis buffer containing protease inhibitors (Santa Cruz Biotechnology, Dallas, TX) was homogenized, and protein levels were quantified by Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA). A known quantity of GSNO (28  $\mu\text{M}$ ) was loaded with coreagents (300  $\mu\text{M}$  NADH and 2 mM glutathione; Sigma-Aldrich, St. Louis, MO) and equivalent protein quantities of frozen lung homogenates in PBS. After incubation for 5 minutes at  $37^{\circ}\text{C}$ , the reaction was quenched by a 1:10 dilution of ice-cold PBS. Uncatabolized GSNO was then measured by 2C/NOA, as previously described in detail (Rogers et al., 2013). Briefly, samples were injected into a temperature-controlled reservoir containing copper cysteine reagent (pH < 6.9) with a continuous flow of blended helium. Gas-phase nitric oxide was liberated from GSNO contained in the injected samples and detected by ozone-based chemiluminescence using an inline nitric oxide analyzer (Seivers 280i; GE Instruments, Boulder, CO). GSNO content was determined by fitting chemiluminescence peaks to a GSNO standard curve and normalizing to sample protein levels. Enzyme kinetics were further derived from a Lineweaver–Burke double-reciprocal plot utilizing a total of three loading doses of GSNO (14, 28, and 56  $\mu\text{M}$ ).

**Western Blot.** Harvested snap-frozen lungs from 3-week-old mice were homogenized in ice-cold radioimmunoprecipitation assay lysis buffer containing protease inhibitors (Santa Cruz Biotechnology), and protein levels were determined by BCA assay (Thermo Scientific). Samples of 50  $\mu\text{g}$  protein were separated by electrophoresis with 4–15% Mini Protean TGX precast gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes (P:0 on iBlot; Invitrogen, Rehovot, Israel). Membranes were blocked with 5% milk or bovine serum albumin (BSA; Sigma-Aldrich) and incubated in GSNOR primary antibody (1:1000 in milk; observed band 40 kDa; Proteintech, Rosemont, IL), endothelial NOS primary antibody (1:1000 in BSA; observed band 140 kDa; BD Transduction Laboratory, San Jose, CA), iNOS primary antibody (1:1000 in BSA; observed band 145 kDa, Abcam, Cambridge, MA), or neuronal NOS primary antibody (1:500 in milk; expected band 161 kDa; Abcam) overnight at  $4^{\circ}\text{C}$  and then horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:3000; Santa Cruz, Dallas, TX) or anti-mouse antibody (1:3000; Santa Cruz), as appropriate, for 1 hour at room temperature. As a loading control, membranes were stripped (Pierce Restore; Thermo Scientific) and reprobed with  $\beta$ -actin primary antibody (1:2000 in milk; observed band 42 kDa, Abcam) and anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam). Band intensities were quantified and normalized to  $\beta$ -actin using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). Relative intensities were measured using densitometry software (Image J, NIH).

**Immunohistochemistry.** After terminal anesthesia (ketamine/xylazine), lungs of 3-week-old mice were inflated with intratracheal 10% formalin at 25 cm  $\text{H}_2\text{O}$ ; tissue was saline perfused with PBS (pH 7.4) and then formalin. The right lung was postfixed in 10% formalin

at 4°C for >24 hours, tissue was paraffin embedded, and 5- $\mu$ m-cut sections were processed. Tissue sections were immunoblotted with GSNOR primary antibody (1:200; Proteintech) at 4°C overnight and then biotinylated goat anti-rabbit secondary antibody (1:10,000; Vector Laboratories, Burlingame, CA) using Vectastain ABC kit, and next counterstained with methylene blue (Sigma-Aldrich), as previously described (Marozkina et al., 2012). Primary antibody was omitted as a negative control. Airways were similarly imaged (Rolera XR CCD camera; Q Imaging, Surrey, Canada).

**miR Microarray.** RNA was extracted from saline-perfused snap-frozen lungs of 3-week-old mice preserved in RNAlater-ICE reagent using a miRVana column isolation kit (Life Technologies, Carlsbad, CA). RNA was quantified by Nanodrop spectroscopy (Thermo Scientific), and microarray analysis of all mature mouse probes from the miRBase V21 library were compared between groups (LC Sciences, Houston, TX). Utilizing a gene-miR interaction search (Dweep et al., 2011, 2014) for the 3' untranslated region binding site of GSNOR mRNA (gene id: *adh5*, alcohol dehydrogenase 5), the most predicted miR candidates were cross-referenced with the microarray results, and high-probability miRs were selected and confirmed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

**qRT-PCR.** RNA was extracted from frozen lungs of 3-week-old mice using TRIzol reagent (Life Technologies) and quantified by Nanodrop spectroscopy (Thermo Scientific). cDNA was generated from 1  $\mu$ g RNA by reverse transcription using qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). Real-time quantitative polymerase chain reaction was performed on a StepOne PCR system (Applied Biosystems, Foster City, CA) using TaqMan probes (Life Technologies) for GSNOR (Mm00475804\_g1) compared with 25% diluted  $\beta$ -actin control (Thermo Scientific) with PerfeCTa qPCR FastMix, UNG, ROX (Quanta Biosciences, Gaithersburg, MD). For microRNA qRT-PCR, RNA was similarly extracted as in the miR microarray studies. cDNA was generated using TaqMan primer-specific assays and MicroRNA Reverse Transcription kit, and real-time quantitative polymerase chain reaction was performed using TaqMan MicroRNA assays for microRNA-342-3p (2260, Thermo Scientific) compared with snRNA-U6 control (001973, Thermo Scientific) with TaqMan Universal Master Mix, No AmpErase UNG (Life Technologies). Fold changes are reported utilizing 2<sup>- $\Delta\Delta$ CT</sup> method and StepOne software v2.3 (Applied Biosystems).

**Transfection with mmu-miR-342-3p and Cytomix Activation of RAW 264.7 Cells.** RAW 264.7 macrophage cells (American Type Culture Collection, Manassas, VA) were cultured in Gibco Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies). Cells were transfected with 20 nM miRIDIAN miR mimic for mmu-miR-342-3p or with a miR mimic transfection control, cel-miR-67 (Dharmacon, GE Lifesciences, Lafayette, CO) by AMAXA electroporation utilizing Nucleofector Kit V (Lonza Group, Basel, Switzerland), per manufacturer's instructions. After 48 hours, cells were harvested for protein or RNA studies, and pellets were snap frozen. Protein levels were determined by BCA assay, and protein was equivalently loaded for gel electrophoresis and Western blot analysis of GSNOR; $\beta$ -actin, as described above. RAW 264.7 qRT-PCR for miR-342-3p was similarly performed on transfected cells, as described above, to confirm increased gene expression resulting from transfection. Additionally, untransfected RAW 264.7 cells in culture media were incubated with cytomix (10 ng/mL each interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , and lipopolysaccharide; Sigma-Aldrich) or vehicle for 10 hours to measure changes in GSNOR expression in the activated macrophage (Tan et al., 2013).

**Synthesis of GSNO.** GSNO was synthesized in-house adapted from a protocol, as previously published (Hart, 1985). Using a nitrogen sparge at 4°C in light-protective conditions, reduced L-glutathione (2 g) in purged hydrochloric acid (2 N) and purged ultrapure water was S-nitrosylated with sodium nitrite (455 mg) over 30–60 minutes (Sigma-Aldrich). The resulting pink GSNO solution was vacuum filtered, mixed with 10 mL purged 50% acetone for 10–20 minutes,

and filtered again. Samples were lyophilized and stored at –80°C. Concentration was confirmed by Saville assay (Gaston et al, 1994).

**GSNOR Inhibitor Administration.** N6022 is a selective small-molecule reversible inhibitor of GSNOR (Green et al., 2012; Blonder et al., 2014). Powdered N6022 (Nivalis Therapeutics, Boulder, CO, purchased through MedChem Express, Monmouth Junction, NJ) was reconstituted in sterile PBS (pH 7.4) and administered to hyperoxia-exposed mice as a single 1 mg/kg i.p. injection the day prior to testing lung mechanics. Dosage was chosen based upon the published work of Blonder et al. (2014).

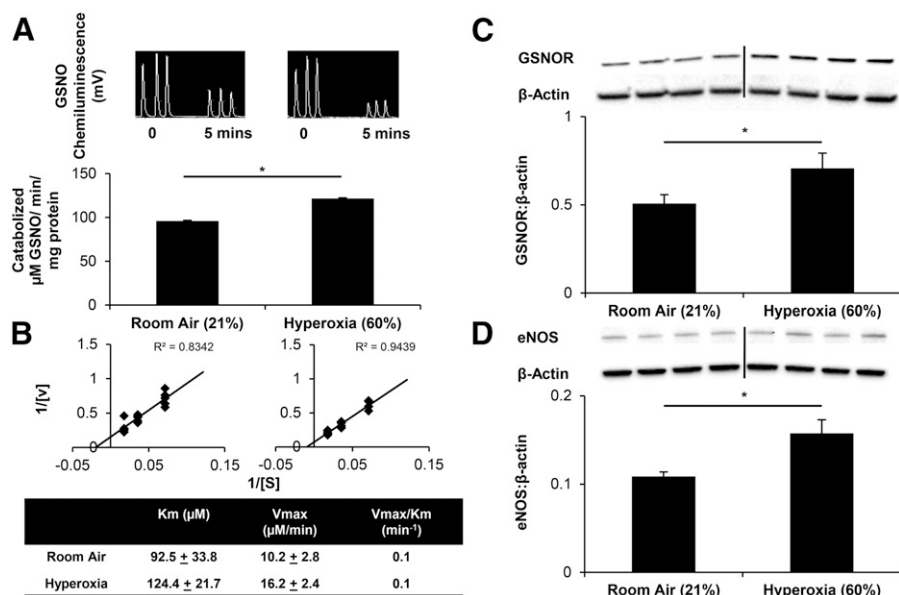
**Lung Mechanics.** Under general anesthesia (i.p. ketamine/xylazine), mice were placed supine on a heated surgical table, tracheostomized, and ventilated via a 19-gauge blunt-tip cannula with a commercial rodent ventilator (flexiVent; SCIREQ, Montreal, Canada). Animals were paralyzed (i.p. pancuronium bromide; Sigma-Aldrich) and ventilated at default settings: tidal volume of 10 mL/kg, a rate of 150 breaths/min, a positive end expiratory pressure of 3 cm H<sub>2</sub>O, and a FiO<sub>2</sub> of 50%. Following two recruitment deep inflations of sustained inspiration up to a pressure of 30 cm H<sub>2</sub>O for 3 seconds, 10 mM GSNO or saline vehicle was aerosolized over 10 seconds using an ultrasonic nebulizer (Aeroneb; SCIREQ) diverted into the ventilator's inspiratory flow. Inhaled GSNO concentration was chosen based upon the published studies in ventilated guinea pigs (Bannenberg et al., 1995) and human trials in cystic fibrosis (Snyder et al., 2002). After 5 minutes had elapsed, two recruitment deep inflations were again delivered, and increasing methacholine doses of 0, 12.5, 25, 50, 100, and 200 mg/mL were similarly aerosolized over 10 seconds to generate a dose-response curve. Using computer software (flexiWare 5.1, Version 7.2, SCIREQ), five measurements of respiratory system resistance (Rrs) were calculated by a 2.5 Hz single-frequency forced oscillation maneuver (Snapshot 150) (Shalaby et al., 2010), and an average was reported for each methacholine dose. Respiratory mechanics were measured in both 3-week-old mice immediately following sustained hyperoxia exposure and separate 6-week-old mice that were recovered in room air following the initial 3 weeks of hyperoxia exposure.

**Statistics.** Data are expressed as means  $\pm$  S.E.M. A minimum of two litters or experiments was used for each study; *n* represent individual animals or cell transfections. Data containing two groups were first tested for normality and variance and then analyzed by two-sample Student *t* test, Welch's *t* test, or Mann–Whitney *U* test, as appropriate. For multiple comparisons, analysis of variance with Tukey–Kramer post hoc test was used. Alterations in airway reactivity with increasing doses of methacholine were compared by two-way analysis of variance repeated-measures analysis with Tukey–Kramer post hoc comparisons using a fixed-sequence method from highest to lowest methacholine dose. *P* < 0.05 was considered statistically significant.

**Materials.** If not otherwise stated, all reagents and chemicals were purchased from Sigma-Aldrich and were of an analytical grade.

## Results

**GSNO Catabolism Is Increased after Neonatal Hyperoxia.** As described in asthma, increased expression of GSNOR causes loss of the endogenous bronchodilator, GSNO, and increased bronchial hyperreactivity (Fang et al., 2000; Que et al., 2009). Using 2C/NOA, we have shown that GSNOR activity (NADH-dependent GSNO catabolism/min/mg protein) in the lungs of 3-week-old mice raised in neonatal hyperoxia was higher than that of room air controls (Fig. 1A). The Lineweaver–Burke plots of estimated maximum velocity and Michaelis–Menton constant tended to be increased among the hyperoxia-exposed group (Fig. 1B), yet the ratio of maximum velocity/Michaelis–Menton constant was similar between groups. Although these kinetic findings could indicate loss of



**Fig. 1.** Increased GSNOR activity and expression in 3-week-old mice after neonatal hyperoxia exposure. (A) GSNOR activity was assessed by timed GSNO catabolism in lung homogenates, normalized to protein. GSNOR activity was increased in hyperoxia. Representative nitric oxide analyzer tracings in triplicate are shown. Data were normally distributed with equal variance, so a two-tailed Student *t* test was used.  $n = 5$ .  $*P < 0.05$ . (B) GSNOR kinetics were estimated by generating a Lineweaver–Burke plot at differing GSNO substrate loads. Maximum velocity/Michaelis–Menton constant did not differ between groups. Data were normally distributed with equal variance, so a two-tailed Student *t* test was used.  $n = 12$ .  $*P < 0.05$ . (C) Representative Western blot bands from the same gel are shown. Relative expression of GSNOR: $\beta$ -actin ratio was increased in hyperoxia. Data were normally distributed with unequal variance, so a two-tailed Welch’s *t* test was used.  $n = 12$ .  $*P < 0.05$ . (D) Representative Western blot bands from the same gel are shown. Relative expression of eNOS: $\beta$ -actin was increased in hyperoxia. Data were normally distributed with equal variance, so a two-tailed Student *t* test was used.  $n = 4$ .  $*P < 0.05$ .

a noncompetitive inhibitor, the most likely explanation was increased GSNOR expression in hyperoxia. GSNOR activity was also measured by 2C/NOA in the lung homogenates from 6-week-old mice who were exposed to 3 weeks of hyperoxia and then recovered in room air. GSNOR activity remained significantly increased in the hyperoxia-exposed room air-recovered mice, compared with 6-week-old room air controls ( $11.84 \pm 0.22$  versus  $11.08 \pm 0.17$   $\mu\text{M}/\text{min}/\text{mg protein}$ , respectively,  $P < 0.05$ ), albeit with less catabolic activity per mg protein than at 3 weeks of age.

**GSNOR Expression Is Increased after Neonatal Hyperoxia.** Consistent with the GSNOR kinetic data in 3-week-old mice, the relative protein expression of GSNOR was increased in the lungs of 3-week-old mice raised in hyperoxia when compared with room air controls, as assessed by Western blot (Fig. 1C).

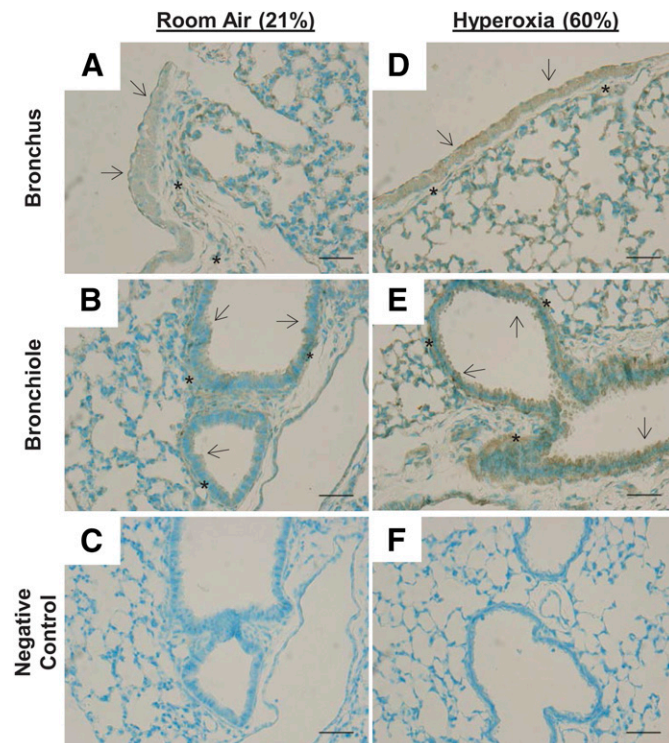
**eNOS Expression Is Increased after Neonatal Hyperoxia.** The relative protein expression of eNOS was increased in the lungs of 3-week-old mice raised in hyperoxia when compared with room air controls, as assessed by Western blot (Fig. 1D). iNOS expression was not significantly different between groups, and neuronal NOS was not detected in the lungs of either group by this Western blot preparation (data not shown).

**GSNOR Gene Expression Is Not Increased after Neonatal Hyperoxia.** To determine whether differences in GSNOR expression were transcriptionally mediated, we performed qRT-PCR on lung homogenates from 3-week-old mice raised in hyperoxia or room air. GSNOR mRNA expression did not differ between groups.

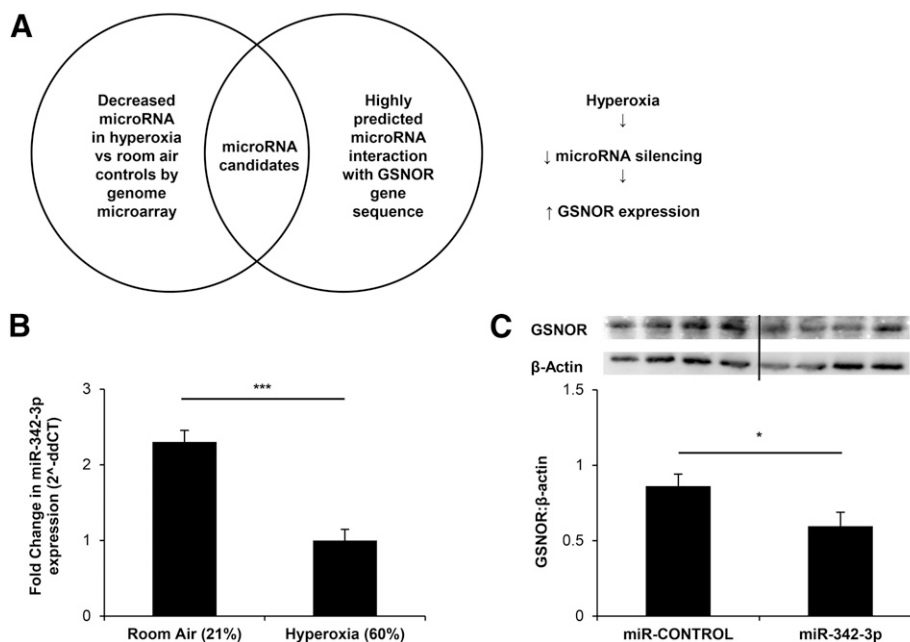
**GSNOR Immunohistochemistry.** GSNOR immunostaining was prominent in the hyperoxia-exposed 3-week-old mice and, consistent with previous findings (Marozkina et al., 2012, 2015), staining was localized to the epithelium and smooth muscle of the airways (Fig. 2).

**mmu-miR-342-3p Gene Expression Is Decreased after Neonatal Hyperoxia.** Because GSNOR mRNA expression did not explain differences in GSNOR protein expression, we next investigated whether microRNA gene silencing regulates its expression. Microarray analysis performed on

lung homogenates from individual 3-week-old animals identified miR candidates found to have decreased expression in hyperoxia. MicroRNA candidates were then cross-referenced with the highest predicted gene-miR interactions to *adh5*, the GSNOR gene (Fig. 3A). *mmu-miR-342-3p* showed trends toward decreased expression in hyperoxia by microarray and



**Fig. 2.** GSNOR immunostaining following hyperoxia in lungs of 3-week-old mice. Representative immunohistochemical probe for GSNOR (brown) of inflation-fixed lung sections showed prominent staining of airway epithelium (arrows) and smooth muscle (\*) in the bronchus (A, D) and bronchioles (B, E) of both groups. Sections were counterstained with methylene blue. Primary antibody was omitted as a negative control (C, F). Scale bar = 50  $\mu\text{m}$ .



**Fig. 3.** microRNA-342-3p regulates post-transcriptional expression of GSNOR. (A) Approach for identifying miR candidates most likely to interact with GSNOR post-transcriptional expression. miR candidates that displayed decreased expression in hyperoxia by microarray analysis were identified. Candidates were then cross-referenced with the highest predicted GSNOR gene-miR interactions using results of multiple prediction algorithms. miR-342-3p had trends toward decreased expression in hyperoxia by microarray analysis ( $n = 2$ ) and was highly predicted to interact with GSNOR mRNA (6 of 10 prediction algorithms). (B) qRT-PCR was performed on lung homogenates from 3-week-old mouse pups to confirm significant changes in miR-342-3p expression. Fold decreases in miR-342-3p expression were observed in hyperoxia compared with room air controls. Data were normally distributed with equal variance, so a two-tailed Student  $t$  test was used.  $n = 6$ . \*\*\* $P < 0.001$ . (C) Mouse macrophage RAW 264.7 cells were transiently transfected with a miR-342-3p mimic or a miR mimic control (cel-miR-67) to assess miR silencing of GSNOR protein expression. Western blot analysis for GSNOR was performed on lysed cells 48 hours after transfection. Representative Western blot bands from the same gel are shown. Relative expression of GSNOR:β-actin ratio was decreased in cells overexpressing miR-342-3p. Data were normally distributed with equal variance, so a two-tailed Student  $t$  test was used.  $n = 8$ . \* $P < 0.05$ .

was predicted by six different prediction data sets to interact with the 3' untranslated region of GSNOR (Dweep et al., 2011, 2014). mmu-miR-342-3p was confirmed by qRT-PCR to be significantly underexpressed in the lungs of hyperoxia-exposed 3-week-old mice when compared with room air controls (Fig. 3B).

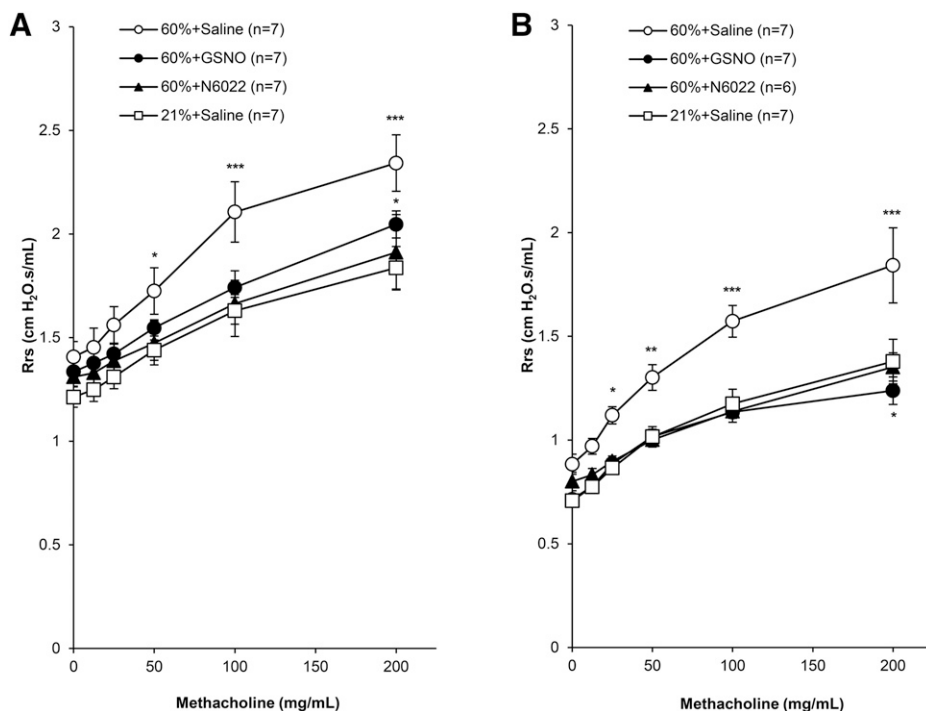
**Transfection with miR-342-3p Decreases GSNOR Expression.** We next showed that miR-342-3p decreases protein expression of GSNOR using mouse macrophage RAW 264.7 cells. RAW 264.7 cells endogenously express GSNOR (confirmed by Western blot in naive cells and those activated with cytomix; no significant difference was observed with cytomix treatment) and were one of the original cell lines used to isolate and describe GSNOR (Liu et al., 2001). RAW 264.7 cells transiently transfected with a miR-342-3p mimic had decreased GSNOR protein expression compared with cells transfected with a miR mimic control (Fig. 3C). We confirmed miR-342-3p overexpression following transfection by qRT-PCR.

**Hyperoxic Changes in Respiratory Mechanics Are Attenuated by Pretreatment with a GSNO Aerosol or by GSNOR Inhibition.** We have shown that neonatal hyperoxia increased GSNOR activity and expression. Therefore, we tested whether GSNO repletion or GSNOR inhibition could reverse the airway hyperreactivity observed in our BPD model. Responses to methacholine-provoked airway hyperresponsiveness were characterized by measuring Rrs, an indicator of airway hyperreactivity. Compared with room air controls, 3-week-old mice raised in hyperoxia displayed elevated Rrs in response to aerosolized methacholine challenge (Fig. 4A). Pretreatment of the hyperoxia-exposed mice

with a 10-second GSNO aerosol attenuated these changes, such that this group was no longer significantly different from room air controls, except at the highest methacholine dose (200 mg/mL). Pretreatment of the hyperoxia-exposed mice with an i.p. injection of a selective inhibitor of GSNOR activity, N6022 (Green et al., 2012), attenuated these changes as well, such that this group was no longer significantly different from room air controls at all methacholine doses. Next we show in room air-recovered six-week-old animals that neonatal hyperoxia-exposed mice continued to have elevated Rrs in response to aerosolized methacholine challenge when compared with room air-raised controls (Fig. 4B). In these six-week-old mice exposed to neonatal hyperoxia, both pretreatment with GSNO aerosolization and GSNOR inhibition with N6022 remained effective in attenuating the hyperoxia-induced airway hyperresponsiveness. At both ages, pretreatment with GSNO in room air-exposed mice did not significantly change Rrs when compared with room air saline-treated controls (data not shown), and baseline Rrs prior to aerosolizations was not statistically different between groups (data not shown).

## Discussion

In this neonatal mouse model of BPD, hyperoxia exposure was associated with increased GSNO catabolism through increased lung expression of GSNOR. Hyperoxia exposure also increased expression of lung eNOS, which has been shown to increase activity, but not protein expression of GSNOR in adult models. We have shown that one mechanism for



**Fig. 4.** GSNO aerosol or GSNOR inhibition attenuates hyperoxia-induced airway hyperresponsiveness to methacholine challenge. Aerosolized methacholine dose responses were compared in (A) 3-week-old mouse pups raised from birth in room air (21%) or hyperoxia (60%) and in (B) adult 6-week-old mice raised in room air or recovered in room air after the initial 3-week hyperoxia exposure. Mice were pretreated with saline vehicle aerosol, 10 mM GSNO aerosol, or 1 mg/kg N6022 GSNOR inhibitor injection. Rrs was significantly increased in hyperoxia at 3 weeks and after room air recovery at 6 weeks of age; pretreatment with GSNO or N6022 attenuated these changes. Comparisons were made to 21% + saline control. Two-way analysis of variance with fixed sequence Tukey–Kramer post hoc analysis from highest to lowest methacholine dose was used. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

hyperoxia-induced upregulation of GSNOR protein expression is through altered post-transcriptional regulation by miR-342-3p; we anticipate that this mechanism may be relevant to other pulmonary toxicities associated with hyperoxia exposure. As anticipated, neonatal hyperoxia increased methacholine-induced airway hyperresponsiveness in both juvenile mice immediately following hyperoxia exposure and room air-recovered adult mice. Remarkably, these detrimental changes in respiratory mechanics were attenuated at both ages by supplementation with a single dose of exogenous GSNO or by administration of a GSNOR inhibitor. These data indicate that GSNOR is an important modulator of neonatal hyperoxia-induced airways reactivity and that it may be a promising new drug target for BPD treatment.

The most immediate respiratory complication following premature birth is respiratory distress syndrome. Newborns with this condition frequently require oxygen supplementation (Carlo et al., 2010), yet this life-saving intervention increases the infant's risk for developing longer-term respiratory morbidities such as BPD (Laughon et al., 2011) and childhood wheezing (Grishkan et al., 2004; Halvorsen et al., 2005; Stevens et al., 2010). In our murine model, moderate hyperoxia elicited increased airway hyperresponsiveness, consistent with other models of moderate hyperoxia (FiO<sub>2</sub> 40–70%) in newborn rodents (Takeda et al., 2009; Wang et al., 2014). Moderate neonatal hyperoxia (65% for just 7 days) may cause lasting changes in bronchiolar smooth muscle, alveolar attachments, and airway tethering, potentially predisposing adult mice to increased airways reactivity (O'Reilly et al., 2014). We show in this work that GSNO-based treatments reverse hyperoxia-induced airway hyperresponsiveness in both juvenile and adult BPD mice. In the room air-recovered BPD adult mouse, elevated GSNOR activity is somewhat attenuated, yet GSNO and GSNOR inhibition remain effective therapies in attenuating airway hyperreactivity. We speculate that GSNO's protective effects on airway

hyperreactivity may persist well into adulthood and need to be studied further.

GSNO and GSNOR inhibition has been shown by our group and others to relax airway smooth muscle and attenuate methacholine-induced bronchoconstriction through a variety of tested pathways. GSNO acts in both a cGMP-dependent soluble guanylate cyclase/protein kinase G pathway (Mayer et al., 1998) and a cGMP-independent manner via GSNO-mediated S-nitrosylation of intracellular Cys thiols that relaxes airway smooth muscle and is reversed by the thiol-reducing agent DL-dithiothreitol (Perkins et al., 1998). GSNO decreases calcium sensitivity of airway smooth muscle, thus reducing regulatory myosin light chain phosphorylation (Pabelick et al., 2000); and striated muscle myosin heavy chains have been shown to be S-nitrosylated in vivo (Evangelista et al., 2010). Additionally, SNOs alleviate bronchoconstriction through cGMP-independent activation of the voltage- and calcium-dependent potassium (BK) channels (Abderrahmane et al., 1998; Janssen et al., 2000) and inhibition of the ADP-ribose cyclase activity of CD38 (White et al., 2002). Pathways relevant in neonatal hyperoxia induced smooth muscle reactivity (Mhanna et al., 2004; Smith et al., 2007; Sopi et al., 2007; Ali et al., 2012; Britt et al., 2015).

In a rodent model of severe neonatal hyperoxia, continuous treatment with an S-nitrosylating gas, ethyl nitrite, improved lung compliance, decreased inflammation, and promoted lung development (Auten et al., 2007). Note that ethyl nitrite inhalation leads to S-nitrosothiol production in vivo (Moya et al., 2001); our current data regarding elevated GSNOR activity in neonatal hyperoxia and potential benefits of GSNO repletion may help to explain these previous results. Additionally, GSNO can improve vascular development (Lima et al., 2009), which could also be of benefit to patients with BPD (Thébaud and Abman, 2007). As such, chronic GSNO repletion on lung parenchymal and vascular development following neonatal hyperoxia may be important to investigate.

Although the phenotype has evolved (Baraldi and Filippone, 2007; Jobe, 2011) since Northway et al. (1967) first described BPD five decades ago, little has changed in symptomatic therapies for the persistent breathing problems experienced by these survivors of premature birth (Allen et al., 2003; Iyengar and Davis, 2015). Despite transient bronchodilation with  $\beta$ 2-adrenergic agonists (Gomez-Del Rio et al., 1986; Wilkie and Bryan, 1987), premature survivors show variable responses (Yuksel and Greenough, 1993; De Boeck et al., 1998; Baraldi et al., 2005), and chronic therapy with these agents potentially creates tolerance (Motoyama et al., 1987; Subbarao and Ratjen, 2006) without decreasing the incidence of BPD (Denjean et al., 1998). This may reflect tachyphylaxis caused by loss of  $\beta$ 2-adrenergic receptors in experimental BPD (Raffay et al., 2014). In both pulmonary and myocardial models, S-nitrosothiols protect against this tachyphylaxis (Whalen et al., 2007). Specifically, in both cases, loss of the GSNO catabolic enzyme, GSNOR, is protective. We speculate that GSNO-based therapies may have additional benefits in BPD through attenuating  $\beta$ 2-adrenergic receptor desensitization.

To our knowledge, this is the first demonstration that elevated GSNOR content and activity contribute to neonatal hyperoxic increases in airway hyperresponsiveness. GSNOR has been studied extensively in clinical and experimental asthma, cystic fibrosis, and pulmonary hypertension. It has been observed that transgenic mice unable to express GSNOR are protected from ovalbumin-induced asthmatic reactivity (Que et al., 2005). Treatments with GSNOR inhibitors (Ferrini et al., 2013; Blonder et al., 2014) have likewise been shown to be protective of adult murine asthmatic hyperreactivity. Asthma patients with respiratory failure are depleted of airway GSNO (Gaston et al., 1998), and those with elevated GSNOR have more severe airways reactivity and earlier onset of symptoms (Que et al., 2009; Marozkina et al., 2015). Cystic fibrosis patients have decreased airway levels of GSNO (Grasemann et al., 1999) and show improved oxygenation when administered exogenous GSNO (Snyder et al., 2002); as such, pharmacologic GSNOR inhibition is undergoing clinical investigation as a cystic fibrosis treatment (Tait and Miller, 2014). Lastly, infants with persistent pulmonary hypertension of the newborn have shown improved oxygenation after administration of a gaseous S-nitrosylating agent (Moya et al., 2002). These studies suggest that GSNOR is, in general, an important modulator of various conditions of pulmonary stress.

In this study, we describe a novel mechanism of dysregulated post-transcriptional GSNOR protein expression via miR silencing. Although eNOS expression has been shown to increase the catabolic activity of GSNOR (Brown-Steinke et al., 2010), GSNOR protein expression remained unchanged. The elevated eNOS expression in association with hyperoxia exposure shown here - and previously by our group (Potter et al., 1999) - does not fully explain the observed kinetics, protein, and immunostaining for GSNOR. MicroRNAs are small, noncoding RNAs that function through complementary binding to target sequences in mRNA. This interferes with translation, resulting in decreased protein expression (Bhaskaran and Mohan, 2014). As previously described in a different hyperoxic BPD model (Bhaskaran et al., 2012), we also observed decreased expression of miR-342-3p in the mouse cohort that was exposed to hyperoxia. This miR was

highly predicted to interact with GSNOR mRNA and, indeed, in vitro overexpression of miR-342-3p resulted in decreased GSNOR protein levels. Although control of protein expression under conditions of hyperoxia is multifactorial, altered miR post-transcriptional regulation is a compelling epigenetic mechanism by which GSNOR can be regulated. Future in vivo studies of miR-342-3p are important to further the understanding of the miR's role in neonatal hyperoxia.

Despite their widespread use in premature infants and children (Mhanna et al., 2009; Slaughter et al., 2014, 2015), limited evidence exists for the efficacy of asthma therapies, such as inhaled corticosteroids (Pelkonen et al., 2001; Gupta et al., 2012) and bronchodilators (Pantalitschka and Poets, 2006; Ng et al., 2012). Furthermore, there is a paucity of recommendations for the adult management of this growing patient population (Bolton et al., 2015). Targeted replacement of depleted GSNO stores in the developing lung exposed to hyperoxia, directly or through inhibition of GSNOR, may provide an exciting new treatment modality for this high-risk population of infants and children.

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#### Authorship Contributions

*Participated in research design:* Raffay, Khalil, MacFarlane, Martin, Gaston.

*Conducted experiments:* Raffay, Dylag, Smith, Einisman, Li, Lakner.

*Contributed new reagents or analytic tools:* Dylag.

*Performed data analysis:* Di Fiore.

*Wrote or contributed to the writing of the manuscript:* Raffay, Gaston.

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