ORIGINAL RESEARCH

Bronchopulmonary Dysplasia and Pulmonary Hypertension
The Role of Smooth Muscle *adh5*

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Abstract

Bronchopulmonary dysplasia (BPD) is characterized by alveolar simplification, airway hyperreactivity, and pulmonary hypertension. In our BPD model, we have investigated the metabolism of the bronchodilator and pulmonary vasodilator GSNO (*S*-nitrosoglutathione). We have shown the GSNO catabolic enzyme encoded by *adh5* (alcohol dehydrogenase-5), GSNO reductase, is epigenetically upregulated in hyperoxia. Here, we investigated the distribution of GSNO reductase expression in human BPD and created an animal model that recapitulates the human data. Blinded comparisons of GSNO reductase protein expression were performed in human lung tissues from infants and children with and without BPD. BPD phenotypes were evaluated in global ($adh5^{-/-}$) and conditional smooth muscle (smooth muscle/$adh5^{-/-}$) *adh5* knockout mice. GSNO reductase was prominently expressed in the airways and vessels of human BPD subjects. Compared with controls, expression was greater in BPD smooth muscle, particularly in vascular smooth muscle (2.4-fold; $P = 0.003$). The BPD mouse model of neonatal hyperoxia caused significant alveolar simplification, airway hyperreactivity, and right ventricular and vessel hypertrophy. Global $adh5^{-/-}$ mice were protected from all three aspects of BPD, whereas smooth muscle/$adh5^{-/-}$ mice were only protected from pulmonary hypertensive changes. These data suggest *adh5* is required for the development of BPD. Expression in the pulmonary vasculature is relevant to the pathophysiology of BPD-associated pulmonary hypertension. GSNO-mimetic agents or GSNO reductase inhibitors, both of which are currently in clinical trials for other conditions, could be considered for further study in BPD.

Keywords: *S*-nitrosoglutathione; neonate; alveolarization; airway hyperreactivity; alcohol dehydrogenase-5

Clinical Relevance

The gene product of *adh5* (alcohol dehydrogenase-5), *S*-nitrosoglutathione reductase, is upregulated in the bronchopulmonary dysplasia lung. Deletion of murine *adh5* ameliorated hyperoxic bronchopulmonary dysplasia with smooth muscle–specific effects on pulmonary hypertension. *S*-nitrosoglutathione reductase is a novel target for future bronchopulmonary dysplasia therapies.

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Over 10% of babies are born prematurely and the number of premature births is projected to increase (1). Bronchopulmonary dysplasia (BPD) is a major morbidity resulting from prematurity (2), with an estimated 10,000 new diagnoses of severe BPD made each year in the United States alone (3). Infants and children with BPD continue to have both increased mortality and prolonged, expensive hospital stays (4, 5). Bronchospastic obstructive airway disease persists into adulthood (6), and there are approximately 1 million BPD survivors in the United States (7).

No effective BPD therapies have been developed for decades. Lung protective ventilation strategies and adjunct medical therapies unfortunately have not improved the overall rates of BPD in most premature infants, which have remained relatively unchanged over the last two decades (8, 9). Hyperoxic injury in the vulnerable premature lung results in alveolar simplification, airway hyperreactivity (AHR), and pulmonary hypertension (PH)—hallmarks of the BPD phenotype (9). Infants with BPD are at risk for the development of longer-term PH (10). Indeed, there is right ventricular hypertrophy (RVH) and dysfunction in as many as 25–40% of infants diagnosed with BPD (11), and infants with established BPD that is complicated by PH have a substantially higher risk of death in early childhood (12, 13). Abnormal pulmonary vascular development, together with lung parenchymal simplification, are believed to contribute to the development of BPD-related PH (7, 11, 13, 14), but it has not previously been possible to distinguish the pathophysiology of the parenchymal and airway disease from that of the pulmonary vascular disease.

S-nitrosothiols are signaling molecules in the human lung that are produced by nitric oxide synthase isoforms and other metalloproteins (15, 16). One such compound is GSNO (*S*-nitrosoglutathione), an endogenous smooth muscle (SM) relaxant (17, 18) with angiogenic properties (19). GSNO reductase (GSNOR), the primary enzyme responsible for GSNO catabolism (20), is encoded by the *adh5* (alcohol dehydrogenase-5) gene. GSNOR expression and activity are increased in the airways of patients with asthma (21) and globally in an adult murine PH model (22). Inhaled GSNO and its precursor drug, ethyl nitrite, have been in clinical trials for PH (23), cystic fibrosis (24), and asthma (clinicaltrials.gov—NCT03926741), as have GSNOR inhibitors, such as Cavosonstat (25). We have shown GSNOR expression and activity are increased in our murine hyperoxic BPD model, in part mediated by microRNA 342-3p (26). Moreover, both inhaled GSNO and GSNOR inhibition reverse airway hyperreactivity in our murine model (26).

Here, we have studied the expression and distribution of GSNOR in the lungs of human infants with BPD. We report that expression is increased in airway and pulmonary vascular SM. To translate these findings, we created an SM conditional knockout (SM/$adh5^{-/-}$) mouse. This mouse develops BPD with no PH, demonstrating the essential, specific role of SM *adh5* in BPD-related PH, and providing a novel model to distinguish BPD in the airway and parenchyma alone from BPD-related PH. Strikingly, the global knockout ($adh5^{-/-}$) is also protected not only from PH but also from alveolar simplification and airway hyperreactivity. These data suggest the possibility that a novel class of therapies could be studied in BPD in general and BPD-related PH in particular.

Methods

Recovery of excess human biopsy specimens was approved by Seattle Children's Hospital Institutional Review Board. Animal protocols conforming to National Institutes of Health guidelines were approved by Case Western Reserve University Institutional Animal Care and Use Committee.

Measurements

Detailed in data supplement.

Human Lung Tissues

Deidentified fixed specimens sectioned at 5 μm were provided by the Seattle Children's Research Institute.

Double Labeling in Human Specimens

Immunofluorescence performed using anti–α-ACTA2 primary (1:400; Sigma-Aldrich) with Alexa Fluor-488 secondary (1:500; Invitrogen) and DAPI nuclear counterstaining (Vector Laboratories). Second labeling by immunohistochemistry of the same slide was performed as described below. Images were visualized by light microscopy and then immunofluorescence excitation with a Rolera XR CCD camera (Q-Imaging) mounted on a microscope (Leica Microsystems).

Immunohistochemistry in Human Specimens

Anti-GSNOR (1:1,000; Proteintech) and biotinylated secondary (1:1,000; Jackson ImmunoResearch) immunohistochemistry performed as previously reported (21). The indirect ABC method (Vectastain Elite; Vector Laboratories) was performed with diaminobenzidine chromogen (Vector Laboratories) with methylene blue counterstain (ScyTek). Negative controls were run by omitting the primary antibodies and with respective isotype controls to Rabbit (Proteintech). Qualitative masked scoring of GSNOR staining in human BPD and control tissues was performed as described in asthma (21). Masked technicians obtained multiple digital images per subject, and then two masked investigators independently scored the deidentified airway epithelium, airway SM, vessel endothelium, and vessel SM for GSNOR presence and intensity compared with isotype negative control (0 = none; 1 = weak, few cells; 2 = moderate, most cells; 3 = moderate, all cells; 4 = intense, all cells).

Animals

Work has been described previously (26). Pregnant wild-type C57BL/6 mice, global $adh5^{-/-}$ or conditional SM/$adh5^{-/-}$ colonies were fostered at Case Western Reserve University. Transgenics, hyperoxic exposure (60%), lung morphometry, staining, nitrogen assays, and respiratory mechanics are detailed in the data supplement.

RVH and Pulmonary Artery Pressure

The ratio of the right ventricle to left ventricle + septum mass was calculated (Fulton's Index) by a masked technician.

Pulmonary arterial pressures in response to the hypoxemic challenge were performed in wild-type mice ($n = 5$ control mice and 4 BPD mice). Mice were anesthetized with intraperitoneal Avertin, placed supine on a heated surgical bed, intubated orally, and ventilated (Mini-Vent 845; Harvard Apparatus). Hair was removed and the anterior chest was opened with a midline sternal incision and the right ventricle was exposed. A Mikro-Tip Catheter (SPR-1000; Millar Instruments) was inserted through a small

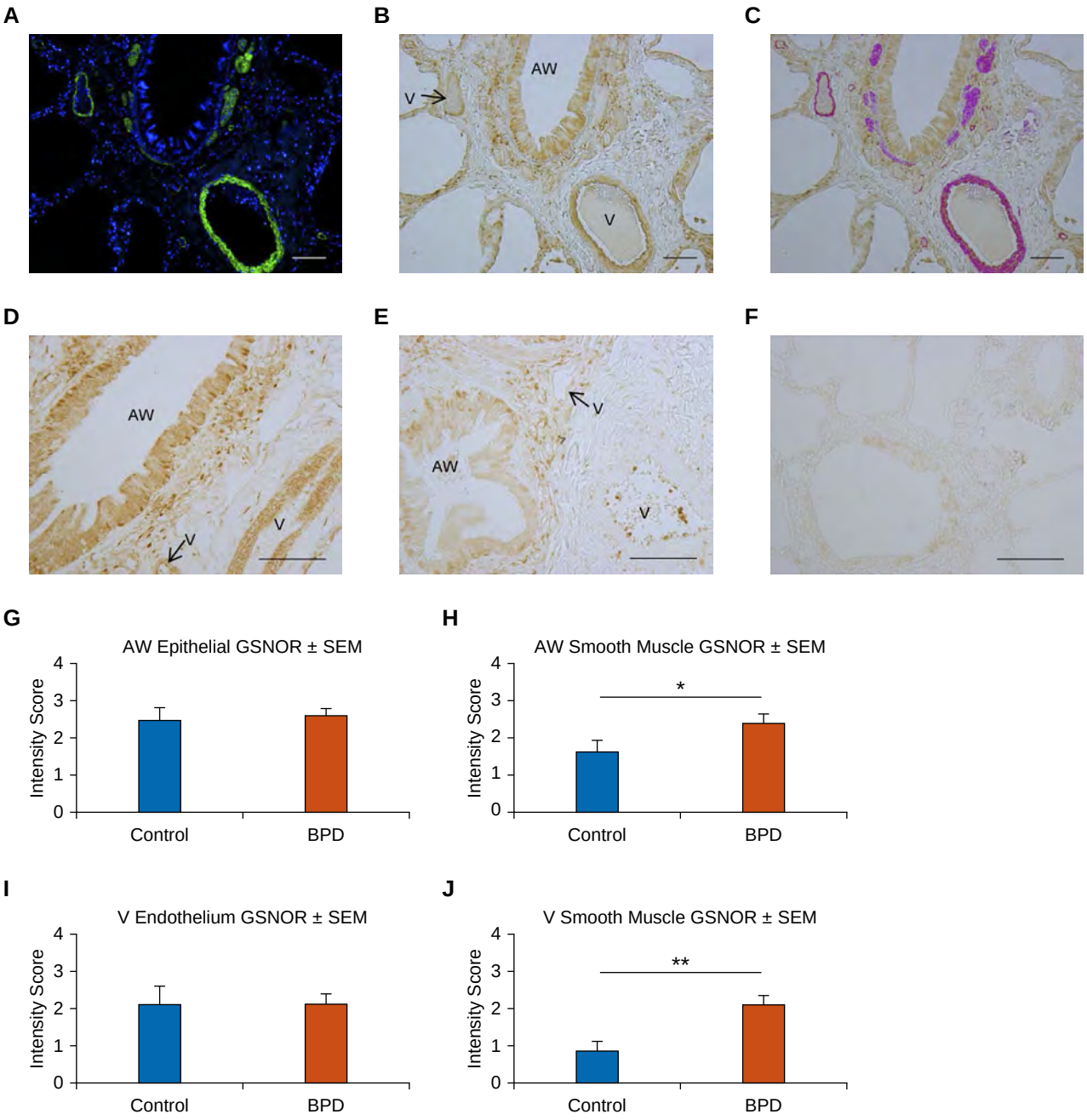

Figure 1. Smooth muscle *S*-nitrosoglutathione reductase (GSNOR) expression in human bronchopulmonary dysplasia (BPD). (*A–C*) Immunofluorescent staining of biopsied lung tissue for α-ACTA2 (green) and DAPI (blue) (*A*) and immunohistochemical staining for GSNOR (brown) (*B*) in the same slide shows GSNOR expression in the muscular airway and vessel (purple: merged α-ACTA2 and GSNOR) (*C*) at 3 years of age in a former premature patient with BPD and pulmonary hypertension. (*D*) Immunohistochemical staining for GSNOR (brown) from a patient with BPD, demonstrating GSNOR in the airway and vessel. (*E*) Referenced to a non-BPD control acquired from a motor vehicle accident autopsy specimen that shows modest GSNOR expression in the airway and arterial vessel smooth muscle. (*F*) Rabbit IgG isotype control in human tissue. (*G–J*) Qualitative masked scoring of GSNOR presence and intensity in the airway epithelium (*G*), airway smooth muscle (*H*), arterial vessel endothelium (*I*), and arterial vessel smooth muscle (*J*) in matched non-BPD controls ($n = 5$) and subjects with BPD ($n = 7$) between 2 months and 4 years of age (Score: 0 = none to 4 = intense). $^{*}P < 0.05$ and $^{**}P < 0.01$ by two-tailed Student's *t* test or Mann-Whitney rank-sum test. Scale bars, 100 μm. AW = airway; V = arterial vessel.

puncture into the right ventricle, then advanced into the pulmonary artery. After a stabilization period, hemodynamic parameters were recorded in response to inspired room air (21%) and hypoxia (15% and 10%) using PowerLab Data Acquisition System and LabChart Software (ADInstruments).

Statistics

Results expressed as means (±SEM). Data were analyzed by Student's *t* test or a Mann-Whitney rank test for two groups, or an

Table 1. Patient Demographics of Analyzed Human Lung Specimens

Gestational Age	Biopsy Age	Diagnosis	Primary Condition
36 wk	3 mo	Control	CPAM resection
Term	9 mo	Control	CPAM resection
Term	9 mo	Control	CPAM resection
Term	3 yr	Control	Accidental-Demise
Term	4 yr	Control	Motor Vehicle Accident-Demise
32 wk	2 mo	BPD	BPD/PIG
29 wk	4 mo	BPD	BPD/PH
30 wk	5 mo	BPD	BPD/PH
29 wk	8 mo	BPD	BPD-Demise
25 wk	8 mo	BPD	BPD/Pulmonary vein stenosis/ASD
23 wk	22 mo	BPD	BPD/PH
32 wk	3 yr	BPD	BPD/PH-Demise

Definition of abbreviations: ASD = atrial septal defect; BPD = bronchopulmonary dysplasia; CPAM = congenital pulmonary airway malformation; PH = pulmonary hypertension; PIG = pulmonary interstitial glycogenosis.
Non-BPD controls were late preterm or term at birth with no known history of asthma. Preterm BPD subjects ranged from 23 to 32 weeks gestation at birth. Indications and findings on biopsy are noted.

ANOVA with *post hoc* Tukey test for multiple groups using statistical software (*SigmaPlot* 12.0; Systat Software). $P < 0.05$ was the significance threshold.

Results

SM GSNOR Expression Is Increased in the BPD Lung

Colocalization staining for α-ACTA2 and GSNOR demonstrates that GSNOR is present in the muscular airways and pulmonary arterial vessels of fixed lung specimens of infants and children diagnosed with BPD and PH (Figures 1A–1C). We compared specimens from patients with BPD with age-matched controls without airway or cardiovascular disease (Table 1) using techniques previously described (21). Blinded analysis by two investigators revealed that GSNOR expression is increased in BPD airway SM ($P = 0.03$) and pulmonary vascular SM ($P = 0.003$), but not in airway epithelium or pulmonary vascular endothelium (P = not significant) (Figures 1D–1J).

Colocalization staining for α-ACTA2 and GSNOR shows that GSNOR is also present in the SM of murine BPD airways and vessels and can be conditionally deleted from the SM in SM/*adh5*$^{-/-}$ transgenic mice (Figures 2A and 2B). Note that GSNOR staining of the epithelium is preserved in the SM/*adh5*$^{-/-}$ airways as in the wild-type airways. We have previously shown in this mouse model that GSNOR whole lung expression and activity was increased after newborn hyperoxia exposure, with GSNOR localizing to the SM of 3-week old pups (26). We now show in room air–recovered 6-week old mice with BPD that GSNOR continues to be prominently expressed in the airways and vessels of wild-type mice, with absent staining in the global *adh5*$^{-/-}$ (Figures 2C and 2D).

Global *adh5*$^{-/-}$ Mice Are Protected from Alveolar Simplification after Neonatal Hyperoxia Exposure

Postnatal hyperoxia exposure in the developing lung results in long-term parenchymal alveolar simplification (27, 28), which can be attenuated with exogenous *S*-nitrosothiols (29). Inflation-fixed murine lung sections were stained with hematoxylin and eosin and imaged. Consistent with human BPD, wild-type and SM/*adh5*$^{-/-}$ mice raised in neonatal hyperoxia had significantly decreased radial alveolar counts ($P < 0.001$) and increased mean linear intercepts ($P < 0.001$), indicating alveolar simplification that persisted after room air recovery (Figure 3). Strikingly, global *adh5*$^{-/-}$ mice were completely protected from the BPD-mimetic effects of hyperoxia exposure and did not significantly differ from room air controls. Room air *adh5*$^{-/-}$ and SM/*adh5*$^{-/-}$ did not significantly differ from wild-type room air controls.

Hyperoxic Changes in Respiratory Mechanics Are Attenuated in Global *adh5*$^{-/-}$ Mice

GSNO is an airway SM relaxant (17, 30), and *adh5*$^{-/-}$ mice have previously been shown to be protected from asthmatic AHR (31). Responses to provoked methacholine bronchoconstriction were characterized in anesthetized 6-week old mice in our BPD model by measuring changes in respiratory system resistance (Rrs) and Newtonian airway resistance (Rn). All groups responded to aerosolized methacholine with significant increases in Rrs and Rn (Figure 4). Compared with wild-type room air controls, 6-week wild-type and SM/*adh5*$^{-/-}$ mice raised in neonatal hyperoxia displayed elevated Rrs ($P < 0.001$) and Rn ($P < 0.001$) in response to methacholine challenge up to 200 mg/ml. Rrs and Rn curves of hyperoxia-exposed *adh5*$^{-/-}$ did not significantly differ from room air controls at any methacholine dose. Methacholine dose–responses of room air *adh5*$^{-/-}$ and SM/*adh5*$^{-/-}$ groups did not significantly differ from wild-type room air controls. Secondary analyses showed a significant interaction between exposure group and sex for Rrs measurements but not Rn (*see* Figure E1 in the data supplement).

Global *adh5*$^{-/-}$ and SM/*adh5*$^{-/-}$ Are Protected from End-Organ Pulmonary Hypertensive Changes after Neonatal Hyperoxia Exposure

Both GSNOR inhibition and exogenous GSNO relaxes arterial vessels (18, 32) and elevated GSNO catabolism occurs in adult PH models (22, 33). Masked measurements of the Fulton's Index, a measure of RVH, were significantly elevated in hyperoxia exposed wild-type mice ($P < 0.001$), but global *adh5*$^{-/-}$ and SM/*adh5*$^{-/-}$ were protected from hyperoxic RVH changes (Figure 5A). Secondary analyses did not show a significant interaction between exposure group and sex; comparisons within male or female subgroups showed hyperoxia-exposed wild-type mice had a significantly increased Fulton's Index compared with room air wild types (*see* Figure E2). Neonatal hyperoxia exposure increased the medial wall thickness of pulmonary arteries in wild-type mice ($P < 0.05$), whereas hyperoxia exposure in global *adh5*$^{-/-}$ and SM/*adh5*$^{-/-}$ mice did not significantly differ from room air controls

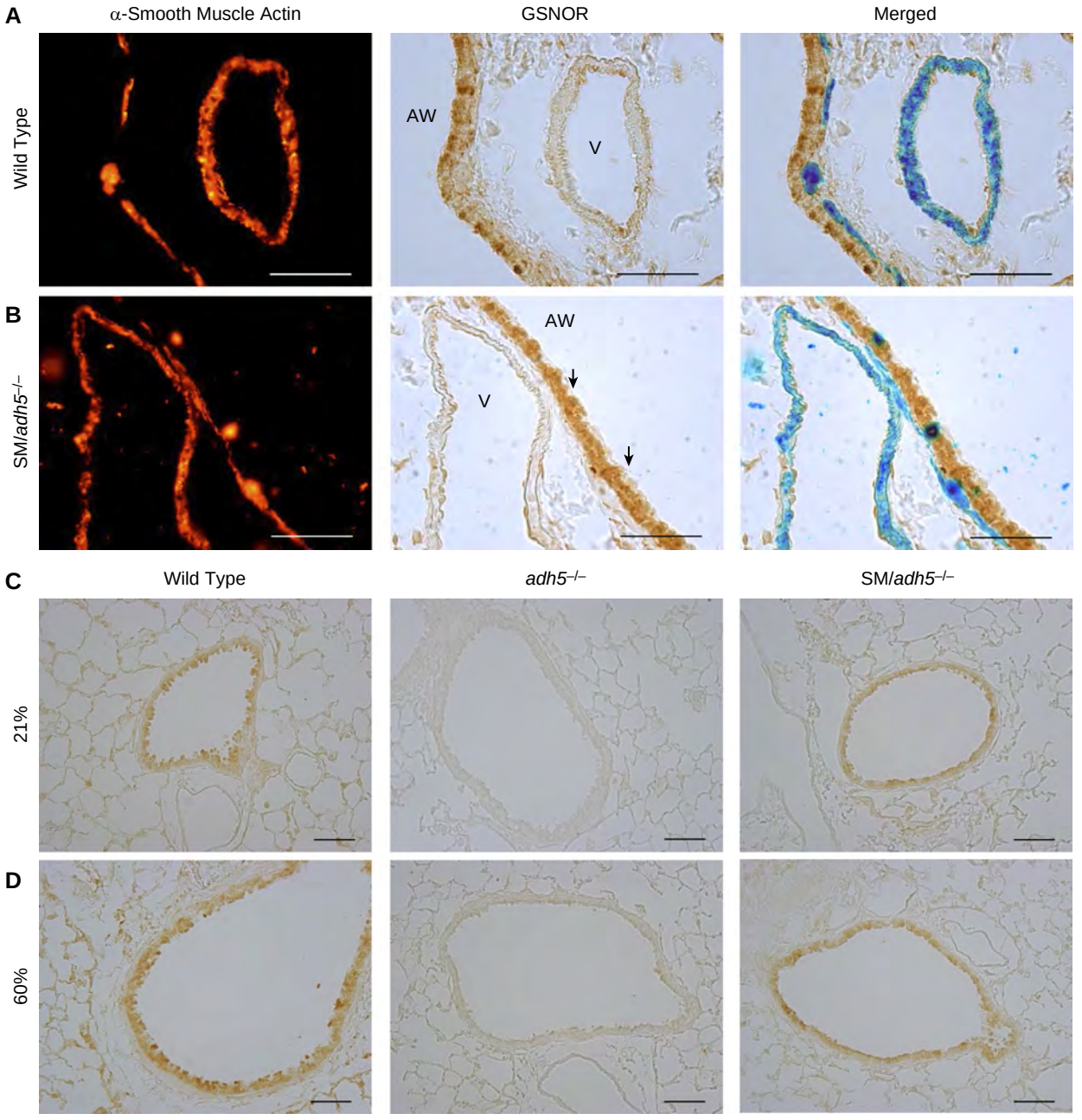

Figure 2. *S*-nitrosoglutathione reductase (GSNOR) expression in murine bronchopulmonary dysplasia (BPD). (*A*) Immunofluorescent staining for α-ACTA2 (red, left; blue, merged right) and immunohistochemical staining for GSNOR (brown, middle, and merged right) shows GSNOR colocalizing in the airways and arterial vessels (indigo, merged right) of wild-type BPD mice. (*B*) Cross-breeding of *sm22α*$^{[Cre]}$ and *adh5*$^{[flox]}$ transgenic mice (SM/*adh5*$^{-/-}$) shows an absence of GSNOR staining of the airway and vascular smooth muscle on colocalization studies; however, GSNOR continues to be prominently expressed in the BPD airway epithelium (arrows). (*C* and *D*) Immunohistochemical staining for GSNOR (brown) of airways and vessels of room air controls (*C*) and neonatal hyperoxia-exposed (60% · 3 wk) (*D*) 6-week recovered wild-type (left), global *adh5* knockout (*adh5*$^{-/-}$, middle), and conditional smooth muscle *adh5* knockout (SM/*adh5*$^{-/-}$, right) mice. Scale bars, 50 μm. *adh5* = alcohol dehydrogenase-5; SM = smooth muscle.

(Figure 5B). Vessel density was not significantly different between hyperoxia-exposed wild-type and SM/*adh5*$^{-/-}$ groups; however, room air *adh5*$^{-/-}$ had increased vessel density counts compared with all groups ($P \leq 0.034$ vs. all) (Figure 6). Direct measurements of mean arterial pressures (MAP) of the pulmonary artery by a transcardiac inserted catheter in anesthetized and ventilated wild-type control ($n = 5$) and BPD ($n = 4$) male mice were not well tolerated with arrhythmias, cyanosis, and mortality in BPD mice. There were no differences in baseline MAP (21% inspired oxygen; $P = 0.46$) and

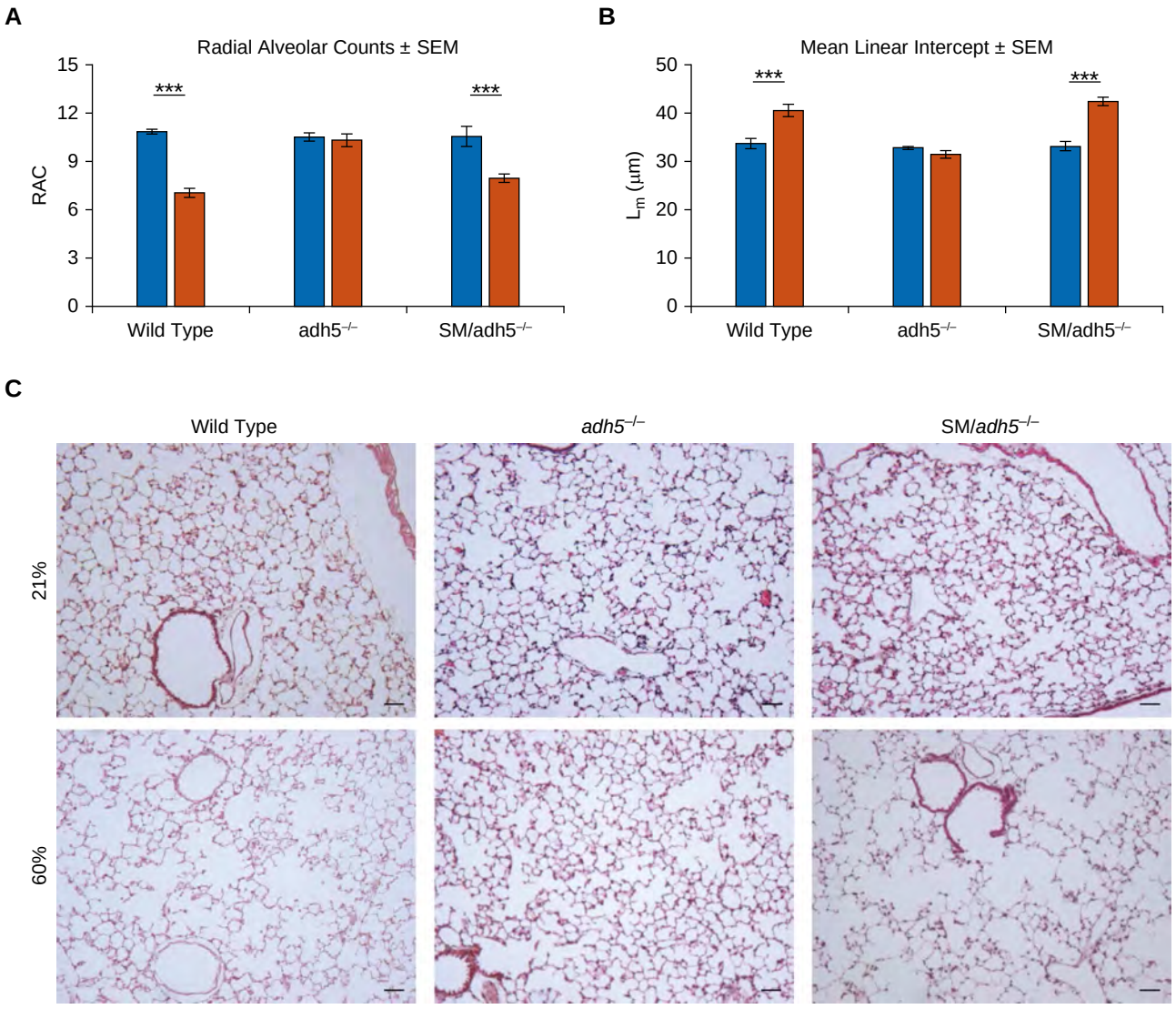

Figure 3. Global deletion of *adh5* protects against bronchopulmonary dysplasia alveolar simplification. Neonatal hyperoxia exposure (60% · 3 wk, red bars) with room air recovery resulted in significantly (*A*) decreased RAC and (*B*) increased L_m in wild-type and conditional smooth muscle *adh5* knockout mice (SM/*adh5*$^{-/-}$) compared with room air controls (21%, blue). The global *adh5* knockouts (*adh5*$^{-/-}$) were protected from alveolar simplification with neonatal hyperoxia exposure. (*C*) Representative photomicrographs of hematoxylin and eosin–stained sections are shown. n = 6–7/group. ***$P < 0.001$, by ANOVA with *post hoc* Tukey comparisons. Scale bars, 50 μm. L_m = mean linear intercepts; RAC = radial alveolar counts.

responses to hypoxic challenge (15% oxygen) did not reach statistical significance in survivors (14.0 ± 0.1% vs. 26.6 ± 0.1% increases in MAP from baseline; wild-type controls vs. BPD; $P = 0.34$). No BPD mice survived challenges with 10% oxygen.

Global *adh5*$^{-/-}$ Mice Do Not Exhibit Elevated Lung Nitrotyrosine after Neonatal Hyperoxia Exposure

We have previously shown that neonatal hyperoxia increases endothelial nitric oxide synthase (eNOS) in wild-type whole lung homogenates immediately harvested from supplemental oxygen (26). This is important because eNOS has been shown to regulate GSNOR activity (33). Although nitrogen oxide levels did not differ in wild-type and *adh5*$^{-/-}$ mice in an adult asthma model (31), the effects of hyperoxia have not yet been reported. Here we compare eNOS and nitrogen metabolites in the lungs of wild-type and global *adh5*$^{-/-}$ 6-week mice (detailed in data supplement). Room air–exposed wild-type and *adh5*$^{-/-}$ mice did not significantly differ in eNOS expression. Neonatal hyperoxia did not significantly increase eNOS expression at 6 weeks. Lung nitrite levels did not significantly differ between groups. Nitrotyrosine, a marker of protein modification by reactive nitrogen species, was significantly increased in hyperoxic wild-type lungs (2.38-fold increase; $P < 0.05$); whereas oxygen-exposed *adh5*$^{-/-}$ were protected (*see* Figure E3).

Discussion

No new treatments have been developed for BPD and associated PH for decades (34, 35). Inhaled GSNO and ethyl nitrite, or in

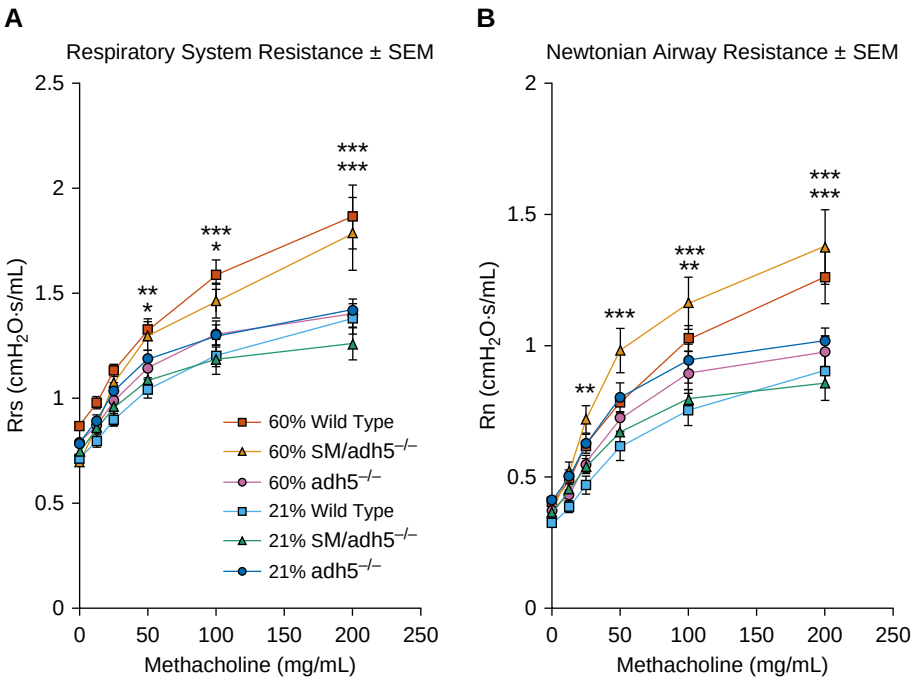

Figure 4. Global deletion of *adh5* protects the airway from bronchopulmonary dysplasia hyperreactivity, but selective smooth muscle deletion does not. Neonatal hyperoxia exposure (60% · 3 wk) with room air recovery resulted in significantly increased (*A*) Rrs and (*B*) Rn responses to aerosolized methacholine in wild-type (red squares) and conditional smooth muscle *adh5* knockouts (SM/$adh5^{-/-}$, orange triangles) compared with room air wild-type controls (21%, blue squares). Hyperoxia-exposed global *adh5* knockout mice ($adh5^{-/-}$, purple circles) were protected from airway hyperreactivity, such that they did not differ from room air wild-type controls. All groups significantly responded to methacholine doses. n = 10/group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, versus room air wild-type controls by two-way ANOVA with *post hoc* Tukey comparisons by group and dose. Rn = Newtonian airway resistance; Rrs = respiratory system resistance.

some cases the GSNOR inhibitor, Cavosonstat have been in clinical trials for pulmonary diseases, including newborn PH (23–25). We therefore have been conducting studies on the role of the GSNO/GSNOR axis in BPD pathophysiology. In our newborn mouse model, hyperoxia exposure increases GSNOR lung expression, in part through microRNA 342-3p, and acute inhalation of GSNO or systemic GSNOR inhibition can reverse the hyperoxic AHR (26). We now show that GSNOR is expressed in the human BPD airways and pulmonary vessels, and that expression is selectively increased in the pulmonary vascular SM of patients with BPD.

Strikingly, we also show here that genetic ablation of the murine gene encoding GSNOR, *adh5*, protects against all detrimental cardiopulmonary BPD outcomes observed after neonatal hyperoxia. These data suggest that GSNOR has an important role in BPD pathophysiology in general and that this pathway might be considered for therapeutic targeting by agents currently available. In addition, the data demonstrate that GSNOR expression in SM is of central importance to BPD-related PH, providing a model for studying BPD lung disease independent of PH, and suggesting that GSNOR inhibition might be valuable for the treatment of BPD-related PH.

Because of functional and structural lung immaturity, premature newborns frequently have respiratory insufficiency and require oxygen supplementation to treat hypoxemia (36). However, oxygen use is associated with an increased risk for morbidities such as BPD (37), childhood wheezing (38), and BPD-related PH (39). Consistent with other hyperoxia/room air recovery studies in newborn rodents, hyperoxia elicited sustained alveolar simplification (28, 29, 40, 41), increased airway reactivity (28, 42), and resulted in PH changes (41, 43).

The hyperoxia exposed $adh5^{-/-}$ and SM/$adh5^{-/-}$ mice were protected from PH changes. However, loss of $adh5^{-/-}$ from SM alone (SM/$adh5^{-/-}$) did not protect against AHR in this model. Thus, the conditional SM knockout provides a unique model for separating airway and vascular effects of hyperoxic BPD. It appears from our data to be unlikely that AHR contributes to PH by causing regional hypoxia or through other mechanisms (11, 13, 44): AHR in the SM/$adh5^{-/-}$ mouse occurs independent of and thus does not lead to PH changes. That is, AHR with frequent bronchospasm and regional hypoxia is not required for the development of RVH; the two processes appear to be causally distinct. These findings will likely be important for future studies of the causes, natural history, and prevention of BPD-related PH.

Several factors could account for the fact that $adh5^{-/-}$ were protected from AHR whereas SM/$adh5^{-/-}$ were not. The most obvious possibility is that GSNO catabolism is regulated in the airways primarily by the epithelium and inflammatory cells, not in the SM alone. Immunohistochemical and immunoblot data from human asthma, which also show elevated GSNOR lung expression, would support this possibility (21, 45). Further supporting a role of the epithelium in BPD AHR, we have recently shown that epithelial debridement of murine BPD intrapulmonary bronchioles prevents relaxation when a GSNOR inhibitor is applied (30).

Loss of intrapulmonary airway support, resulting from decreased alveolarization, has also been proposed as a cause for obstruction and AHR in BPD; thus, moderate neonatal murine hyperoxia (65%) has been shown to cause lasting alveolar simplification with diminished airway tethering (40). We interpreted the decreased radial alveolar counts and increased mean linear intercepts as simplification of alveolar structures as opposed to emphysematous changes seen in high-dose supplemental oxygen and double-hit models (27). The SM/$adh5^{-/-}$ mouse had both alveolar simplification and AHR, suggesting that decreased alveolarization was associated with AHR independently of PH SM changes, such that

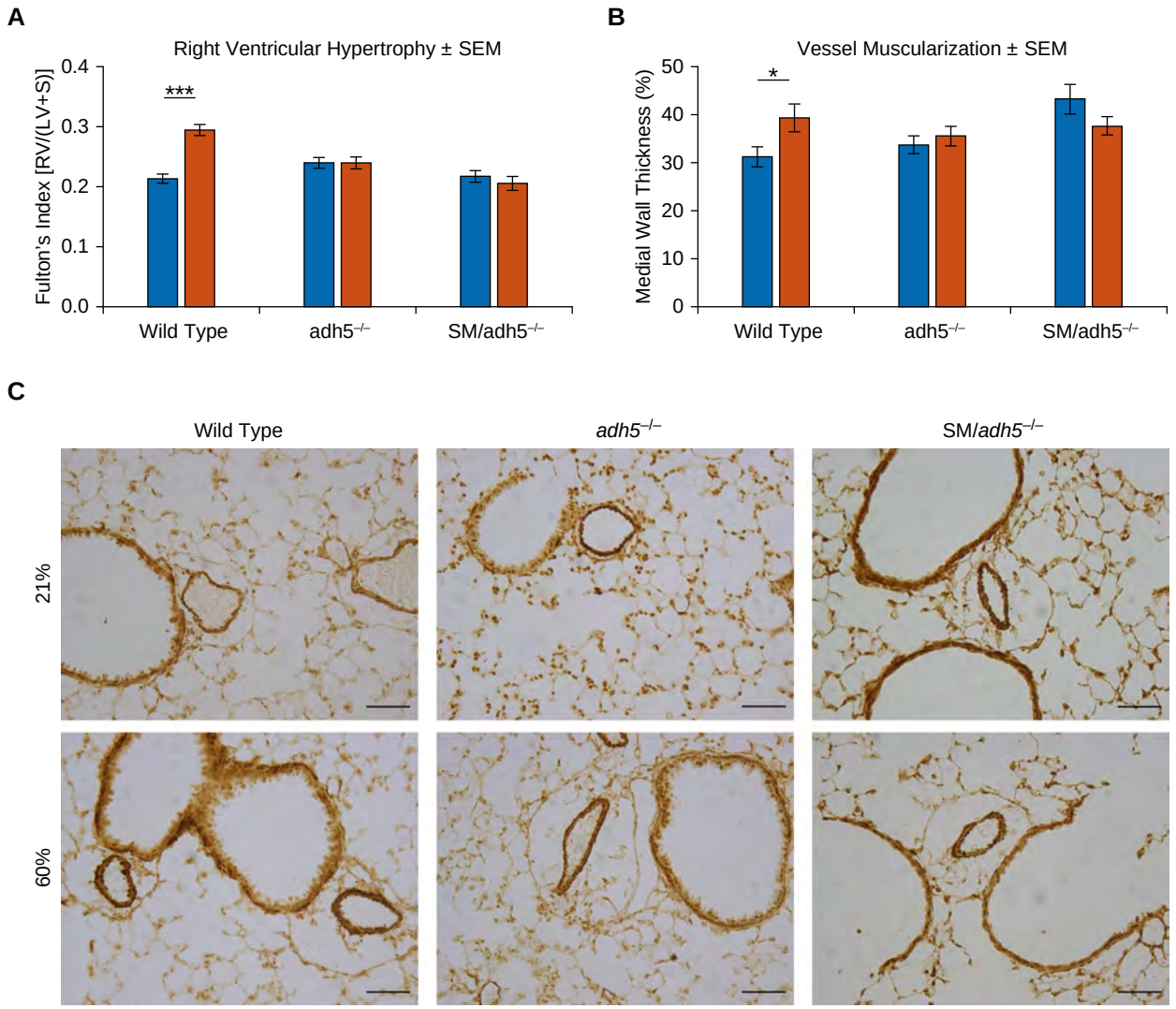

Figure 5. Both global and selective smooth muscle deletion of *adh5* protects against bronchopulmonary dysplasia pulmonary hypertensive changes. Neonatal hyperoxia exposure (60% · 3 wk, red bars) with room air recovery resulted in significantly increased (*A*) Fulton's index and (*B*) medial wall thickness in wild-type mice compared with room air controls (21%, blue). Hyperoxia-exposed global *adh5* knockout mice ($adh5^{-/-}$) and conditional smooth muscle *adh5* knockout mice (SM/$adh5^{-/-}$) were protected from these changes. (*C*) Representative photomicrographs of α-ACTA2 stained vessels are shown. Fulton's index n = 10–19/group, photomicrographs n = 6–7/group. *$P < 0.05$ and ***$P < 0.001$, by ANOVA with *post hoc* Tukey comparisons. Scale bars, 50 μm. LV = left ventricular; RV = right ventricular; S = septum.

decreased parenchymal support could be an SM-independent explanation for AHR (40). Furthermore, the absence of GSNOR in fibroblasts and myofibroblasts of SM lineage did not protect against hyperoxic alveolar simplification.

If alveolar simplification is a central issue underlying AHR in the hyperoxic model, the question is: why would the global loss of GSNOR ($adh5^{-/-}$ mouse) protect against alveolar simplification? Moderate hyperoxia has been shown to impair the growth and function of mesenchymal stromal and endothelial colony-forming cells from human infants (46, 47) and persistently impair lung growth in neonatal rodents (27, 28). In a rodent model of severe neonatal hyperoxia (95%), continuous treatment with an *S*-nitrosylating gas, ethyl nitrite, promoted alveolar development, improved baseline compliance changes, attenuated nitrotyrosine accumulation, and decreased inflammation (29). Ethyl nitrite inhalation *in vivo* results in robust *S*-nitrosothiol production (48). As with ethyl nitrite, the $adh5^{-/-}$ mouse appeared to be protected from oxidant-driven nitrotyrosine modifications in the lung. To the best of our knowledge, this is the first model in which airway and vascular effects of BPD can be separated.

Increased right heart work caused by pulmonary vascular disease is commonly associated with BPD, and pulmonary vascular disease contributes significantly to BPD-related morbidity (10, 11). However, the underlying pathophysiology of BPD-related RVH and pulmonary vascular disease is unclear. Here, we have identified a gene in the pulmonary vascular SM that is necessary for the development of end-organ PH changes in our murine BPD

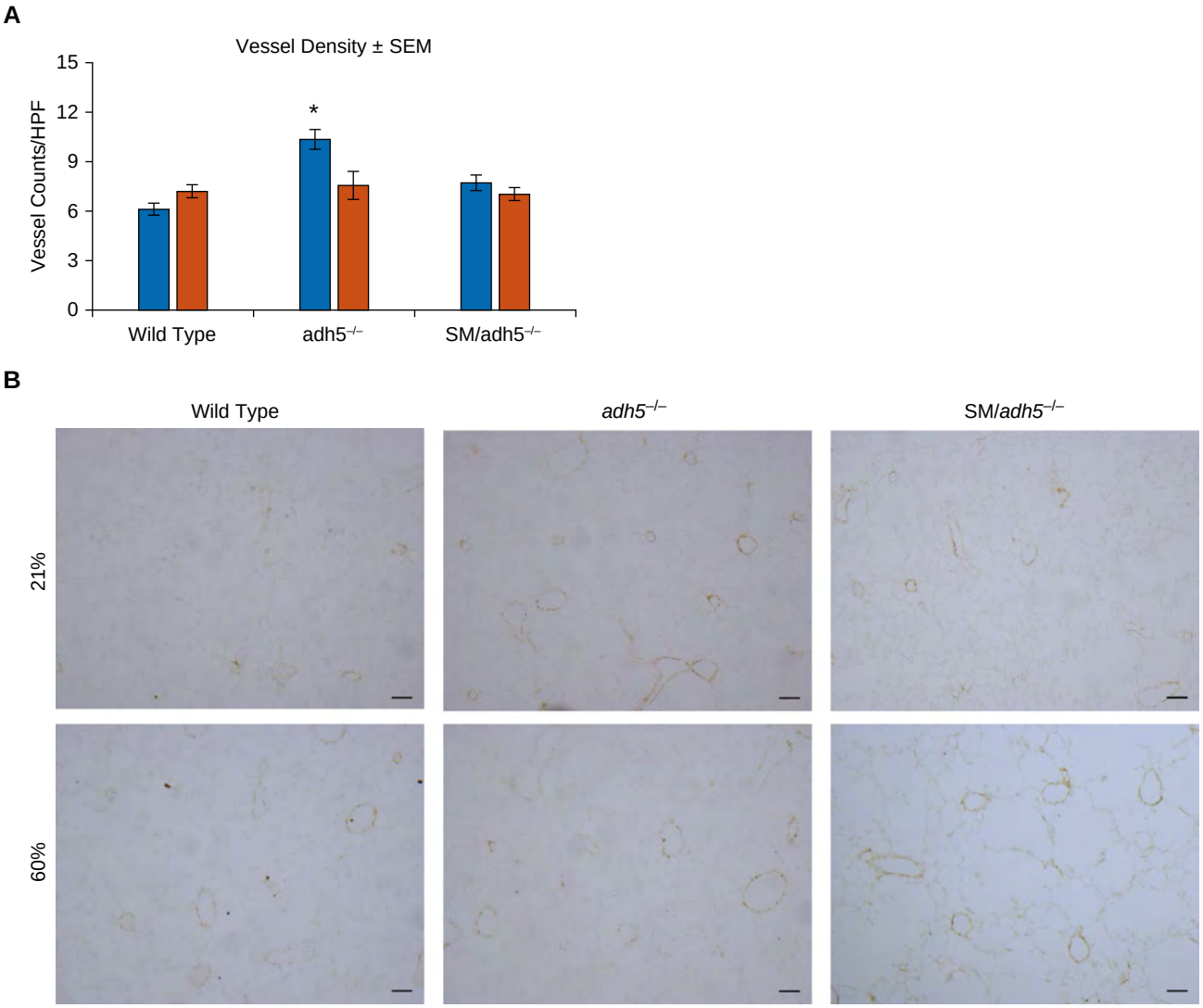

Figure 6. Global deletion of *adh5* in room air increases vessel density. Neonatal hyperoxia exposure (60% · 3 wk, red bars) with room air recovery resulted in (*A*) no significant difference in vessel density in wild-type or SM/$adh5^{-/-}$ animals; however, room air $adh5^{-/-}$ had increased vessel density compared with all groups ($P \leq 0.034$). (*B*) Representative photomicrographs of von Willebrand Factor stained vessels are shown. n = 6–7/group. *$P < 0.05$, by ANOVA with *post hoc* Tukey comparisons. Scale bars, 50 μm. HPF = high-powered field.

model. GSNO prevents pulmonary vasoconstriction (18) and promotes angiogenesis (19), in part through stabilization of hypoxia transcription factors. We show that both global $adh5^{-/-}$ and the conditional SM SM/$adh5^{-/-}$ mice were protected from these PH end-organ changes, with the room air $adh5^{-/-}$ also displaying increased pulmonary vessel counts. These contrasting $adh5^{-/-}$ and SM/$adh5^{-/-}$ findings suggest upregulation of GSNOR during hyperoxia exposure (26) causes loss of pulmonary vascular SM GSNO, increasing pulmonary arterial tone and right heart work. These data suggest that GSNO normally present in the healthy lung helps to maintain low pulmonary vascular tone, consistent with work from our laboratory and others (33, 49).

Nitrogen oxide metabolism is critical to cardiopulmonary physiology in health and disease (16). Here, our work specifically looked at the results of reducing endogenous *S*-nitrosothiol catabolism through the use of a global $adh5^{-/-}$, which displays a fourfold decrease in timed catabolism in this model (30). Loss of GSNOR does not appear to increase baseline nitrosative stress, eNOS expression, or total nitrite levels. Thus, the effect of global deletion of GSNOR is not to increase total nitrogen oxides production, but rather to increase *S*-nitrosothiols, which are higher in $adh5^{-/-}$ than wild-type mice (31). It is of interest that there also appears to be a beneficial effect with loss of GSNOR attenuating protein nitration in hyperoxia. That is, GSNO may be a nitrogen oxide that prevents oxidative and/or nitrosative stress. This is an interesting general point that will certainly require further investigation in the future.

Our sustained moderate hyperoxia model increased GSNOR expression and activity (26) but also encompasses multiple stages of murine lung development—from the saccular through early and bulk alveolarization (27). The human lung

equivalent would be a severe course of BPD/PH with supplemental oxygen requirements from preterm birth into early childhood. Other exposure intensities and durations may yield alternative findings. Indeed, although alveolar simplification has mostly been shown to be dose dependent regarding oxygen exposures (27, 28, 41), severe hyperoxia (>70%) results in minimal AHR when compared with mild and moderate hyperoxia (28, 42). Most hyperoxic rodent PH models use severe hyperoxia for days or weeks (41, 43), whereas moderate hyperoxia requires longer durations or secondary insults to develop PH changes (41, 50, 51). We intended to study the role of GSNOR in the SM while balancing alveolar simplification, AHR, and PH RVH; however, further optimization of the hyperoxic model may better highlight these translational findings.

This work has limitations. Quantitative measures of GSNOR activity from the matched human BPD specimens and controls were not possible. Furthermore, human pathology is heterogeneous, so we cannot exclude the possibility that GSNOR expression is altered in the control lungs or account for the multiple antemortem exposures experienced by the BPD subjects, which may have impacted GSNOR expression. The BPD mice were quite labile and did not tolerate right heart catheterization, so direct measurements of pulmonary arterial pressure responses to hypoxemia could not be accomplished in all animals and thus may have been underpowered to detect differences. Closed chest studies such as rodent echocardiogram have been reported (43, 50, 51) and may be an alternative approach in studying these labile mice. Also, despite observed hyperoxic alveolar simplification and increased medial wall thickness, vessel density did not significantly differ in wild-type BPD mice. Although identifying the *adh5* gene product, GSNOR, as a critical enzyme for the development of hyperoxic BPD is novel, much work is still required to evaluate the multiple mechanisms implicated in the development of BPD and associated PH.

Although BPD has evolved (2) since Northway and colleagues (52) first described the disease more than five decades ago, few therapies exist for the breathing problems and cardiovascular issues survivors experience (34, 35). Although many premature infants respond to β2 agonist bronchodilators, children with BPD show variable responses with a potential to develop tolerance during chronic therapy, and β2 agonists do not decrease BPD incidence in randomized trials. Treatment of BPD-related PH is challenging because current therapies are limited to various classes of pulmonary vasodilators (34), which do not address underlying fixed obstructions and parenchymal simplification during the development of BPD (13, 14), aspects from which the global $adh5^{-/-}$ were protected.

In summary, global *adh5* expression is required for hyperoxic BPD in this mouse model. Expression of *adh5* in the vascular SM is necessary for the development of RVH and increased pulmonary vascular muscularization. The SM conditional knockouts were not protected from alveolar simplification or AHR. These distinctions provide a unique system for studying the separate development of different forms of heart and lung pathology in BPD. The findings in human infant lung specimens were consistent with the murine data, suggesting that GSNOR has a central role—and could be a new target in the treatment of BPD-associated PH. Because pulmonary vascular disease and right ventricular dysfunction are major contributors to BPD-related morbidity and mortality, the human infant data also suggest that interventions could be investigated for use early in the course of neonatal lung disease to attempt to prevent and treat these complications by repletion of GSNO and/or inhibition of GSNOR. ■

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