

RESEARCH ARTICLE

Caveolin-1 scaffolding domain peptide prevents hyperoxia-induced airway remodeling in a neonatal mouse model

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Vogel ER, Manlove LJ, Kuipers I, Thompson MA, Fang YH, Freeman MR, Britt RD Jr, Faksh A, Yang B, Prakash YS, Pabelick CM. Caveolin-1 scaffolding domain peptide prevents hyperoxia-induced airway remodeling in a neonatal mouse model. *Am J Physiol Lung Cell Mol Physiol* 317: L99–L108, 2019. First published May 1, 2019; doi:10.1152/ajplung.00111.2018.—Reactive airway diseases are significant sources of pulmonary morbidity in neonatal and pediatric patients. Supplemental oxygen exposure in premature infants contributes to airway diseases such as asthma and promotes development of airway remodeling, characterized by increased airway smooth muscle (ASM) mass and extracellular matrix (ECM) deposition. Decreased plasma membrane caveolin-1 (CAV1) expression has been implicated in airway disease and may contribute to airway remodeling and hyperreactivity. Here, we investigated the impact of clinically relevant moderate hyperoxia (50% O₂) on airway remodeling and caveolar protein expression in a neonatal mouse model. Within 12 h of birth, litters of B6129SF2J mice were randomized to room air (RA) or 50% hyperoxia exposure for 7 days with or without caveolin-1 scaffolding domain peptide (CSD; caveolin-1 mimic; 10 μ l, 0.25 mM daily via intraperitoneal injection) followed by 14 days of recovery in normoxia. Moderate hyperoxia significantly increased airway reactivity and decreased pulmonary compliance at 3 wk. Histologic assessment demonstrated airway wall thickening and increased ASM mass following hyperoxia. RNA from isolated ASM demonstrated significant decreases in CAV1 and cavin-1 in hyperoxia-exposed animals while cavin-3 was increased. Supplementation with intraperitoneal CSD mitigated both the physiologic and histologic changes observed with hyperoxia. Overall, these data show that moderate hyperoxia is detrimental to developing airway and may predispose to airway reactivity and remodeling. Loss of CAV1 is one mechanism through which hyperoxia produces these deleterious effects. Supplementation of CAV1 using CSD or similar analogs may represent a new therapeutic avenue for blunting hyperoxia-induced pulmonary damage in neonates.

airway smooth muscle; asthma; caveolin-1; extracellular matrix; hyperoxia; neonatal

INTRODUCTION

Reactive airway diseases including wheezing and asthma are significant sources of pulmonary comorbidity in neonatal and

pediatric patients (3, 4, 6, 24). Preterm infants are disproportionately exposed to perinatal insults that can contribute to the development of wheezing and asthma. Postnatal supplemental oxygen is a common perinatal exposure that can contribute to development of reactive airway disease as well as other pulmonary comorbidities. The effects of supplemental oxygen are broad and include inflammatory responses and alveolar arrest in addition to reactive airway disease that particularly affects the bronchial airways (10, 19, 33, 40).

The contribution of hyperoxia to airway hyperreactivity and airway remodeling, characterized by increased airway smooth muscle (ASM) mass and extracellular matrix (ECM) deposition, has been demonstrated in a number of animal studies (5, 17, 26, 41). These studies have demonstrated that perinatal oxygen exposure can result in increased airway contractility, epithelial hyperplasia, increased ASM mass, and increased collagen deposition in the airway wall. In addition, the effects of hyperoxia are long lasting, with increased airway reactivity and ASM noted in mice at 21 days of age (41) and as late as 10 mo of age even after a few days of initial hyperoxia, indicating that early, perinatal oxygen exposure could have long-term consequences for airway structure and function (27). Thus the mechanisms and effects of initial hyperoxia on long-term structural and functional changes in developing bronchial airways become important.

With a focus on alveolar changes (e.g., in the context of bronchopulmonary dysplasia), many previous studies, however, have been performed in the setting of higher levels of oxygen (>80% O₂) than are currently clinically relevant. Recognition of the deleterious effects of supplemental oxygen has led to clinical practice changes, and more moderate levels of hyperoxia (40–60% O₂) are now the clinical standard. Intriguingly and unfortunately, these moderate levels of oxygen may actually predispose to a greater extent of bronchial airway remodeling than higher levels of oxygen. One study found that 40% oxygen increased respiratory system resistance, decreased compliance, and increased ASM mass while mice exposed to higher levels of oxygen (70%) did not significantly differ from room air control mice, in terms of these parameters (41). Similarly, we have previously demonstrated that fetal ASM (fASM) proliferation is increased with moderate levels of hyperoxia (40–50% O₂) while oxygen >60% results in apoptosis (16). These studies demonstrate that the

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effects of oxygen are likely dose dependent, with higher levels predisposing to alveolar damage while moderate levels contribute to bronchial airway remodeling and reactive airway disease.

Despite data demonstrating the deleterious effects of moderate hyperoxia on the developing lung, the mechanisms behind hyperoxia effects remain poorly elucidated. Since supplemental oxygen is a necessary clinical tool to support the preterm lung, finding mechanisms of hyperoxia that could be intervened upon is particularly important and appealing. The plasma membrane protein caveolin-1 (CAV1) could represent one such mechanism. We and others have previously found caveolae in ASM of different species and demonstrated the importance of CAV1 in lung diseases such as asthma (8, 12, 13, 30, 31). There has been increasing interest in the cavin family of proteins that are key to caveolae formation (cavin-1), shape (cavin-2), trafficking (cavin-3), and interactions with intracellular structures (cavin-4) (7, 15, 25). However, their cell-specific function or role in lung disease is not known. Decreased CAV1 expression has been shown to increase cellular proliferation and ECM deposition in a number of fibrotic diseases, including pulmonary fibrosis (12, 35, 36, 42). In addition, we have previously demonstrated that CAV1 knockout mice exhibit airway hyperreactivity (2) and that hyperoxia results in decreased expression of CAV1 in human fASM cells as well as increased fASM deposition of ECM components (39). In our study, pretreatment of fASM cells with caveolin-1 scaffolding domain peptide (CSD), a mimic of caveolin-1, prevented hyperoxia-induced changes in ECM deposition. Intriguingly, CSD is active when administered in vivo and has been shown to ameliorate alveolar collagen deposition, alveolar damage, and inflammation in a murine bleomycin model of adult pulmonary fibrosis (37).

In light of our in vitro data demonstrating decreased CAV1 as a contributor to fASM remodeling as well as the in vivo applications of CSD, we investigated the impact of moderate hyperoxia on airway remodeling and caveolar protein expression in a neonatal mouse model. We expected hyperoxia exposure to result in decreased CAV1 expression in the ASM and that this would contribute to increased ASM mass and ECM deposition in the airway wall. We also anticipated that hyperoxia-exposed pups would demonstrate increased airway reactivity with methacholine challenge. We hypothesized that concomitant treatment of hyperoxia-exposed pups with CSD would prevent these structural and physiologic changes.

METHODS

Animals and exposures. All animal study protocols were approved by the Mayo Clinic Institutional Animal Care and Use Committee and conducted in accordance to guidelines derived from the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Studies were conducted using B6129SF2J mice, which are the appropriate wild-type strain for CAV-1 knockout mice that we have previously used (2) (Jackson Laboratories, Bar Harbor, ME). Mice were bred following a minimum of 1 wk of acclimation to the vivarium. Within 12 h of birth, each litter of newborn mice was randomized to room air (RA) or 50% hyperoxia exposure with or without CSD treatment (Fig. 1). Thus the four groups were RA/PBS, O2/PBS, RA/CSD, and O2/CSD.

CSD with the Antennapedia Internalization Sequence linked to the caveolin-1 NH₂ terminus was used to help cell permeability (Enzo Life Sciences; Farmingdale, NY). Each litter was paired with a second litter receiving the same treatment protocol, and the pups were pooled and randomly redistributed. Oxygen levels were monitored continuously. To prevent oxygen toxicity, nursing dams were rotated between paired RA and oxygen-exposed litters every 24 h. The pups randomized to the CSD groups were administered 10 μ l of 0.25 mM CSD daily via intraperitoneal injection. The remaining pups received intraperitoneal injection of PBS with 5% DMSO as a vehicle control (CSD was prepared as a 1 mM stock solution in 20% DMSO, which was diluted to 0.25 mM in 5% DMSO). Intraperitoneal injection of CSD was chosen due to technical limitations given the size of the pups. Following 7 days of exposure, all pups were returned to RA for 2 wk of normoxic recovery. At 21 days of age, pups were euthanized and pulmonary tissue was collected. Lungs were randomly distributed to be either flash frozen for laser capture microdissection (LCM) processing or inflated under 25 cmH₂O with 4% paraformaldehyde for histologic assessment.

Assessment of pulmonary function. At 21 days of age, pulmonary function parameters were assessed using a FlexiVent system (SCIREQ, Montreal, Canada). The mice were anesthetized with intraperitoneal ketamine (20 mg/kg) and xylazine (2 mg/kg). Tracheal cannulation was performed with 20-gauge blunt Luer cannulas, and positive pressure ventilation was initiated. Baseline pulmonary resistance and compliance were assessed (38). Following baseline assessments, increasing doses of nebulized methacholine (PBS 0, 6.3, 12.5, 25, and 50 mg/ml; Sigma, St. Louis, MO) were administered and dose response of the baseline pulmonary parameters were measured.

Histology and morphometric evaluation. At 21 days, lungs inflated at 25 cmH₂O with 4% paraformaldehyde. Paraffin embedded samples were sectioned at 10 μ m and stained with hematoxylin and eosin (H&E) or Masson trichrome stains using standard protocols. Stained sections were assessed at $\times 200$ and $\times 400$ magnification for changes in airway and alveolar architecture using previously described techniques (18, 28). Representative images and measurements were taken

Fig. 1. Experimental protocol. Within 12 h of birth, litters of B6129SF2J mice were randomized to either room air (RA) or hyperoxia (50% O₂) exposure with or without caveolin-1 scaffolding domain (CSD) peptide treatment (10 μ l of 0.25 mM CSD or PBS vehicle; intraperitoneal). After 7 days, all pups were allowed to recover for 14 days in RA before pulmonary function testing and tissue processing at 21 days of age. Thus, the four groups were RA/PBS, RA/CSD, O2/PBS, and O2/CSD. PND, postnatal day; AHR, airway hyperresponsiveness; LCM, laser capture microdissection.

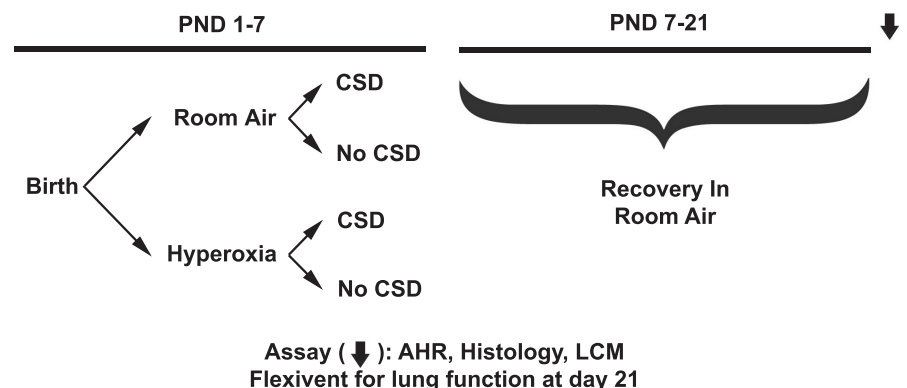


Table 1. Baseline characteristics

	RA/PBS (n = 21)	RA/CSD (n = 27)	O2/PBS (n = 21)	O2/CSD (n = 25)	P Value
No. of female (male)	9 (12)	12 (15)	10 (11)	14 (11)	0.315
Average weight (SE)	11.74 (0.28)	11.26 (0.26)	11.08 (0.23)	10.44 (0.21)	0.01

All experimental groups contained comparable numbers of male and female mice. The O2/caveolin-1 scaffolding domain (CSD) group was noted to have a significantly lower weight when compared with the other groups. This did not result in significant impact in the data as evidenced by the lack of difference between the O2/CSD group and room air (RA)/PBS (control) groups throughout the remaining data.

from a minimum of three airways per section with two sections per animal. Airway thickness and diameter, alveoli number, and alveolar size were quantified using ImageJ software. These measurements and analysis were performed by a blinded investigator.

Immunohistochemistry. Ten-micrometer cross sections of paraffin-embedded lungs were deparaffinated and rehydrated in preparation for immunostaining. Samples were blocked in 4% normal donkey serum with 0.3% Triton Tris-buffered saline (TBS) for 30 min before overnight incubation in TBS (for unstained control) of 1 μ g/ml monoclonal anti- α -smooth muscle actin (A2547; Sigma-Aldrich, St. Louis, MO), polyclonal caveolin-1 (AB18199; Abcam, Cambridge, MA), polyclonal cavin-1 (NB100-60635; Novus, Littleton, CO), polyclonal cavin-3 (AB76676; Abcam), or polyclonal fibronectin (AB2413; Abcam) primary antibodies. After washing, slides were incubated for 2 h in 1:200 diluted Alexa 555 anti-mouse secondary antibody (Invitrogen, Carlsbad, CA). Nuclei were visualized using DAPI stain. A Nikon Eclipse Ti microscope was used to visualize slides under an oil immersion lens. Immunofluorescence was quantified using ImageJ software. ASM abundance was quantified by measuring the area of smooth muscle actin with fluorescent intensity over a set threshold.

Caveolin-1, cavin-1, cavin-3, and fibronectin abundance was quantified by measuring fluorescent intensity within the airway wall. All images were captured under identical fluorescence and camera settings to allow for semiquantitative comparisons between groups.

Laser capture microdissection. Flash frozen, RNase-free lungs were sectioned at 10 μ m and mounted on prechilled clean slides. Following ethanol dehydration, samples were visualized using a Veritas microdissection system (Arcturus, Molecular Devices, Sunnyvale, CA) under light microscopy and small airways (300- to 350- μ m diameter) were identified. An infrared laser was used to microdissect ASM regions of interest onto CapSure Macro LCM caps, and total RNA was isolated from the caps for quantitative RT-PCR (qRT-PCR) as previously described (2). Tissue purity and homogeneity were determined using qRT-PCR for quantification of smooth muscle actin and epithelial marker E-cadherin.

Quantitative real-time PCR. Total RNA was isolated from the LCM caps using an RNeasy micro kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized using standard methods. RT-PCR was performed using an LC480 LightCycler system (ABI, Carlsbad, CA). Each sample was run in triplicate.

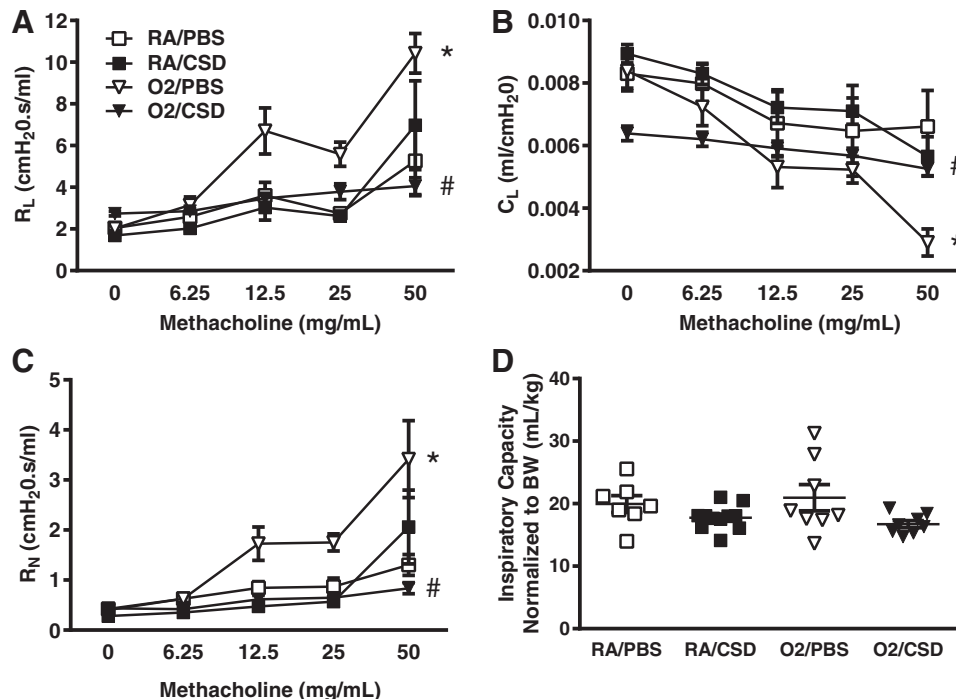


Fig. 2. Pulmonary resistance and compliance with methacholine dose response. A: O2/PBS exposed mice demonstrated increased pulmonary resistance (R_L) at 21 days compared with room air (RA)/PBS control ($*P < 0.05$). Mice in the O2/caveolin-1 scaffolding domain (CSD) group had significantly decreased pulmonary resistance compared with O2/PBS mice ($\#P < 0.05$). There was no difference noted between the RA/PBS, RA/CSD, and O2/CSD groups. B: O2/PBS mice had significantly lower compliance (C_L) than RA/PBS ($*P < 0.05$). O2/CSD mice had a significantly higher compliance than O2/PBS mice ($\#P < 0.05$). There was no difference in compliance noted between the RA/PBS, RA/CSD, and O2/CSD groups ($P < 0.05$). C: Newtonian resistance (R_N) as a measurement of resistance of the central and conducting airways demonstrated a significant increase in R_N in the O2/PBS group compared with RA/PBS control ($*P < 0.05$) while O2/CSD mice had a significant decrease in R_N in comparison to O2/PBS mice ($\#P < 0.05$). D: there was no significant difference in inspiratory capacity when normalized to body weight (BW) between treatment groups. Data are presented as means $\pm$ SE; $n = 7$ –11 pups in each group. Two-way ANOVA with Tukey's multiple comparison test was used for statistical analysis.

mRNA of interest was normalized to housekeeping gene GAPDH. Relative fold change was calculated by using the $\Delta\Delta C_t$ method. Primers of interest included collagen IA2, collagen IIIA3, caveolin-1, cavin-1, and cavin-3.

Statistical analysis. Two to four litters were assigned to each of the experimental groups. All functional and histologic assessments included a minimum of two litters. Statistical analyses were performed using GraphPad software statistical package (GraphPad Software, La Jolla, CA). Data were analyzed by unpaired Student's *t*-test or two-way ANOVA with statistical significance set at $P < 0.05$. Numbers of animals are indicated in figure legends.

RESULTS

Baseline characteristics. Baseline characteristics, including weight and sex, of all pups included in the study were assessed before the terminal experiment (Table 1). There was no statistical difference in the sex distribution of pups across groups. Male and female pups were included in all assessments in comparable numbers. All pups were weighed at 21 days, before lung function assessment and tissue harvesting. The pups in the O₂/CSD group showed body weights that were significantly lower than for the other three groups ($P < 0.05$).

Respiratory mechanics. Compared with RA/PBS mice, O₂/PBS mice demonstrated significantly increased total lung resistance (R_L ; $P < 0.05$, Fig. 2A). O₂/PBS mice also demonstrated decreased dynamic compliance (C_L) when compared

with RA/PBS mice ($P < 0.05$, Fig. 2B). Treatment with CSD abrogated these changes in both resistance and compliance. No significant difference was found between either RA/CSD- or O₂/CSD-treated mice and the RA/PBS control group (Figs. 2A and 2B). Using input impedance, we also examined Newtonian resistance (R_N ; Fig. 2C) as a measurement of resistance of the central and conducting airways and found again a significant increase in R_N in the O₂/PBS group that was abolished with CSD treatment ($P < 0.05$, Fig. 2C). There was no significant difference in inspiratory capacity when normalized to body weight between the different treatment groups. These results show the significant effect of hyperoxia per se on airway function and the alleviating effect of CSD.

Histology and lung morphometry. Standard lung morphometric techniques were used to determine ASM thickness corrected for airway diameter. Examination of H&E sections demonstrated significantly increased ASM thickness in the O₂/PBS group compared with RA/PBS control. This increase was significantly reduced with CSD treatment ($P < 0.05$, Fig. 3, A and B). Measurement of airway epithelial thickness normalized to airway diameter and epithelial metaplasia (measured as DAPI count and normalized to airway diameter) demonstrated no difference between treatment groups (Fig. 3, C and D). These results demonstrate the effect of hyperoxia and/or CSD specifically on the ASM layer per se.

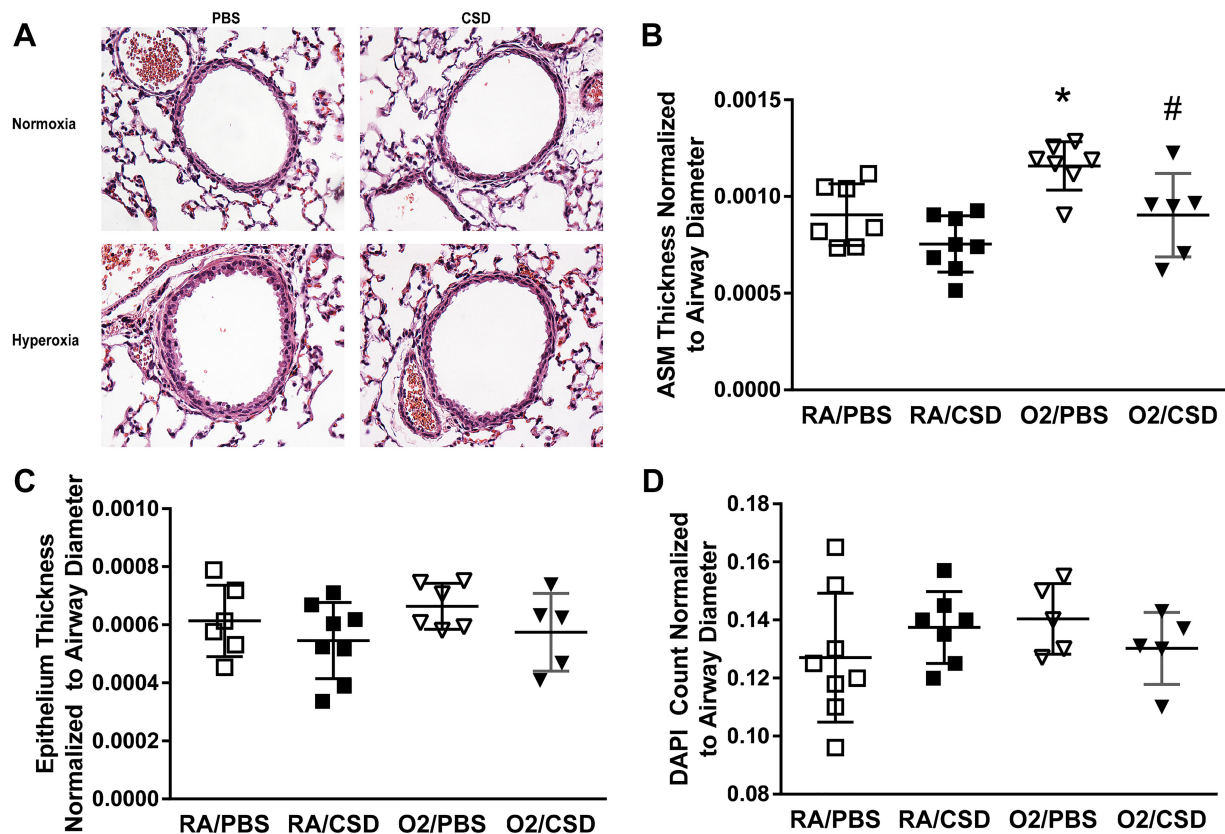


Fig. 3. Caveolin-1 scaffolding domain (CSD) prevents hyperoxia-induced increase in airway thickness. A: O₂/PBS exposure resulted in airway wall thickening characterized by a thicker airway smooth muscle (ASM) layer by hematoxylin and eosin staining. B: quantitative analysis showed significant increase in ASM thickness/airway diameter when compared with room air (RA)/PBS mice ($*P < 0.05$). CSD treatment abrogated these remodeling changes, resulting in a significant decrease in airway thickness/airway diameter in O₂/CSD mice when compared with O₂/PBS mice ($#P < 0.05$). C and D: average airway epithelial thickness/airway diameter and epithelial metaplasia (expressed as DAPI count) showed no significant difference between treatment groups. Data are presented as means $\pm$ SE; $n = 6-8$ in each group. One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis.

Extracellular matrix deposition (ECM) was assessed using Masson trichrome staining for collagens (Fig. 4A) and immunohistochemistry for fibronectin, with the latter used to perform quantitative analysis of ECM deposition within the ASM layer (Fig. 4B). Hyperoxia increased ECM deposition, which was blunted in the presence of CSD as suggested by the Masson trichrome stains (Fig. 4A) and the fibronectin quantification ($P < 0.05$ for both O₂ and CSD effects; Fig. 4B).

Examining H&E sections for alveolar changes (Fig. 5) demonstrated no difference in alveolar size or number between treatment groups (Fig. 5, A–C), further demonstrating the significant effect of hyperoxia and CSD treatment specifically on the bronchial airways. These findings in combination with the findings in Fig. 4 further demonstrate the effects of moderate hyperoxia on ASM and conversely the alleviating effects of CSD. In addition, the neonatal hyperoxia model demonstrates value for exploring airway changes rather than alveolar modifications as in bronchopulmonary dysplasia.

ASM area was further assessed through immunofluorescent staining for α -smooth muscle actin. Airways from O₂/PBS

mice demonstrated significantly increased α -smooth muscle actin-positive areas compared with RA/PBS control ($P < 0.05$, Fig. 6). These changes were abrogated with CSD treatment ($P < 0.05$, Fig. 6).

Expression of mRNA for caveolae-associated proteins. mRNA assessment was performed by RT-PCR on ASM isolated by LCM to assess changes in caveolar gene expression between all groups at 21 days of life. CAV1 was significantly decreased in O₂/PBS mice and additional CSD treatment did not affect this change. Interestingly, RA/CSD mice also showed decreased CAV1 expression. Cavin 1 mRNA expression was decreased in O₂/PBS mice without further change with CSD. In contrast, cavin-3 mRNA significantly increased with hyperoxia and CSD significantly reduced this change ($P < 0.05$; Fig. 7). In regard to protein expression, we used immunohistochemistry to measure CAV1 and cavin-1 and -3 expression (Fig. 8). CAV1 expression was significantly decreased in the O₂/PBS group but was restored in the O₂/CSD group. Cavin-1 significantly increased with RA/CSD. In addition, its expression was increased in the O₂/CSD group in comparison with O₂/PBS. Cavin-3 demonstrated a significant

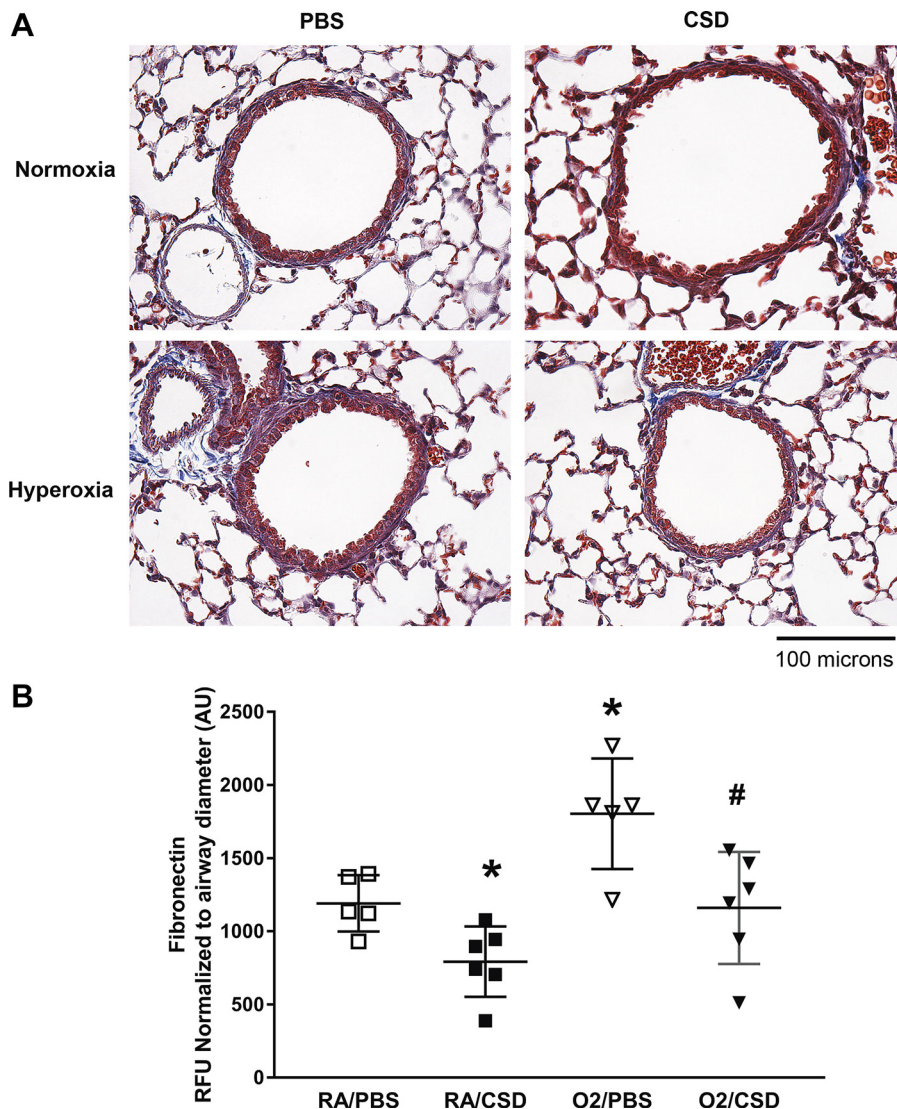


Fig. 4. Caveolin-1 scaffolding domain (CSD) reduces extracellular matrix deposition in hyperoxia-treated mice. **A:** Masson trichrome staining showed greater collagen staining in the airways of 21-day-old O₂/PBS mice, which was reduced by CSD. **B:** assessment of fibronectin deposition in immunohistochemically stained sections showed that compared with room air (RA)/PBS mice, O₂/PBS mice have significantly greater fibronectin deposition ($*P < 0.05$). This increase was substantially reduced by treatment with CSD ($\#P < 0.05$); $n = 5-6$ in each group. One-way ANOVA was performed for statistical analysis. RFU, relative fluorescence units.

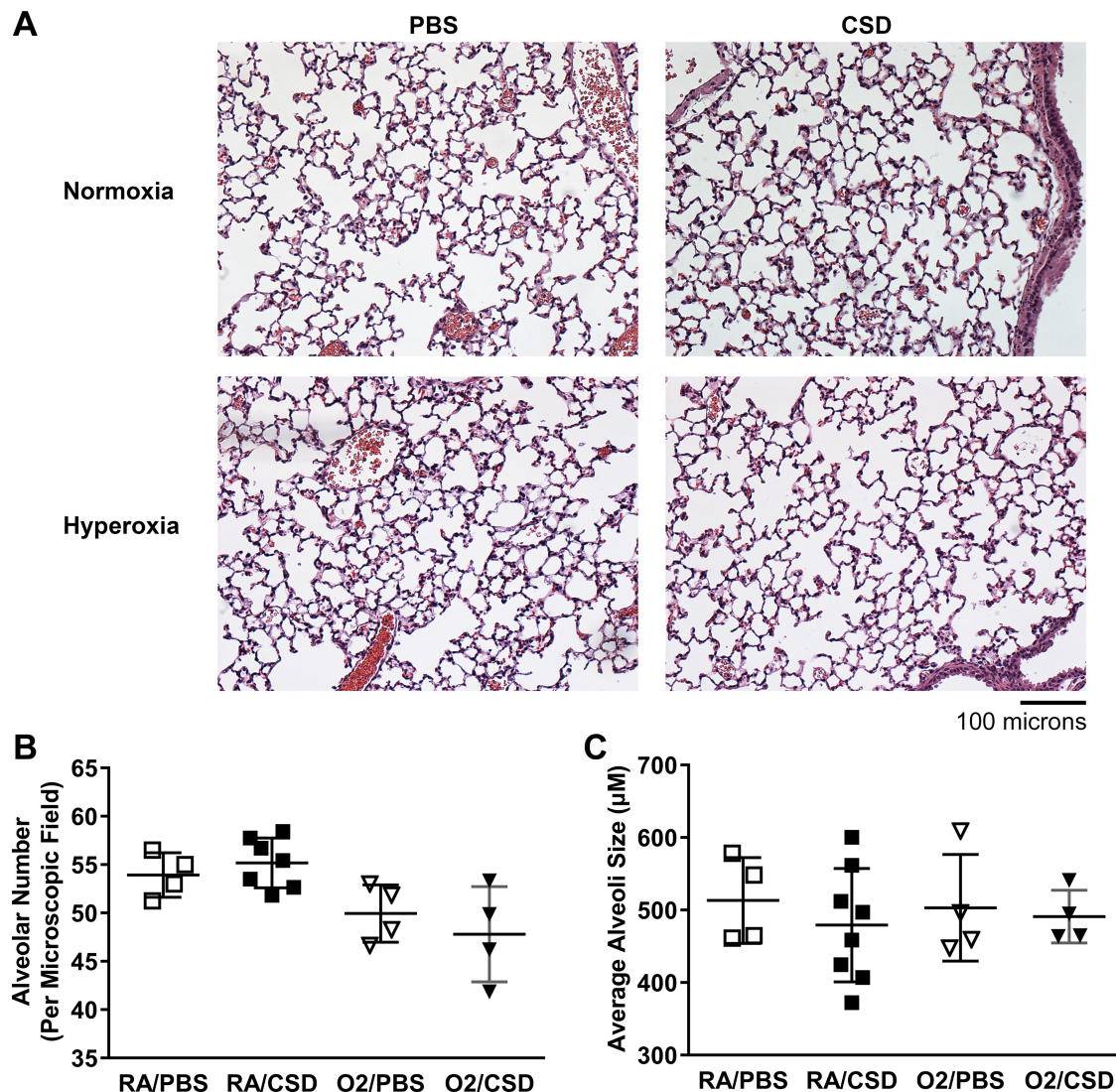


Fig. 5. Hyperoxia does not affect alveolar number and size. There was no significant difference in the appearance of alveolar structures (A), number (B), or alveolar size (C) between treatment groups. Data are presented as means $\pm$ SE; $n = 4-8$ in each group.

increase in the RA/CSD and O2/CSD groups in comparison to RA/PBS ($P < 0.05$, Fig. 8).

DISCUSSION

Perinatal exposure to supplemental oxygen in the context of prematurity constitutes a significant risk factor for subsequent development of reactive airway diseases such as asthma. In this regard, the role of ASM in altered contractility and airway structure via remodeling is becoming evident, although the mechanisms underlying oxygen effects in the context of persistent airway remodeling and hyperreactivity remain poorly understood. In this study, we demonstrate hyperoxia-induced changes in ASM caveolar proteins and the alleviating effect of CSD supplementation as a potential intervention. Overall, exposure to hyperoxia resulted in increased airway resistance and decreased compliance with methacholine challenge. Hyperoxia also caused changes in airway structure, including increased ASM thickness and increased ECM deposition. CSD treatment abrogated these hyperoxia-induced changes.

Reactive airway diseases, such as asthma, involve both functional and structural changes. Airway remodeling, characterized by increased ASM mass (reflecting changes in cell proliferation and size) and increased ECM deposition, is the primary structural change, and it ultimately results in stiffer, more fibrotic airways (9, 20, 32). Remodeling induced airway wall-thickening decreases airway caliber, resulting in an obstructive pathology (9, 29, 32). In this study, we found significant changes in both ASM layer thickness and ECM deposition with moderate hyperoxia exposure. These results are consistent with prior murine and cellular studies that have demonstrated significant remodeling with moderate hyperoxia (16). In previous *in vitro* work using fetal ASM cells, we found a dose-dependent effect of hyperoxia on cellular proliferation (16). Moderate levels of hyperoxia (40–60%) resulted in increased ASM proliferation while high levels of hyperoxia resulted in apoptosis. Further work in fetal ASM cells found that moderate hyperoxia also increases ECM deposition (39). Several studies have found increased collagen deposition in the

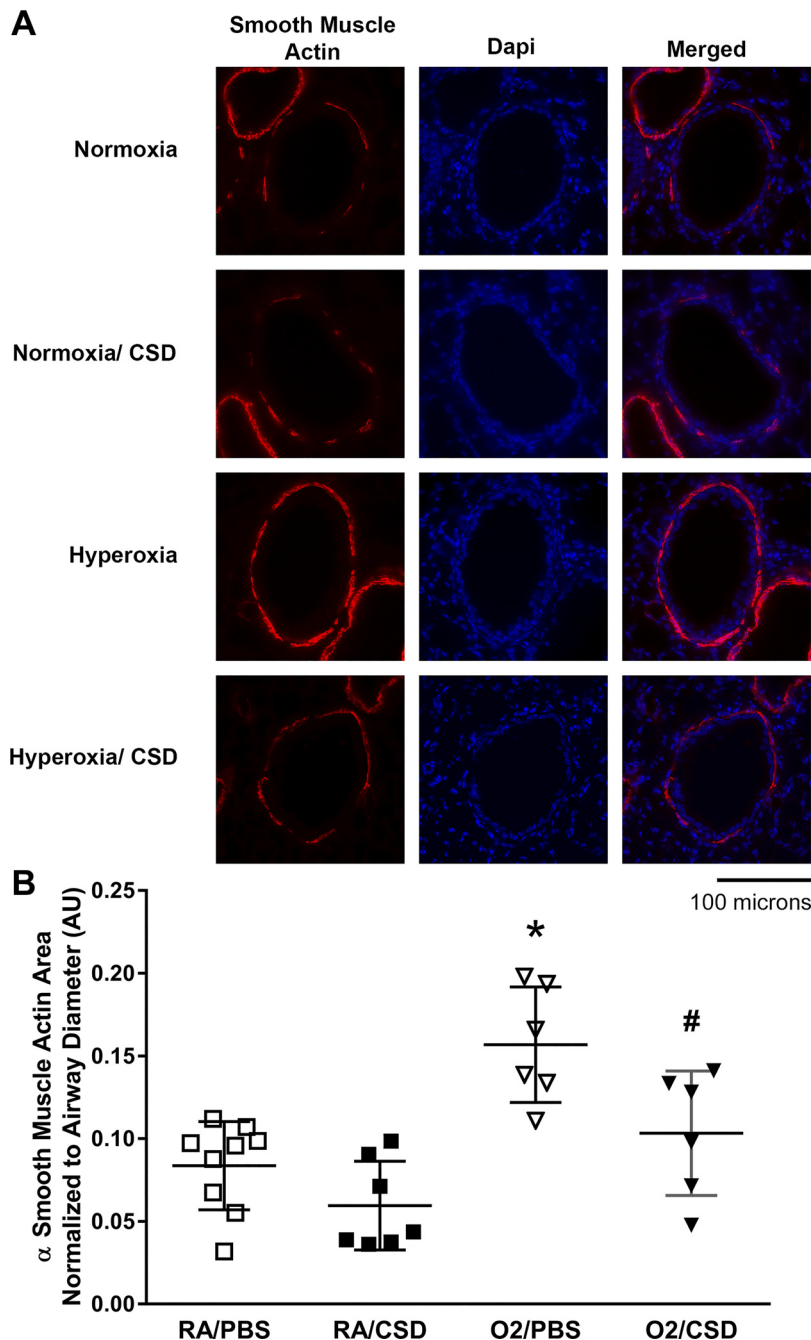


Fig. 6. Caveolin-1 scaffolding domain (CSD) attenuates hyperoxia-mediated increase in airway α smooth muscle actin area. **A:** imaging of immunofluorescently tagged smooth muscle actin demonstrated increased area in O₂/PBS mice at 21 days. **B:** O₂/PBS smooth muscle actin expression was significantly increased from room air (RA)/PBS control ($*P < 0.05$). CSD-abrogated hyperoxia-mediated increase and O₂/CSD airways showed significantly less smooth muscle actin expression than O₂/PBS airways ($\#P < 0.05$). There were no significant differences between RA/PBS, RA/CSD, and O₂/CSD groups. Data are presented as means $\pm$ SE; $n = 6-9$ in each group. One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis. AU, arbitrary units.

airways of mice exposed to 40–70% oxygen in the perinatal period (41). The persistence of these structural changes is of particular relevance, extending well beyond the immediate perinatal period.

The underlying cause of structural and functional changes and possible interventions to prevent detrimental effects of supplemental oxygen therapy remain elusive. Certainly increasing care has been taken to minimize perinatal exposure to excessive supplemental oxygen. However, since even moderate levels of oxygen are associated with detrimental pulmonary effects, additional interventions require further investigation. Supplementation of CAV1 represents one possible intervention.

CAV1 is a ubiquitous cellular mediator involved in numerous molecular pathways including cellular signaling, fibrosis, and cellular proliferation (11, 12, 14, 23, 30, 34). It is the primary constituent molecule of caveolae, which are flask-shaped cellular membrane invaginations that represent key sites for cellular regulation and cross-talk for cellular signaling pathways (12, 34). Decreased expression of CAV1 has been associated with pulmonary fibrosis and increased ECM deposition in both cellular and murine models of pulmonary disease (12, 34, 37). In addition, CAV1 knockout mice demonstrate increased airway reactivity as well as airway remodeling (2). In a previous study, we found that CAV1 expression was significantly decreased in fetal ASM cells exposed to moderate

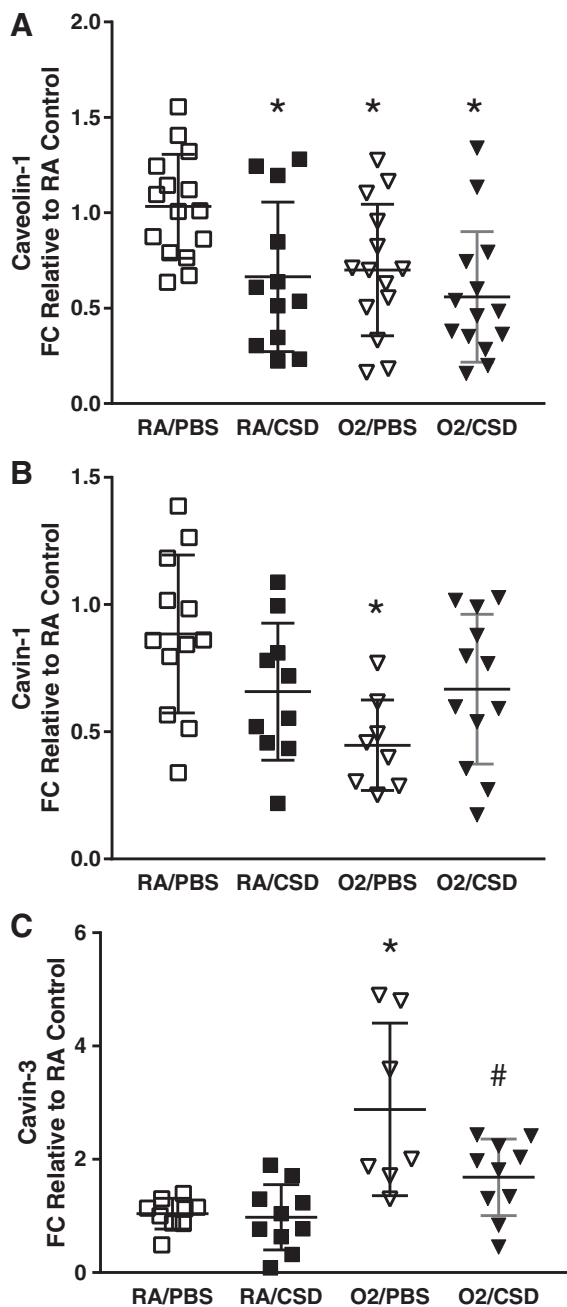


Fig. 7. Airway smooth muscle (ASM) mRNA expression of caveolar proteins with hyperoxia exposure. mRNA expression changes were assessed in ASM isolated using laser capture microdissection. A: caveolin-1 expression was significantly reduced in all treatment groups in comparison to RA/PBS ($*P < 0.05$). B and C: while O2/PBS significantly decreased cavin-1 ($*P < 0.05$; B), O2/PBS treatment significantly increased cavin-3 ($*P < 0.05$; C); the effect was alleviated in O2/caveolin-1 scaffolding domain (CSD) animals ($\#P < 0.05$). Data are presented as means $\pm$ SE; $n = 8$ –14 pups per group. One-way ANOVA with Tukey's multiple comparisons test was used for data analysis. FC, fold change.

hyperoxia. Here, we similarly found that CAV1 mRNA expression was decreased in the ASM of neonatal mice exposed to hyperoxia, even weeks after the exposure ended. CAV1 protein expression within ASM by immunofluorescent evaluation was similarly decreased with hyperoxia exposure, while CSD treatment prevented this change. We additionally found

that caveolar regulators such as cavin-1 were decreased while cavin-3 expression was increased with hyperoxia exposure. Both Cavin-1 and cavin-3 expression increased with CSD treatment. These findings are intriguing because cavin-1 is involved in insertion of CAV1 into the cellular membrane while cavin-3 is involved in removal and internalization of CAV1 from its active site in the membrane (12, 21, 22, 34). Supplementation with CSD not only prevented the hyperoxia-mediated decrease in CAV1 expression but also increased the expression of cavin-1, which could result in more efficient insertion of CAV1 into its active position in the cellular membrane. In light of these findings, we further explored whether supplementation of CAV1 through use of CSD, an analog of the active portion of CAV1, could abrogate the changes in airway structure and function.

CSD peptide is a stable analog of the active portion of CAV1 that can be used for in vivo and in vitro applications. In this study, repletion of CAV1 function through intraperitoneal administration of the CSD peptide did result in abrogation of the hyperoxia-mediated changes in airway structure and function. Airway reactivity, ASM thickness, and ECM deposition were all decreased in mice treated with CSD during hyperoxia exposure. Of note, room air mice treated with CSD (RA/CSD group) did not demonstrate any significant change in pulmonary function, ASM, or ECM deposition compared with control mice. This implies that CSD supplementation offsets the effects of hyperoxia but does not result in decreased ECM deposition or ASM changes in a normal airway not exposed to a hyperoxic insult. The data are consistent with a study that showed that CSD treatment ameliorated fibrotic remodeling in an adult bleomycin model of lung injury (42). However, the impact of hyperoxia on in vivo caveolar protein expression and the utility of CSD in treating hyperoxia-induced airway remodeling presented here has not been previously demonstrated.

In our study, we used intraperitoneal administration of CSD as initial proof of principle for efficacy of activating CAV-1. We recognize that one limitation is the lack of lung specificity for CSD in this approach. Furthermore, an evaluation of the precise pharmacodynamics of the CSD peptide and the manner in which intraperitoneal administration resulted in the described pulmonary effects was beyond the scope of our current study. Here, several scenarios are possible. One could be prolonged accumulation of the peptide in pulmonary tissue (with expected accumulation in other tissues as well), the other being effects of ongoing/persistent impact following early initiation of CSD-mediated signaling cascades. Certainly, an indirect effect on the lung due to CSD effects on other tissues (given the broad expression of CAV-1) cannot be ruled out. Thus it may be necessary to perform additional studies exploring brief CSD applications at different phases of the hyperoxia versus normoxia exposure, as well as the potential to provide CSD via the intrapulmonary route, which would enhance translational appeal.

While supplementation of CAV1 with CSD prevents hyperoxia-mediated changes, the exact downstream pathways involved in this process remain unclear. Caveolar proteins are certainly involved in numerous cellular signaling pathways responsible for cellular proliferation and fibrosis and can furthermore be themselves modulated by factors such as inflammation, making it difficult to explore individual pathways. Conversely, hyperoxia can enhance ROS and inflammatory

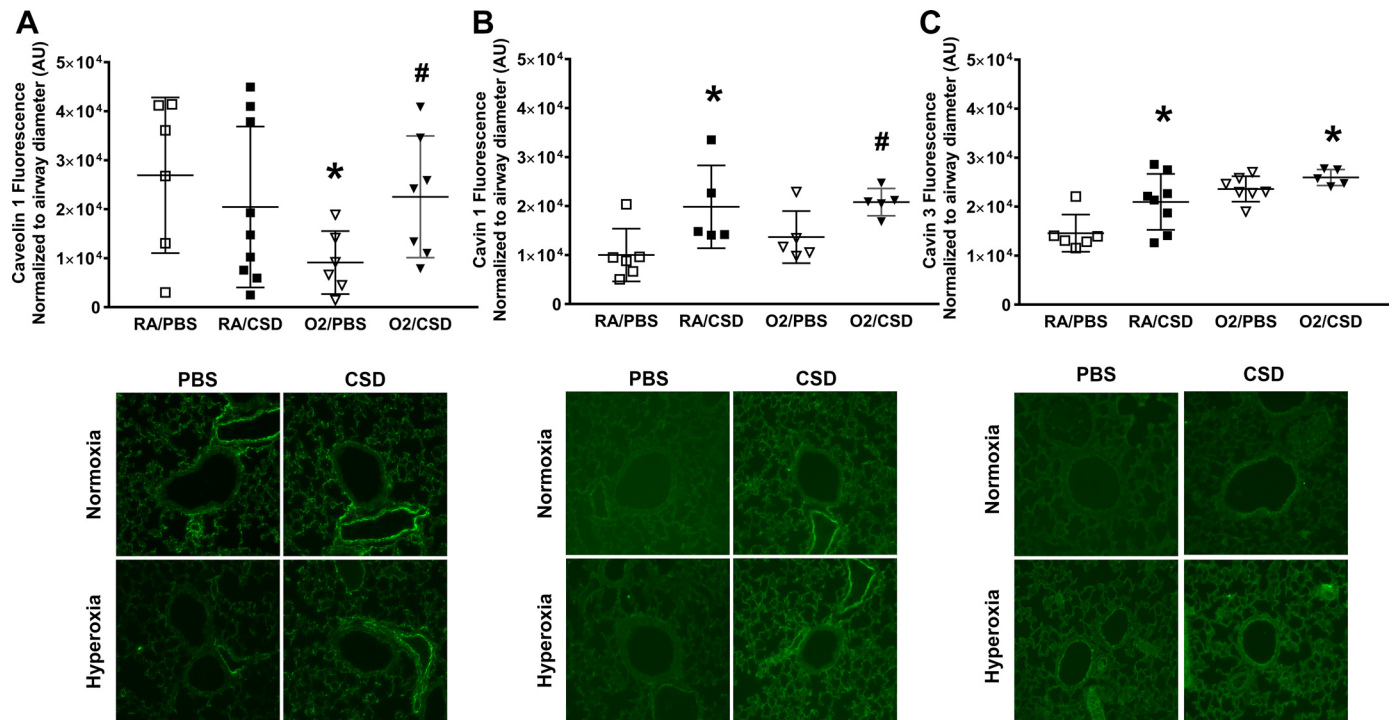


Fig. 8. Caveolin-1 (CAV1) and cavin-1 and -3 protein expression changes with hyperoxia exposure. Changes in protein expression were assessed in tissue samples using semiquantitative immunofluorescence with normalization to airway diameter. A: O₂/PBS significantly decreased CAV1 expression (* $P < 0.05$) while O₂/caveolin-1 scaffolding domain (CSD) reversed this effect (# $P < 0.05$). B: while cavin-1 did not change with O₂/PBS, CSD did increase cavin-1 in both RA (* $P < 0.05$) and O₂ (# $P < 0.05$) subgroups. C: cavin-3 expression also showed a pattern similar to cavin-1 (* $P < 0.05$). Data are presented as means $\pm$ SE; $n = 5$ –9 pups per group. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons test.

cascades leading to complex effects. Nonetheless, it is tempting to suggest that transforming growth factor- β (TGF- β)-mediated pathways are involved as TGF- β is known to be a key regulator of cellular proliferation and ECM deposition (1, 43) and can further be relevant to airway growth. Such mechanisms remain to be explored.

Overall, our data demonstrate that hyperoxia-induced airway changes may, at least in part, be mediated through decreased CAV1 activity. It appears that a decrease in CAV1 protein expression or function results in alterations in airway structure and function that may be ameliorated with administration of a CAV1 analog. Based off of our data, the cells most affected by the hyperoxia-mediated decrease in CAV1 are ASM cells. Our data demonstrate a hyperoxia-mediated increase in ASM as well as increased airway reactivity, both of which are abrogated by CSD treatment. Epithelial and alveolar architecture was not significantly influenced by moderate hyperoxia or by CSD treatment. CSD peptide or other CAV1 analogs may represent a potential therapeutic avenue for prevention and treatment of hyperoxia-induced pulmonary damage in neonates, especially if techniques can be developed for intrapulmonary administration to limit systemic effects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.M.P. conceived and designed research; E.R.V., L.J.M., I.K., M.A.T., Y.-H.F., M.R.F., R.D.B., A.F., and B.Y. performed experiments; E.R.V., L.J.M., I.K., M.A.T., Y.-H.F., M.R.F., R.D.B., A.F., and B.Y. analyzed data; E.R.V., I.K., M.A.T., Y.-H.F., M.R.F., R.D.B., A.F., Y.S.P., and C.M.P. interpreted results of experiments; E.R.V., L.J.M., B.Y., and C.M.P. prepared figures; E.R.V., L.J.M., Y.S.P., and C.M.P. drafted manuscript; E.R.V., L.J.M., I.K., M.A.T., Y.-H.F., M.R.F., R.D.B., A.F., Y.S.P., and C.M.P. edited and revised manuscript; E.R.V., L.J.M., I.K., M.A.T., Y.-H.F., M.R.F., R.D.B., A.F., B.Y., Y.S.P., and C.M.P. approved final version of manuscript.

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