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Hydrogen sulfide, oxygen, and calcium regulation in developing human airway smooth muscle

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Abstract

Preterm infants can develop airway hyperreactivity and impaired bronchodilation following supplemental O₂ (hyperoxia) in early life, making it important to understand mechanisms of hyperoxia effects. Endogenous hydrogen sulfide (H₂S) has anti-inflammatory and vasodilatory effects with oxidative stress. There is little understanding of H₂S signaling in developing airways. We hypothesized that the endogenous H₂S system is detrimentally influenced by O₂ and conversely H₂S signaling pathways can be leveraged to attenuate deleterious effects of O₂. Using human fetal airway smooth muscle (fASM) cells, we investigated baseline expression of endogenous H₂S machinery, and effects of exogenous H₂S donors NaHS and GYY4137 in the context of moderate hyperoxia, with intracellular calcium regulation as a readout of contractility. Biochemical pathways for endogenous H₂S generation and catabolism are present in fASM, and are differentially sensitive to O₂ toward overall reduction in H₂S levels. H₂S donors have downstream effects of reducing [Ca²⁺]_i responses to bronchoconstrictor agonist via blunted plasma membrane Ca²⁺ influx: effects blocked by O₂. However, such detrimental O₂ effects are targetable by exogenous H₂S donors such as NaHS and GYY4137. These data provide novel information regarding the potential for H₂S to act as a bronchodilator in developing airways in the context of oxygen exposure.

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AUTHOR CONTRIBUTIONS

C.M. Bartman, M. Schiliro, M. Helan, Y.S. Prakash, D. Linden, and C. Pabelick designed research; C.M. Bartman, M. Schiliro, and M. Helan performed research and analyzed data; D. Linden contributed new reagents or analytic tools; C.M. Bartman, Y.S. Prakash, D. Linden, and C. Pabelick wrote manuscript drafts. All authors edited and approved the final version.

CONFLICT OF INTEREST

None.

Keywords

calcium; contractility; fetal airway; oxygen; prematurity

1 | INTRODUCTION

Supplemental oxygen (hyperoxia) is unfortunately necessary in the context of premature birth and underdeveloped lungs to maintain oxygenation and ventilation. However, despite advancements in neonatal care, even moderate levels of hyperoxia (30%-60% O₂) can contribute to detrimental airway changes¹⁻⁴ that have not only immediate, but also long-term effects on airway hyperreactivity and impaired bronchodilation toward wheezing and asthma in children.⁵⁻⁸ In this regard, airway smooth muscle (ASM), a critically important cell type for airway contractility and relaxation,^{5,7,9} is a likely prime target of O₂. Accordingly, understanding the mechanisms that contribute to O₂-altered contractility and relaxation of developing ASM becomes important.

The mechanisms of ASM contractility have been well studied in adults,^{9,10} but are relatively less known in developing airways. Nonetheless, using isolated ASM cells from lungs of 18-22 week fetuses, we have demonstrate their suitability for studying regulation of [Ca²⁺]_i and proliferation,¹¹ showing that fASM cells express [Ca²⁺]_i regulatory proteins and respond to bronchoconstrictor agonists such as acetylcholine (ACh) and histamine with robust [Ca²⁺]_i responses. Using this model, we previously found that O₂ enhances fASM [Ca²⁺]_i¹¹ as well as cellular contractility,¹² and O₂-induced increases in airway contractility have also been observed in neonatal mouse models of hyperoxia.^{8,13} Compared to these findings, O₂ effects on bronchodilation per se are largely unknown, although clinical data do show that neonates exposed to O₂ have impaired bronchodilation.¹⁴ In fact, while in adult airways bronchodilation is induced by cAMP (beta agonists) or cGMP (nitric oxide; NO),¹⁵ in developing ASM hyperoxia disrupts the NO-sGC-cGMP axis^{16,17} and NO is clinically less effective,¹⁸ while there is less responsiveness to cAMP.^{1,19} Accordingly, there is need to identify novel bronchodilatory mechanisms in the context of O₂.

Hydrogen sulfide (H₂S) has been known as a toxic gas with the distinct odor of rotten eggs, but over the past two decades, H₂S has been recognized as a vitally important endogenous gaseous signaling molecule akin to NO or CO.²⁰⁻²⁴ H₂S is synthesized via cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) with a third pathway of 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteine aminotransferase (CAT).^{20,24-26} Conversely, intracellular catabolism of H₂S involves the mitochondrial enzymes sulfur quinone oxidoreductase (SQOR) and sulfur dioxygenase (ETHE1). Thus, under normal conditions, the level of intracellular H₂S is carefully controlled by the overall activity of these enzymes. Both endogenous H₂S and that induced using exogenous H₂S donors such as the short-acting, rapid-release donor NaHS, and the slow-release donor GYY4137 have been shown to be anti-inflammatory and cytoprotective, modulating plasma membrane pathways (especially channels) and intracellular signaling pathways to promote vasodilation and resistance to oxidative stress.^{20,21,24,27} Pathways by which H₂S can influence the vasculature are also important in ASM, for example, K_{ATP} and BK_{Ca} channels, MAPK and

PI3/Akt, and mitochondria.^{23,27,28} Additionally, in an adult mouse model of asthma, lack of CSE increases AHR and inflammation.²⁹ Data in adult vasculature point toward effects of H₂S on muscle tone, inflammation, metabolism, proliferation, and fibrosis^{27,28,30–32}; pathways highly relevant to airway disease. There is limited evidence suggesting a link between H₂S production and oxygen availability, although the focus has been directed toward the effect of hypoxic insults on H₂S synthesis (increase in H₂S) and the cytoprotective role of H₂S in hypoxia.³³ However, it is also clear that H₂S biology is complex, with cell-and context-dependent effects.

There is currently little to no data regarding endogenous H₂S or its effects in developing airway or with hyperoxia. Previous studies have shown that in experimental models of bronchopulmonary dysplasia, the slow-releasing H₂S donor GYY4137 restores arrested alveolarization,³⁴ while knockout of CSE or CBS prevents alveolarization,³⁵ overall suggesting a protective role of H₂S in developing lung. CBS is predominantly expressed in brain, and CSE in peripheral tissues, including lung. However, systemic CSE is lower in premature infants and newborns,^{36,37} justifying the idea that prematurity represents a state of deficient endogenous H₂S. Cellular H₂S levels vary inversely with O₂ (hypoxia increases H₂S), which then acts to induce protective responses. Whether hyperoxia reduces endogenous H₂S is not known.

In the present study, we explored the expression of key H₂S synthesis and oxidation enzymes in fASM compared to adult ASM, investigated the effect of O₂ on endogenous H₂S machinery, and uncovered mechanisms of H₂S mediated [Ca²⁺]_i regulation that are targetable by the exogenous H₂S donors, NaHS, and GYY4137, with the hypothesis that a normally protective endogenous H₂S system is detrimentally influenced with prematurity and/or O₂ resulting in impaired/altered ASM functionality.

2 | MATERIALS AND METHODS

2.1 | Human ASM cells

Human fetal ASM (fASM) were enzymatically dissociated from tracheobronchial trees of 18-22 week gestation fetuses following demise, as previously described.^{11,38} fASM cells are de-identified, considered exempt by Mayo Institutional Review Board, and not considered Human Subjects Research. Our studies included nine female and three male fASM as identified by RT-PCR for SRY gene. We previously published characteristics of these cell types including expression of key smooth muscle markers and elements involved in [Ca²⁺]_i regulation.^{7,11,39} Adult human ASM cells were enzymatically isolated from 3rd-6th generation bronchi from surgical specimens of patients undergoing elective thoracic surgery at Mayo Clinic⁴⁰ under Mayo IRB approval (Mayo IRB #08-002518). Lung samples were typically from lobectomies for focal cancers, and airways distal to the tumor were collected for cell isolation, while bulk lung samples were processed for paraffin embedding. Our studies included adult ASM from four female and two male patients.

2.2 | Human neonatal lung tissue

FFPE blocks were obtained from the Mayo specimen biorepository after approval from Mayo Clinic Institutional Review Board (#15-007416).¹²

2.3 | Cell culture, treatments, and drugs

Fetal or adult ASM were cultured < passage 7 in Dulbecco's modified Eagle's medium/F12 (Life Technologies, Rockville, MD) supplemented with 10% FBS and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA). About 24 hours prior to experimentation, fetal and adult ASM were serum-starved in medium containing 0.5% or 0% FBS, respectively. Hyperoxia exposures lasted 24-48 hours in a humidified incubator set to 40% O₂. Inhibitors of H₂S synthesis or oxidation enzymes were purchased from Tocris (Bristol, UK): SHIP2A (6101); and Sigma-Aldrich (St. Louis, MO): DL-Propargylglycine (PAG; P7888; 0.1, 0.5, 100 mM) and Antimycin A (AA; A8674). H₂S donor NaHS was purchased from Sigma-Aldrich: sodium hydrosulfide hydrate (NaHS; 161527). Slow-releasing H₂S donor GYY4137 was purchased from Tocris Bioscience (3658; 50 μM). Calcium channel blockers were purchased from Tocris: Pyr3 (3751; 10 μM), (-)-Xestospongine C (1280; XeC; 1 μM); and Sigma-Aldrich/Millipore: AnCoA4 (532999; 10 μM), and Glibenclamide (G0639; 1 μM).

2.4 | siRNA transfections

siRNAs were purchased from Dharmacon Inc (Lafayette, CO). fASM were transfected following manufacturer's instructions at ~70% confluency with either CSE/CTH ON-TARGETplus SMARTPool human siRNA (L-003481-00-0005), CBS ON-TARGETplus SMARTPool human siRNA (L-008617-00-0005), or "Scrambled" ON-TARGETplus Non-targeting Control Pool human siRNA (D-001810-10-05). Lipofectamine RNAiMAX Transfection Reagent was used throughout (ThermoFisher Scientific, Scotts Valley, CA). 48 hours post transfection, fASM were used for calcium imaging experiments.

2.5 | Immunofluorescence

Paraffin-embedded human neonatal or adult lung sections were de-paraffinized and immunostained using standard procedures and three-color immunofluorescence. The antibodies used and their concentrations and specificity have been characterized previously.^{41,42} Images were obtained on a Nikon Eclipse Ti2 fluorescence microscope at 400× using established techniques, with fixed illumination settings via a Thorlabs LED light source and image acquisition parameters above background controls at each wavelength.

2.6 | Western blot

Antibodies used for Western blot were purchased from Abcam (Cambridge, MA): CBS (ab96252; RRID:AB_10678974); Novus Biologicals (Centennial, CO): SQRDL (NBP1-84510; RRID:AB_11027088); Invitrogen (Carlsbad, CA): CSE (PA5-29725; RRID:AB_2547199), and ETHE1 (PA5-56040; RRID:AB_2641135); and Cell Signaling Technology (Danvers, MA): GAPDH (2118; RRID:AB_561053). The Criterion Western Blot system (Bio-Rad) and Li-Cor Odyssey XL were used to measure protein expression. Image Studio software was used to quantify band intensity and blots were normalized to

GAPDH. Fold change of the treatment group from the control group was calculated where appropriate.

2.7 | H₂S detection (probe)

H₂S fluorescent probe, P3 (Sigma-Aldrich/Millipore; 534329)⁴³ was used at a final concentration of 10 μ M (optimal concentration empirically determined). Cells were incubated with the probe for 30 minutes prior to fluorescence measurements using a Flexstation plate reader (Ex/Em: 375/505 nm). Measurements were normalized to cell number, which was quantified using CyQUANT NF assay kit (Invitrogen) following manufacturer's instructions (Ex/Em: 480/520) (cell quantification was done in wells receiving the vehicle control instead of P3).

2.8 | [Ca²⁺]_i imaging

Techniques for imaging fASM cells have been previously published.¹¹ fASM were loaded with 5 μ M Fura-2/AM (ThermoFisher Scientific, Scotts Valley, CA) for 30 minutes and washed with HBSS (containing 2 mM CaCl₂, 1 mM MgSO₄, HEPES, pH 7.4). Based on the experimental protocol, fASM were treated with either: PBS, H₂O, or DMSO (vehicle), H₂S donor NaHS, H₂S donor GYY4137, Ca²⁺ channel inhibitors Pyr3, Glibenclamide, XeC, or AnCoA4, or H₂S donor +Ca²⁺ channel inhibitors. NaHS treatments were 30 minutes and calcium channel inhibitor treatments were 1 hour total. GYY4137 treatments were done for a total of 72 hours (for hyperoxia exposures, GYY4137 was added for 24 hours prior to additionally introducing 48 hours of hyperoxia). fASM were imaged on an inverted microscope (Nikon Eclipse Ti-U) using filters to detect Fura-2/AM (Ex/Em: 340 and 380/410 nm). Agonist (histamine; 10 μ M or acetylcholine; 10 μ M) was perfused during imaging and baseline, peak, and amplitude of [Ca²⁺]_i responses were assessed.

2.9 | Store-operated Ca²⁺ entry

Techniques for imaging store-operated Ca²⁺ entry (SOCE) in fASM have been previously described.⁴⁴ fASM were loaded with 5 μ M Fura-2/AM for 30 minutes at room temperature and washed with HBSS prior to live cell imaging. Cells were imaged on an inverted microscope (Nikon Eclipse Ti-U) using filters to detect Fura-2/AM (Ex/Em: 340 and 380/410 nm). Cells were perfused for ~2 minutes/condition by first removing extracellular calcium, followed by inhibition of L-type channels with nifedipine (KCl used to stabilize membrane potential), and lastly depletion of SR Ca²⁺ stores by CPA (in zero calcium) before reintroduction of 2 mM extracellular Ca²⁺ in the presence of nifedipine and CPA to induce SOCE. Rate (slope) and peak response after reintroduction of Ca²⁺ were measured.

2.10 | Statistical analysis

All experiments used at least four fASM patient samples ("n" = one patient line). Data were analyzed using either unpaired or ratio paired *t* tests or two-way ANOVA, where appropriate, in GraphPad Prism 8.02 software. Data are represented as mean $\pm$ SEM and *P* < .05 used for statistical significance.

3 | RESULTS

3.1 | Key enzymes involved in H₂S synthesis and oxidation are expressed in developing ASM

Relative gene and protein expression levels of key H₂S synthesis and oxidation enzymes were assessed in fetal and adult ASM. At both mRNA (Figure 1A) and protein levels (Figure 1B), CSE (*CTH*), CBS, SQRDL (*SQOR*), and ETHE1 were all expressed albeit at lower levels in fetal ASM compared to adult, although developmental differences in expression varied depending on the enzyme (Figure 1A,B). Representative blots are shown in Figure 1B. *MPST* gene expression was not detected in fASM samples, and therefore, this avenue was not pursued further. Interestingly, the synthetic enzymes CBS and to a lesser extent CSE were expressed to a higher level in adult suggesting that endogenous H₂S levels may be lower in developing airway. Conversely, ETHE1 was expressed at much higher levels by both fetal and adult ASM while SQRDL was higher in adult ASM, suggesting that endogenous H₂S is likely catabolized to a large extent.

To visualize protein expression in tissue, human lung sections from neonates (who died <30 days post birth) and adults were stained for immunofluorescence microscopy. CSE, CBS, SQRDL, and ETHE1 were expressed and co-localized with α -smooth muscle actin (α SMA) cells of both neonatal and adult human airways (Figure 1C), with the smooth muscle layer showing immunoreactivity particularly for CBS in both age groups but also for ETHE1 (particularly in neonates). The epithelial layer generally expressed all of the enzymes with no discernable differences between age groups.

3.2 | Hyperoxia alters expression of key enzymes involved in H₂S synthesis and oxidation and blunts H₂S production in fASM

Human fASM were exposed for 48 hours to 40% O₂ vs. 21% O₂ in humidified 5% CO₂. Samples were then assessed for gene (Figure 2A) and protein (Figure 2B) expression of the key H₂S enzymes. While statistical significance was absent at the genomic level for *CBS*, *CTH*, *SQOR*, or *ETHE1*, protein levels for CBS were downregulated in hyperoxia compared to normoxia while ETHE1 was also downregulated with a trend for decreased SQRDL (Figure 2B). Representative Western blots are shown in Figure 2B.

To determine the effect of hyperoxia on endogenous H₂S production, fASM were stained with a sensitive and specific H₂S fluorescent probe.⁴³ After 48 hours of hyperoxia, fASM exhibited less H₂S production compared to normoxia (Figure 2C). These data indicate that the endogenous H₂S machinery is functional in fASM and is adversely affected by O₂ toward producing less H₂S.

3.3 | Inhibiting endogenous H₂S increases [Ca²⁺]_i in fASM

We previously showed that hyperoxia increases [Ca²⁺]_i responses to histamine in fASM.¹¹ Since we found decreased H₂S synthesis in hyperoxia (Figure 2C), we first determined whether targeting the endogenous machinery altered H₂S production or oxidation. Using DL-propargylglycine (PAG) to inhibit H₂S production arising primarily through CSE (PAG is more selective for CSE, but can also inhibit CBS at higher concentrations)^{45,46} (Figure

3A) or Antimycin A (AA) to inhibit SQRDL (Figure 3B), we demonstrated decreases or increases in H₂S production, respectively, occurs. To determine whether inhibiting H₂S production would alter [Ca²⁺]_i, fASM were then treated with 0.1, 0.5, or 100 mM PAG prior to [Ca²⁺]_i imaging using Fura-2/AM.^{35,45,46} Blocking H₂S production with PAG resulted in an increased peak and amplitude of [Ca²⁺]_i response to histamine and did not affect baseline (Figure 3C). Representative tracings are shown. The contribution of CSE and CBS in [Ca²⁺]_i regulation was assessed by CSE or CBS siRNA-mediated knockdowns which resulted in increased [Ca²⁺]_i responses to histamine (Figure 3D). Knockdown validation was done by Western blot (representative images shown in Figure 3D). These data support our findings that lack of H₂S synthesis detrimentally impacts [Ca²⁺]_i regulation.

3.4 | Exogenous H₂S decreases [Ca²⁺]_i in fASM

We next determined whether elevating H₂S in fASM could rescue deleterious effects of hyperoxia on [Ca²⁺]_i. We used a rapid-releasing H₂S donor, NaHS, to first determine whether exogenous H₂S increases intracellular H₂S. About 250 μM acute (<30 minutes) NaHS increased H₂S levels in both normoxic and hyperoxia fASM, as measured by H₂S fluorescent probe (Figure 4). Since these H₂S donor effects were maintained in hyperoxia, we were able to determine donor effects on [Ca²⁺]_i under both conditions. Acute exposure (30 minutes) to 50 μM NaHS decreased [Ca²⁺]_i responses to histamine in normoxic fASM with a similar trend in hyperoxia, reducing [Ca²⁺]_i levels to normoxic levels (Figure 5A,B). The concentration of NaHS used for calcium measurements was most likely not detected by the H₂S fluorescent probe due to limits in detection range, but importantly this suggests that even small changes in H₂S production have potent effects on [Ca²⁺]_i.

While the results using NaHS highlighted an alleviating role for H₂S, in the context of chronic hyperoxia effects, it is important to also demonstrate the sustained effects of H₂S. To this end, we used the slow-releasing H₂S donor GYY4137 to investigate whether this could provide a protective effect in hyperoxia. fASM were treated with 50 μM GYY4137 for 24 hours prior to incubation in hyperoxia for 48 hours (72 hours total GYY4137 treatment). We found that GYY4137 decreased [Ca²⁺]_i response to histamine in both normoxia and hyperoxia (Figure 5C,D). We previously showed that hyperoxia also increases [Ca²⁺]_i response to ACh¹¹ and therefore determined whether the slow-releasing H₂S donor would provide broad-based protective effect in response to bronchoconstrictor agonist. We found that GYY4137 decreased [Ca²⁺]_i response to ACh in normoxia and hyperoxia (Figure 6A).

We previously showed that developing ASM has many of the [Ca²⁺]_i regulatory mechanisms as in adult cells.¹¹ Furthermore, we found that hyperoxia enhances plasma membrane Ca²⁺ influx pathways,¹¹ which is consistent with the idea that developing airway cells are likely more dependent on such influx pathways (exacerbated by hyperoxia) given a need for disassembled endoplasmic/sarcoplasmic reticulum during this period of rapid cell division and proliferation. Studies in vascular smooth muscle have suggested a role for modulation of membrane potential by H₂S via activation of K_{ATP} channels²⁷ which would also affect influx. Therefore we focused on exploring influx mechanisms by which H₂S could regulate [Ca²⁺]_i in hyperoxia. Addition of 10 μM of glibenclamide (K_{ATP} channel blocker) did not largely blunt the effect of NaHS on [Ca²⁺]_i responses to histamine (Figure 6B). Similarly,

inhibition of the IP₃ receptor with Xestospongin C (XeC) did not block NaHS effects, either. However, inhibition of TRPC3 with 10 μ M Pyr3⁴⁷ reversed NaHS effects, suggesting at least one influx mechanism targeted by H₂S in fetal ASM (Figure 6B). Separately, using established techniques,⁴⁸ we explore store-operated Ca²⁺ entry (SOCE) involving initial depletion of intracellular Ca²⁺ stores with cyclopiazonic acid (in zero extracellular Ca²⁺) followed by rapid re-introduction of extracellular Ca²⁺ to activate Orai1 channels that mediate SOCE.¹⁰ NaHS itself decreased SOCE as assessed by amplitude and slope in both 21% and 40% O₂ with NaHS slowing the rate of Ca²⁺ influx in hyperoxia more so than the vehicle control in 21% O₂ (Figure 7A–C). Inhibition of Orai1 with AnCoA4 reversed NaHS effects significantly in 21% O₂ with a similar trend observed in 40% O₂ (Figure 7D). The effects of calcium channel inhibitors on NaHS effects on [Ca²⁺]_i highlight the overall effect of H₂S on plasma membrane pathways in fASM (TRPC3 and Orai1).

4 | DISCUSSION

The present study reports on the expression of functional H₂S machinery in developing airway smooth muscle that can reduce [Ca²⁺]_i; effects made dysfunctional by moderate hyperoxia but can be overcome with exogenous H₂S donors. These data point to a potential novel mechanism to induce airway relaxation in a vulnerable age group of premature infants where therapies such as inhaled NO have not been shown to be effective.¹⁸ Furthermore, O₂ in prematurity has the potential to impact airway structure and function beyond the initial period of exposure toward AHR⁴⁹ and childhood asthma.^{50–52} Thus, H₂S effects in the early postnatal period is mechanistically significant for future therapies (Figure 8).

H₂S is a colorless gas known for its toxicity at high concentrations yet has now been established as an important “gasotransmitter” similar to NO and CO, with a range of physiological effects in different cell types including effects on mitochondrial and bioenergetic function^{20–24,26} (we direct the reader to a recent review by Murphy and colleagues⁵³). It is also now recognized that H₂S is synthesized in a majority of mammalian cells via the enzymes CBS and CSE (that metabolize L-cysteine) as well as 3-MST and CAT.^{20,24–26}

Conversely, scavenging systems involving glutathione, and the catabolizing effects of SQRDL and/or ETHE1 serve to limit intracellular H₂S effects and toxicity.²⁶ This endogenous metabolic machinery is strictly regulated at both transcriptional and post-transcriptional level. CBS is mostly constitutively expressed and its activity is modulated through methylation, sumoylation, and glutathionylation.^{54–56} Conversely, CSE expression is induced in response to insults such as oxidative and ER stress, mitochondrial damage, inflammation, or hyperhomocysteinemia.^{57–61} While expression of these enzymes varies, CBS and CSE appear to be the major synthetic enzymes and have been shown to be present in the lung.^{22,62} Interestingly, at least in adults, CBS appears to be predominantly expressed in vasculature (including smooth muscle) and epithelial cells, and CSE in parenchyma.³⁵ In this regard, our data in adult ASM are novel, showing expression of H₂S synthetic enzymes in mesenchymal cells of the airway, with CBS being a likely mechanism for H₂S production. Interestingly, the high level of ETHE1 in adult ASM suggests that at baseline overall H₂S levels could be kept low, especially given that scavenging systems such as glutathione are

also well-developed in the adult airway. In contrast, our data show that developing (fetal) ASM expresses lower levels of the synthetic enzymes but comparable levels of ETHE1 that would suggest perhaps less ability to generate and maintain endogenous H₂S levels. Intriguingly, we were unable to detect the synthetic enzyme 3MST in our fASM samples, which appears to differ from previous findings of 3MST in adult human ASM in the context of COPD.⁶³ The functional significance of absent 3MST is unclear, but given the detection of endogenous H₂S and its alteration by CBS or CSE inhibition, there are sufficient other enzymatic pathways for H₂S production in developing airway.

The role of H₂S in the respiratory system is complex and ranges from poisonous effects at high doses to central control of respiration at physiological concentrations.^{64,65} Altered H₂S metabolism has been described in human and animal models of lung diseases, such as COPD and tobacco-induced emphysema.²² H₂S in adult airways and adult asthma has been explored. Human studies show decreased serum and breath H₂S levels in asthmatic patients.⁶⁶ Lung H₂S levels are lower in an ovalbumin rat model of asthma⁶⁷ and negatively correlate with inflammatory cell portfolios and ASM hyperplasia. In mice, absence of CSE expression promotes AHR.²⁹ Exogenous administration of H₂S has been demonstrated as protective in ventilator-induced lung injury,^{68,69} acute lung injury,^{70–74} hypoxia/hyperoxia environments,^{74–76} and during acute viral illnesses.^{77–79} Compared to these data, there is currently no information on H₂S in the developing bronchial airways per se. Nonetheless, H₂S has been found to be involved in promoting pulmonary vascular development and alveolarization,^{34,35} likely involving CBS and CSE.³⁵ Absence of these enzymes results in blunted vascular and alveolar development.³⁵ In a mouse model of high-O₂-induced bronchopulmonary dysplasia, the slow-releasing H₂S donor GYY4137 partially restores arrested alveolarization.³⁴ These limited data suggest a protective role of H₂S whose absence is detrimental at least for parenchymal development. Correlatively, in humans, prematurity and infancy are associated with lower levels of CSE with a delay in subsequent increase with age.^{36,37} While the effect of early O₂ on postnatal pattern of CSE expression has not been examined, it is interesting to note that lower CSE in neonates is associated with increased risk of respiratory viral infections, again suggesting a protective role for H₂S. In these contexts, our studies show novel data on lower levels of H₂S generating enzymes in premature ASM that are targetable and appear important in terms of reducing [Ca²⁺]_i and may provide protective roles for deleterious effects of moderate hyperoxia.

While H₂S has been shown to have multiple effects in terms of reducing inflammation, cell proliferation, or vascular tone, the mechanisms of H₂S action may be cell- and context-specific.^{23,27,28} For example H₂S can act as a ROS scavenger and modify proteins by S-sulfhydration that have downstream effects on protein function.^{27,80} There have been several reports including in vasculature showing that H₂S effects involve activation of K_{ATP} channels^{31,81,82} leading to plasma membrane hyperpolarization, but glibenclamide, a K_{ATP} channel blocker, does not consistently modulate H₂S effects on vascular tone.^{83,84} Other voltage sensitive potassium channels,⁸⁴ intracellular acidification,⁸³ and activation of large conductance potassium (BKCa) channels⁸⁵ have all been shown to be involved in different vascular beds. Overall, a major theme that emerges is that effects of H₂S involve the plasma membrane. In this regard, many of the mechanisms explored in vasculature are known to be involved in regulating [Ca²⁺]_i and contractility of ASM, albeit in the adult.^{9,10} We have

previously established that fASM cells do express a portfolio of $[Ca^{2+}]_i$ regulatory pathways¹¹ justifying exploration of plasma membrane pathways. In our studies while we did not find a substantial effect of H_2S on K_{ATP} channels, we found at least one mechanism by which H_2S could promote bronchodilation in developing airways. We report novel findings that SOCE via Orai1 channels, as well as nonspecific influx via TRPC3 channels both contribute to H_2S effects. The relevance of these pathways lies in the fact that both TRPC3 and Orai1 are known to have downstream genomic effects beyond acute Ca^{2+} influx in terms of cell signaling and proliferation,⁸⁶ and thus, contribute to genomic H_2S effects as well. Interestingly, H_2S did not have any effects on intracellular $[Ca^{2+}]_i$ pathways such as IP_3 receptors, which may be consistent with a baseline higher proliferation, and thus, a disassembled endoplasmic reticulum of fASM. Nonetheless, further studies are needed to determine whether H_2S can influence other intracellular pathways, particularly mitochondria given known effects of H_2S on ROS and cellular metabolism which could have impact on proliferation and synthetic activities during a period of rapid airway growth.

Our findings that even moderate levels of hyperoxia reduce H_2S in developing ASM in the context of $[Ca^{2+}]_i$ raises the question whether exogenous H_2S can then be used to overcome hyperoxia inhibition of endogenous pathways. Here, hyperoxia seems to impair H_2S production by reducing synthetic machinery, while allowing H_2S catabolism to be maintained (eg, SQRDL levels are unchanged). Thus, the effect of exogenous H_2S at physiological concentrations becomes important. Interestingly, we observed a significant effect of hyperoxia on protein expression of both H_2S synthetic and catabolic enzymes without a defined genomic effect, suggesting a possible posttranscriptional regulation of these enzymes. Because hyperoxia drives cellular ROS and oxidative stress,^{87,88} possible mechanisms by which hyperoxia detrimentally influences key enzymes involved in H_2S synthesis include posttranslational modifications affecting enzyme function and mitochondrial damage affecting mitochondrial H_2S enzyme function. Additional studies are needed to investigate these novel mechanisms.

Our data show that the H_2S donor NaHS is capable of reducing $[Ca^{2+}]_i$ even under hyperoxic conditions. NaHS is commonly used as an H_2S donor, including in lung studies.^{29,67,82,89} Given lack of data on endogenous H_2S levels in the developing airway, we focused on concentrations used in previous studies in other contexts.^{82,89} We recognize a limitation is an inability to control the kinetics of H_2S release. Nonetheless, at the concentrations used, we can detect an increase in H_2S levels using fluorescent probes, and importantly, observe functional effects in terms of reduced $[Ca^{2+}]_i$ in fASM cells. To address the limitation of H_2S release, we additionally used a slow-releasing H_2S donor GYY4137 to confirm our findings of decreased $[Ca^{2+}]_i$ response to histamine. The relevance of slow-release donors such as GYY4137 further lies in their potential use to alleviate longer term and chronic effects of hyperoxia such as enhanced airway remodeling, and thus, therapeutic potential of H_2S modulators.

In terms of mechanisms, the current study found H_2S effects on plasma membrane pathways that are known to contribute to hyperoxia effects in fASM.^{11,17} However, this does not rule out other relevant cellular pathways influenced by H_2S , such as mitochondrial function. Given previous data that hyperoxia promotes mitochondrial fission¹¹ possibly leading to

altered mitochondrial function and increased ROS, exploring the effect of H₂S on mitochondria in the developing lung may provide elucidations on hyperoxia effects in this critical setting. Moreover, H₂S has been described to act as a phosphodiesterase (PDE) inhibitor and PDE inhibition in vascular smooth muscle cells has been suggested as one mechanism responsible for H₂S-mediated vasodilatation.^{90,91} While not a focus of our current study, the relationship between H₂S and PDE may also be relevant in developing airways, given previous data that moderate hyperoxia disrupts the NO-sGC-cGMP axis in fASM^{16,17} and that fASM is less responsiveness to cAMP,^{1,19} highlighting a need to elevate cyclic nucleotides to promote bronchodilation. These alternative/additional mechanisms of H₂S action may be further relevant to the combined use of H₂S donors and mitochondrial or PDE modulators toward alleviating the effects of hyperoxia.

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Abbreviations:

[Ca²⁺]_i	intracellular calcium concentration
AA	antimycin A
AnCoA4	orai1 inhibitor
CBS	cystathionine-β-synthase
CSE	cystathionine-γ-lyase
ETHE1	ethylmalonic encephalopathy protein 1; persulfide dioxygenase
fASM	fetal airway smooth muscle
Glib	glibenclamide, K _{ATP} inhibitor
GY4137	P-(4-Methoxyphenyl)-P-4-morpholinyl-phosphinodithioic acid
H₂S	hydrogen sulfide
IP3R	inositol 1,4,5-trisphosphate receptor
NaHS	sodium hydrosulfide
Orai1	calcium release-activated calcium channel protein 1 subunit
PAG	DL-propargylglycine

PDE	phosphodiesterase
Pyr3	TRPC3 inhibitor
SOCE	store-operated calcium entry
SQRDL	sulfide quinone oxidoreductase
TRPC3	transient receptor potential channel 3
XeC	IP3R inhibitor

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FIGURE 1.

Key H₂S enzymes in developing and adult airways. Relative (A) mRNA or (B) protein expression of CSE (gene = *CTH*), CBS, SQRDL (gene = *SQOR*), and ETHE1 in fetal and adult airway smooth muscle. Gene and protein expression are normalized to GAPDH. Western blots are representative images from independent experiments comparing five individual fetal and five individual adult ASM samples. Statistical significance in gene or protein expression was determined by multiple *t* tests using the Bonferroni-Dunn method between fetal and adult ASM ($\alpha = .05$). Outliers were removed using Grubbs' Test. Data are represented as means $\pm$ SEM; $n = 5$ patients each. C, Airway images from human neonatal and adult lung sections were stained for α -smooth muscle actin (α SMA) and CSE, CBS, SQRDL, or ETHE1. DAPI mounting medium was used to visualize total cells. Red = protein of interest; green = α SMA; blue = DAPI; * = lumen of airway; yellow v = vessel; white arrow = co-localization of α SMA and protein of interest

FIGURE 2.

Hyperoxia alters mRNA and protein expression of key enzymes involved in H₂S synthesis and oxidation and blunts H₂S production in fASM. fASM samples from normoxia were used as control to compare hyperoxic fASM (48 hours 40% O₂) for RNA. mRNA (A) or protein (B) expression analysis of fASM exposed to 48 hours 21% or 40% O₂ was performed by qPCR or Western blot, respectively. Western blots are representative images from independent experiments comparing 5-6 individual fetal ASM samples from normoxic or hyperoxic conditions. Protein expression quantification was determined by densitometry and normalized by dividing the raw values of each protein of interest by the raw values of GAPDH and fold change calculated from the normoxic controls. Statistical significance in protein expression was determined by multiple *t* tests between normoxic and hyperoxic fASM using the Bonferroni-Dunn method ($\alpha = .05$ vs normoxic control). Outliers were

determined using Grubbs' Test. Data are represented as means $\pm$ SEM; n = 5-6 patients each. C, Normoxic or hyperoxia (48 hours 40% O₂) fASM were treated with 10 μ M H₂S-sensitive fluorescent probe (P3) for 30 minutes prior to detection at ex/em 375/505 nm on a Flexstation plate reader. Data were normalized to cell number (CyQuant assay) by dividing raw fluorescence values by total cell number per condition/sample. Fold change was calculated from respective control (normoxia or vehicle-treated). Statistical significance was determined by ratio paired *t* test (*****P* < .0001 vs normoxia. Data are represented as means $\pm$ SEM; n = 5 patients each

FIGURE 3.

fASM H₂S machinery is targetable and inhibiting H₂S synthesis increases [Ca²⁺]_i response to histamine. fASM were treated with (A) 100 mM of the CSE/CBS inhibitor PAG for 30 minutes or (B) SQRDL inhibitor antimycin A (AA) prior to incubation with H₂S fluorescent probe, P3, and analysis. All data were normalized to cell number (CyQuant assay) by dividing raw fluorescence values by total cell number per condition/sample. Fold change was calculated from vehicle-treated control. Statistical significance was determined by ratio paired *t* test (*****P* < .0001 vs vehicle-treated control). Outliers were determined using Grubbs' Test. Data are represented as means ± SEM; n = 5-8 patients each. C, fASM were treated with 0.1, 0.5, or 100 mM PAG for 30 minutes followed by staining with Fura-2/AM for an additional 30 minutes in the presence of PAG (appropriate vehicle control used for these experiments). D, fASM were transfected with CSE or CBS siRNA (or "Scr." Control) for 48 hours prior to assessment of [Ca²⁺]_i response to histamine. Validation of CSE or CBS siRNA-mediated knockdown was done by Western blot. Calcium imaging analysis was performed as described previously¹¹ in response to 10 μM histamine. Baseline and peak of the response were recorded and amplitude calculated thereafter. Representative tracings are shown. Statistical significance of peak response was determined by ratio paired *t* test (**P* < .05 vs vehicle control). Data are represented as means ± SEM; n = 6 patients each

FIGURE 4.

Exogenous H₂S donor NaHS increases H₂S synthesis in 21% and 40% O₂. fASM were serum deprived and exposed to 21% or 40% O₂ for 48 hours. Cells were treated with 250 μM NaHS for 15 minutes prior to 15 minutes incubation with P3 H₂S fluorescent probe and measurements performed on Flexstation plate reader. All data were normalized to cell number (CyQuant assay) by dividing raw fluorescence values by total cell number per condition/sample. Fold change was calculated from vehicle-treated control fASM in 21% O₂. Statistical significance was determined by twoway ANOVA (**P* < .05 and ***P* < .001 compared to vehicle-treated in 21% O₂; #*P* < .001 compared to 250 μM NaHS in 21% O₂; \$*P* < .001 compared to vehicle-treated in 40% O₂). Outliers were determined using Grubbs' Test. Data are represented as means ± SEM; n = 4 patients each

FIGURE 5.

Exogenous H_2S donors NaHS and GYY4137 decrease intracellular calcium response to histamine. For all experiments, fASM were serum deprived and incubated in 21% or 40% O_2 for 24 hours prior to live cell imaging. fASM were then loaded with 5 μM of the fluorescent Ca^{2+} indicator dye, Fura-2-AM, for 30 minutes in HBSS, washed, and treated under the following conditions: A, 30 minutes 50 μM NaHS or vehicle control (PBS) in fASM exposed to 21% O_2 ; B, 30 minutes 50 μM NaHS or vehicle control (PBS) in fASM exposed to 40% O_2 ; C, 72 hours total 50 μM GYY4137 or vehicle control (DMSO) in fASM exposed to 21% O_2 ; or (D) 72 hours total 50 μM GYY4137 or vehicle control (DMSO) in fASM exposed to 40% O_2 . For the fASM treated with slow-releasing H_2S donor GYY4137 for 24 hours prior to 48 hours normoxia or hyperoxia exposure. Representative tracings are shown. Amplitude was calculated from baseline to maximum peak $[Ca^{2+}]_i$ response. Statistical significance was determined by ratio paired t test (* $P < .05$ vs. vehicle). Outliers were removed by Grubbs' Test. Data are represented as means $\pm$ SEM; $n = 5-6$ patients each

FIGURE 6.

Mechanisms of H₂S donors in intracellular calcium responses. For all experiments, fASM were serum deprived and incubated in 21% or 40% O₂ for 24 hours prior to live cell imaging. fASM were then loaded with 5 μ M of the fluorescent Ca²⁺ indicator dye, Fura-2-AM, for 30 minutes in HBSS, washed, and treated under the following conditions: A, 72 hours total 50 μ M GYY4137 or vehicle control (DMSO) in fASM exposed to 21% or 40% O₂ prior to measuring [Ca²⁺]_i response to 10 μ M of Acetylcholine (ACh); or (B) Hyperoxic fASM exposed to 30 minutes calcium channel inhibitors Pyr3 (10 μ M; TRPC3 inhibitor), Glibenclamide (1 μ M; K_{ATP} channel inhibitor), or Xestospongins C (XeC; 1 μ M; IP3R inhibitor) followed by 30 minutes 50 μ M NaHS or vehicle control in the presence of each respective inhibitor. [Ca²⁺]_i response to 10 μ M histamine was recorded. Horizontal dotted lines are representative of the average amplitude of vehicle control fASM in hyperoxia. Amplitude was calculated from baseline to maximum peak [Ca²⁺]_i response. Statistical significance was determined by ratio paired *t* test (**P* < .05 vs vehicle). Outliers were removed by Grubbs' Test. Data are represented as means $\pm$ SEM; n = 5-6 patients each

FIGURE 7.

SOCE influx and rate following SR Ca^{2+} depletion is decreased and slowed in the presence of exogenous H_2S donor NaHS. fASM exposed to either 21% or 40% O_2 for 24 hours were loaded with Fura-2-AM and washed prior to live cell imaging. A, B, fASM were treated for 30 minutes with 50 μM NaHS or vehicle control (PBS). SOCE protocol was performed as previously described (see Methods). Representative SOCE tracing is shown. B, Rate of SOCE was calculated by measuring the slope of the $[\text{Ca}^{2+}]_i$ tracing after reintroduction of extracellular calcium. NaHS slowed the rate of Ca^{2+} influx in 40% O_2 conditions. C, SOCE was calculated between SR Ca^{2+} depletion via CPA and peak response to extracellular Ca^{2+} reintroduction. D, Inhibition of Orai1 with AnCoA4 decreased SOCE as expected, which blunted NaHS effects, in both 21% and 40% O_2 . Inhibition of NaHS effects on SOCE through Orai1 were significant in normoxia. Statistical significance was determined by ratio paired t test ($*P < .05$ vs. vehicle). Outliers were removed by Grubbs' Test. Data are represented as means $\pm$ SEM; $n = 5$ -6 patients each

FIGURE 8.

H₂S pathway modulation of [Ca²⁺]_i in developing airway. Developing airway smooth muscle express intact H₂S machinery and exogenous H₂S donors can modulate [Ca²⁺]_i through plasma membrane pathways in the context of hyperoxia. Schematic was prepared in part using Motifolio Scientific Illustration Toolkits