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REVIEW

PEDIATRIC PULMONOLOGY WILEY

S-Nitroso-L-cysteine and ventilatory drive: A pediatric perspective

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Abstract

Though endogenous S-nitroso-L-cysteine (L-CSNO) signaling at the level of the carotid body increases minute ventilation ($\dot{v}_E$), neither the background data nor the potential clinical relevance are well-understood by pulmonologists in general, or by pediatric pulmonologists in particular. Here, we first review how regulation of the synthesis, activation, transmembrane transport, target interaction, and degradation of L-CSNO can affect the ventilatory drive. In particular, we review L-CSNO formation by hemoglobin R to T conformational change and by nitric oxide (NO) synthases (NOS), and the downstream effects on $\dot{v}_E$ through interaction with voltage-gated K^+ (Kv) channel proteins and other targets in the peripheral and central nervous systems. We will review how these effects are independent of—and, in fact may be opposite to—those of NO. Next, we will review evidence that specific elements of this pathway may underlie disorders of respiratory control in childhood. Finally, we will review the potential clinical implications of this pathway in the development of respiratory stimulants, with a particular focus on potential pediatric applications.

KEYWORDS
apnea, hemoglobin, newborn, S-nitrosoglutathione, S-nitroso-L-cysteine, voltage-gated potassium channel

1 | INTRODUCTION

Recently, pediatric pulmonologists—particularly those in training—have been encouraged to become more familiar with the novel and emerging basic science discoveries relevant to their field of practice.[1] Understanding the complexities of ventilatory control, particularly in the newborn period, is fundamental to understanding childhood respiratory disease. The roles of dissolved gas O_2 and CO_2 tensions, as well the role of of blood pH, in regulating minute ventilation ($\dot{v}_E$) have been studied carefully.[2] The roles of additional neuronal regulatory proteins, like the *PHOX2B* gene product, in childhood respiratory control are also now understood with increasing clarity and are of importance to the pediatric pulmonologist.[3,4] However, many questions remain about the central and peripheral neuronal pathways affecting ventilation. This is particularly important in pediatrics, where issues like the perinatal ventilatory response to hypoxia are clinically important but incompletely understood. Indeed, caffeine remains the only approved ventilatory stimulant for use in newborns. Here, we describe data supporting a novel signaling pathway involving S-nitroso-L-cysteine (L-CSNO); these data began to emerge over two decades ago,[5–8] but have gone largely unnoticed by the pulmonology community in general and by the pediatric

pulmonology community in particular. We will review several aspects of this pathway. Our goals are to inform, to stimulate research, and to encourage the development of novel therapeutics that can make use of this alternate pathway and the drug targets it provides.

2 | FORMATION OF S-NITROSO-L-CYSTEINE (L-CSNO) IN VIVO

S-Nitroso-L-cysteine is a member of a class of signaling molecules relevant to a variety of processes in pulmonary biology, including respiratory control, bronchodilation, and pulmonary blood flow and a spectrum of pulmonary diseases, ranging from apnea to asthma.[2,9–11] Its metabolism will be reviewed here because it is of central importance to new discoveries regarding ventilatory control (see below). It is distinct from nitric oxide (NO) and other redox forms of nitrogen in terms of its chemistry and biology, as recently reviewed[11] (Table 1).

In the blood, in the lungs, and in neuronal tissues, the generation of L-CSNO occurs through several mechanisms relevant to ventilatory control. Most, but not all, L-CSNO synthetic pathways involve metalloproteins, one of which is the class of enzymes known as nitric oxide (NO) synthases (NOSs). Humans have at least three NOS isoforms: neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2), and endothelial NOS (eNOS; NOS3). All three are capable of synthesizing biological S-nitrosothiols (SNOs) like L-CSNO. For example, nNOS can impact physiological responses through the local formation of S-nitrosothiols.[12] In some cases, S-nitrosoglutathione (GSNO) is formed by NOS isoforms and is later converted to L-CSNO (see below).[13,14] Neuronal NOS is an important regulator of respiratory control in the brainstem and carotid body (CB). Nitric oxide itself depresses respiratory drive; paradoxically, S-nitrosothiols increase it.[15–17] Inducible NOS and eNOS also can form S-nitrosothiol moieties.[18–21]

It was a discovery of importance to pediatric pulmonologists that hemoglobin can signal a change in $\dot{v}_E$, independently of signaling mechanisms involved in sensing dissolved blood gas concentrations. L-CSNO can be produced not only by NOS but through a pathway associated with the conformational R-to-T transformation of erythrocytic hemoglobin (Hb).[22] Hemoglobin is a tetrameric protein that is in the relaxed (R) state when fully oxygenated. As it passes through the systemic vasculature, decreasing oxygen saturation, decreasing pH, and increasing CO_2 tensions result in a conformational shift to the tense (T) state of the tetramer. During this shift from R to T, the β-chain cysteine 93 (Cysβ93) is exposed to the surface of the hemoglobin tetramer, whereas the Cysβ93 is sterically shielded from intraerythrocytic solutes when the Hb tetramer is in the R state.[10,23,24] Several assays have now shown that NO binds to Cysβ93 (as NO^+ [nitrosonium]; Table 1), and that binding to the hemoglobin thiol is favored in the R state when the Cysβ93-NO is sterically preventedfrom reacting with intraerythrocytic thiols like glutathione (GSH).[14,25] These various confirmatory assays have included X-ray crystallography, photolysis-chemiluminescence, reduction-chemiluminescence, fluorescence-based assays, impedance-based immunosensor assays, mass spectrometry, and a number of bioassays.[7,14,22,25–32] This chemistry is important in hypoxia signaling, in peripheral blood flow regulation,[10] in pulmonary hypertension,[33] and in ventilatory control;[15,26] but not in eNOS-dependent regulation of blood pressure.[34,35] Additional metalloproteins are involved in S-nitrosothiol formation, including ceruloplasmin;[36] S-Nitrosothiols are also formed from nitrous acid at the low pH in the stomach, in the lung periphery, and in ischemic tissues.[37]

Many endogenous S-nitrosothiols that affect respiratory physiology are labile;[38–40] for example, L-CSNO has a half-life of ~90 s.[4142] L-CSNO levels are nearly undetectable in normoxic blood, but are increased in desaturated blood: rapid detection is required for their measurement.

GSNO is the S-nitrosothiol most commonly characterized in biology (Figure 1). Once formed, GSNO can cross cell membranes,[15,22,30] as can

TABLE 1 S-Nitrosothiols and other biologically active redox states of nitrogen (adapted from Marozkina and Gaston[11]).

Nitrogen oxidation state	Names/examples of biological relevance
3−	Ammonia and amines
1−	Hydroxylamine
0	Nitrogen
1+	Nitroxyl anion and nitrous oxide
2+	Nitric oxide
3+	S-nitroso-L-cysteine and other S-nitrosothiols; additional nitrosonium donating species such as dinitrogen trioxide and nitrous acid
4+	Nitrogen dioxide
5+	Nitrate and nitric acid

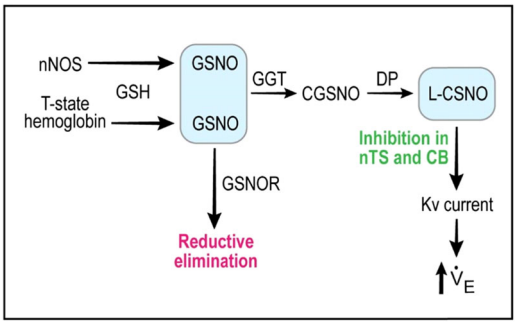
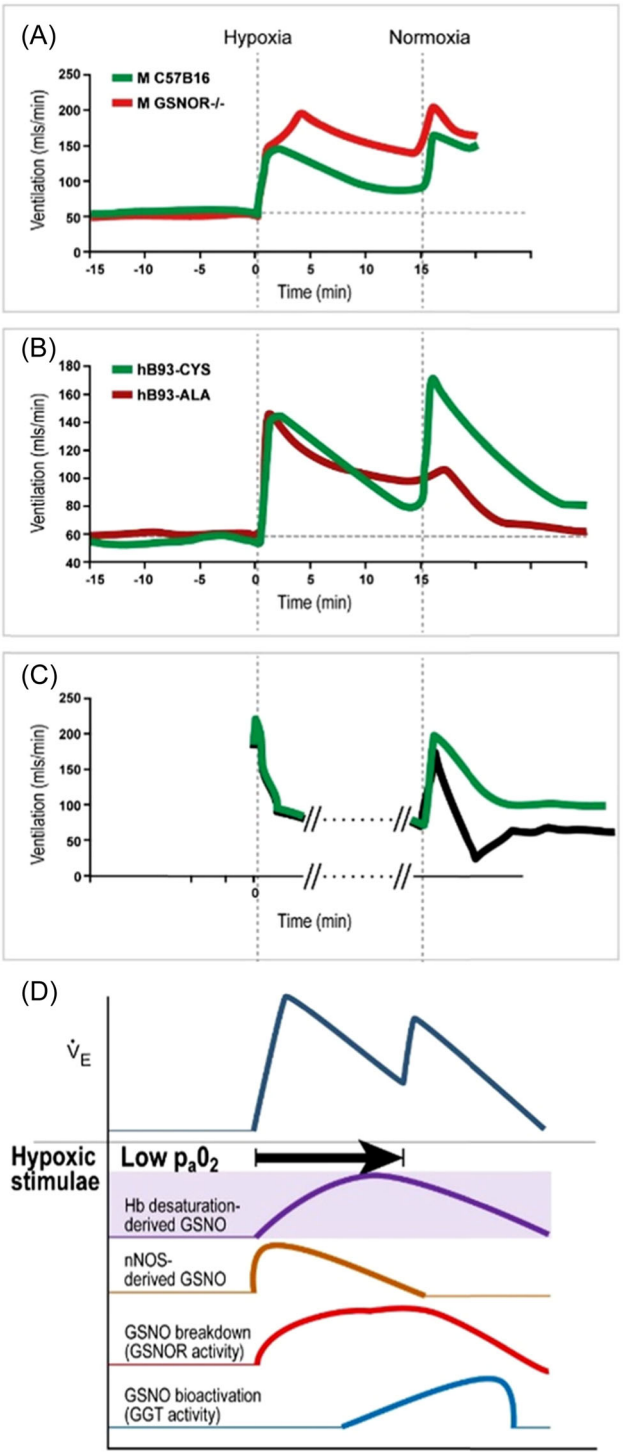


FIGURE 1 Neuronal NOS (nNOS) and T state hemoglobin signal an increase in minute ventilation ($\dot{v}_E$). S-Nitrosoglutathione (GSNO) is generated by deoxygenated (T-state) hemoglobin and nNOS. GSNO is metabolized by γ-glutamyl transpeptidase (GGT) to form S-nitrosocysteinyl glycine (CGSNO) and glutamate. CGSNO is broken down by dipeptidases (DP) to form glycine and S-nitroso-L-cysteine (L-CSNO). One bioactivity of L-CSNO involves inhibition of voltage-gated potassium (Kv) channels in the nucleus tractus solitarius (nTS) and carotid body (CB), increasing $\dot{v}_E$.

other S-nitrosothiols.[33,43] Additionally, S-nitrosothiol/NO^+ equivalents can be transferred across cell membranes by anion exchange protein 1 (AE1), protein disulfide isomerase[44] and, in the case of L-CSNO, by the L-amino acid transporter.[25]

In an important pathway similar to that involved in the metabolism and bioactivation of cysteinyl leukotrienes, GSNO is converted to *S*-nitrosocysteinyl glycine (CGSNO) and glycine by γ-glutamyl transpeptidase (GGT), and CGSNO, in turn, is converted to L-CSNO and glutamate by dipeptidases (Figure 1).[8,45,46] Blocking GGT prevents effects of GSNO in ventilatory regulation and in other signaling processes.[47,48]



3 | EFFECTS OF L-CSNO TO INCREASE MINUTE VENTILATION

We will first provide a brief review of the mechanisms underlying the effects of hypoxia on type 1 (Glomus) cells in the CB. Arterial hypoxia leads to an inhibition of K^+ current, and to augmented Ca^{2+} cell entry. This, in turn, signals afferent nerves in the carotid sinus nerve, e, ultimately transmitting the signal to the respiratory centers of the brainstem. These signals are integrated by the nucleus tractus solitarius (nTS) and other medullary structures. They modulate the output of medullary rhythm generating nuclei and affect efferent respiratory motor signaling. There are several neuronal pathways involved in regulating a number of aspects of ventilatory control, but we will focus here on the CB and nTS, which are relevant to L-CSNO signaling. Note also that acute hypoxia typically is associated with ventilatory roll-off, such that the initial increase in ventilation gradually declines during hypoxia, followed by a short-term potentiation (increased $\dot{v}_E$) upon return to normoxia (see Figure 2). These findings can readily and reproducibly be measured in awake mice and rats using whole-body plethysmography.

The identification of the ventilatory effects of L-CSNO primarily involved plethysmographic studies in awake, pre-instrumented rodents. These demonstrated clearly that L-CSNO increased $\dot{v}_E$ at both levels of nTS and CB.[24,25] Both tidal volume and respiratory rate were increased. The effects were strikingly hypoxia-mimetic, with

FIGURE 2 Proposed schematic integrating genetic murine models regardingthe role of L-CSNO synthesis and degradation in the regulation of ventilation. The initial ventilatory response to hypoxia is shown, followed by roll-off, followed then by posthypoxic facilitation during the return to normoxia. These traces are derived from experiments in the references listed.[15,24,47] (A) $GSNOR^{-/-}$ mice have an exaggerated initial hypoxic response, attenuated roll-off, and increased facilitation: once GSNO is formed, they do not clear it efficiently and its effects are sustained (through L-CSNO).[47] (B) In mice with unable to form L-CSNO or GSNO during hemoglobin deoxygenation, post hypoxic facilitation is diminished; specifically, it is diminished in mice in which Hb-β-Cys93 is replaced with a Hb-βAla93.[24] (C) In $GGT^{-/-}$ mice unable to form L-CSNO from GSNO, posthypoxic facilitation is lost; in fact, recovery from hypoxia can lead to apnea.[4] (D) Low pO2 itself stimulates increased ventilation according to established pathways. GSNO levels increase during the hypoxic challenge as SNO is transnitroated from T state Hb. nNOS-derived GSNO may be formed more quickly than Hb-derived GSNO. Local GSNOR activity increases as a counter-regualtory mechanism, attentuating the increase in GSNO. GGT activity also increases, making L-CSNO bioavailable. CGSNO, S-nitrosocysteinylglycine; GGT, γ-glutamyltranspeptidase; GSH, glutathione; GSNO, S-nitrosyl-glutathione; GSNOR, S-nitrosoglutathione reductase; Hb, hemoglobin, nNOS, neuronal nitric oxide synthase; $\dot{v}_E$, minute ventilation.

post-dosing roll-off that was nearly identical to posthypoxic facilitation.[8] S-Nitrosoglutathione was also active in the nTS, but its effect was ablated by pretreatment with a GGT inhibitor (see Figure 1).[8,26] Though L-CSNO and D-CSNO evolve NO radical at the same rate, D-CSNO was almost completely inactive.[15,26] Considered along with evidence that the effect of NO itself is to decrease ventilatory drive, it was clear that the effect of L-CSNO was independent of—and, in fact, opposite—that of NO.[8,26,49] Remarkably, the effects of L-CSNO were inhibited by the L-CSNO congeners, S-methyl cysteine, and S-phenyl cysteine, suggesting a ligand–receptor type of mechanism.[26] Indeed, the effects were NO- and guanylate cyclase-independent.[8,26,50,51] Thus, L-CSNO appeared to behave as an NO-independent signaling molecule. The reader interested in a more detailed description of how this signaling was discovered is encouraged to read reference [26].

To identify the NO-independent mechanism by which L-CSNO could affect $\dot{v}_E$, twoprotein affinity approaches were used—one gel-based, and the other based on affinity chromatography.[26] L-CSNO-protein interactions were blocked by S-methyl and S-phenyl cysteine. Both chromatography approaches were followed by an unbiased proteomic analysis of L-CSNO-binding proteins. In both cases, voltage-gated potassium channel (Kv) proteins were identified as binding partners for L-CSNO.[26] It had previously been published that specific Kv knockout mice had abnormal respiratory control and responses to hypoxia.[52] In follow-up studies, L-CSNO ventilatory effects were studied in the KCNA (Kv1.1)$^{-/-}$ mouse.[26] Loss of the protein resulted in the loss of L-CSNO binding.[26] Moreover, L-CSNO inhibited Kv current in isolated rat and mouse respiratory control neurons, as well as in Kv-over-expressing Chinese hamster ovary (CHO) cells, but not in control (Kv 1.1$^{-/-}$) neurons or control CHO cells.[21] Next, hydrogen–deuterium exchange and surface plasmon resonance spectroscopy were used to show that L-CSNO bound to specific regions of Kv1.1 and Kvβ2. Finally, the effect of L-CSNO to increase $\dot{v}_E$ when administered at the level of the CB in preinstrumented rats was prevented by preadministration of S-methyl- and S-phenyl-cysteine.[26] The increase in $\dot{v}_E$ was independent of any effect on blood pressure, was not prevented by inhibition of guanylate cyclase, and was not recapitulated by administration of an NO donor other than L-CSNO.[26] Taken together, these data demonstrated that L-CSNO, which can be formed by nNOS and by deoxygenated Hb, increases $\dot{v}_E$ at the level of the CB, as it does and at the level of the nTS. This effect, at least in part, is mediated through interactions with Kv channels; and the effects are NO-independent.

Note also that S-nitrosothiol bioactivities can be regulated catabolically. First, L-CSNO itself is labile, decomposing to NO and cystine rapidly in the neurons, lungs, and blood.[5,11,53] As is the case with certain lipid mediators, this lability makes it quite suitable as a signaling molecule in general, and as a respiratory stimulant in particular: prolonged stability in the blood would cause hyperventilation and other potential side effects. It also makes it difficult to detect, requiring rapid assays.[18,26] Upstream of L-CSNO, GSNO, and other S-nitrosothiols are more stable, and their concentrations in many tissues are catabolically regulated by GSNO reductase (GSNOR), carbonyl reductase, S-nitroso-coenzyme A reductase, and other enzymes.[54–56] Of these, GSNOR appears to be the most active in respiratory control, and mice missing this enzyme have prolonged neuronal exposure to GSNO (and therefore to downstream L-CSNO) and have sustained hyperventilation during and after hypoxia (Figure 2).[24,47] Note in this regard that the Hb beta93$^{-/-}$ the mouse has dramatically impaired posthypoxic facilitation (Figure 2) and, in general, an imparied response to hypoxia.[58] Additionally, the nNOS-deficient mouse also has an impaired ventilatory response to hypoxia.[57] Further, mice lacking GGT have a profoundly impaired - indeed, paradoxical - response to hypoxia, likely because they cannot bioactivate GSNO by forming L-CSNO (Figures 1 and 2).[47] Thus, mice unable to form L-CSNO have decreased ventilation, while mice unable to catabolize GSNO have hyperventilation.[8,47] Taken together, this genetic evidence suggests strongly that hypoxia increases $\dot{v}_E$ not only by conventional pathways, but also by formation of GSNO and, downstream, L-CSNO. Here, we have for the first time proposed how these observations may be integrated into a unified pathway.

Note also that elegant data suggest that H_2S signaling pathways are important in ventilatory control in the CB.[59–61] Though these pathways are separate from L-CSNO-signaling, the two mechanisms do interact. Biochemical interactions between H_2S and S-nitrosothiol signaling have been recently reviewed, and the activity of H_2S-regulatory proteins can be inhibited by S-nitrosylation.[62,63] The interactions between these pathways will be important to study in the future.

4 | POTENTIAL IMPLICATIONS FOR RESPIRATORY CONTROL IN CHILDREN

These studies have several potential implications for childhood respiratory disease.

First, L-CSNO formation by GGT should be studied further to determine whether it has a role in perinatal respiratory drive, a role that could help understand some cases of newborn apnea. The GGT-deficient mouse has a paradoxical (at times apneic) response to hypoxia[4] that is reminiscent of the newborn human's paradoxical response to hypoxia, though it is important to note that there are many models of newborn apnea.[64] Human newborn apneic responses are more prominent and troublesome in infants born prematurely. One goal of this review is to alert pediatric researchers to the potential prenatal role of GGT and related enzymes.

Second, L-CSNO and other S-nitrosothiols appear to also play a role in the perinatal transition to air-breathing.[65] Human HbF carries significant S-nitrosothiol signaling potential, in addition to HbA,[66,67] and because the P_{50} of HbF is lower (its oxygen affinity is higher), the oxygen tension at which NO is transferred to form S-nitrosothiols is higher. Oxygen tensions are quite low before birth, shifting dramatically at birth. Further, S-nitrosothiol concentrations are depleted in human newborn umbilical venous blood in response to

perinatal distress.[60,68] Additional studies are needed on perinatal S-nitrosothiol metabolism and its role in the newborn transition.

Third, the L-CSNO-mediated ventilatory pathway could have important therapeutic implications. Hildebrant et al.[69] showed that *N*-acetyl cysteine is a respiratory stimulant in adults, augmenting hypoxic respiratory drive.[69] Palmer has confirmed the hypoxia-mimetic effect of long-term, high-dose NAC to cause pulmonary vascular remodeling in mice through the hemoglobin-mediated metabolic pathway described above.[33] It has not previously been considered that acute use of NAC could be studied as a clinical substitute for caffeine in apnea of prematurity. Moreover, cysteine esters, bypassing the LAT transporter for access to intracellular targets, are being developed as respiratory stimulants that are safe and effective in animals.[58,70] D-isomers with access to the intracellular space are effective and do not appear to have the side effects of the L-isomer-based esters.[71] These compounds could be among the first ever effective respiratory stimulants for a variety of causes of respiratory depression in children, including respiratory depressant narcotic and benzodiazepine use in the pediatric critical care setting.

Fourth, the activity of the L-CSNO on the Kv ion channel may provide novel therapeutic opportunities.[26] Increasingly, it is appreciated that Kv channels are critical to a range of physiological functions, including hypoxia-sensing[72], and that their dysfunction is relevant to a range of inherited neurological diseases of childhood. Identification of new Kv agonists and antagonists relevant to respiratory control may provide new possibilities for management.[26] Because L-CSNO is a labile mediator in blood, its effects are local at the CB, and it does not remain in plasma during the circulatory cycle. This lability may account for the apparent lack of off-target side effects.[26]

Fifth, this signaling pathway may help to explain abnormal ventilatory control in some disorders of hemoglobin chemistry. The tachypnea followed by apnea that is characteristic of acute methmoglobinemia, for example, could be explained by increased L-CSNO formation followed by erythrocytic NO depletion.[73] Increased CO exposure may be associated with increased sleep-disordered breathing, and certainly can affect CB signaling, with different effects at different levels.[74] There remains much to be investigated regarding the role of abnormal hemoglobin chemistry and hemoglobinopathies in ventilatory control. The signaling pathway described here may provide new insight into these processes.[75–77]

Finally, the discovery of an alternate pathway involved in respiratory control (Figure 2) could generate a range of new hypotheses regarding longstanding questions in pulmonary pathophysiology in general, and in pediatrics in particular. For example, why newborn anemia is a cause of apnea; or how fever causes tachypnea; or how respiratory drive re-sets in infants with cyanotic heart disease, such that they are not chronically tachypneic; or how postnatal respiratory drive re-sets in response to increased pO_2. There are, of course, operant theories to explain all these phenomena; theories that may be partially or completely correct. Here, our goal is simply to alert the pediatric pulmonary community to a novel, alternate set of possibilities.

AUTHOR CONTRIBUTIONS

Dallin Hubbard: conceptualization (equal); visualization (equal); writing—original draft (equal); writing—review & editing (equal). **Kaylee Tutrow**: visualization (equal); writing—original draft 1(equal); writing—review & editing (equal). **Benjamin Gaston**: conceptualization (equal); funding acquisition (equal); project administration (equal); validation (equal); visualization (equal); writing—original draft (equal); writing—review & editing (equal).

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CONFLICT OF INTEREST

Benjamin Gaston is a co-founder of Atelerix Life Sciences, developed to apply discoveries discussed here to clinical medicine.

DATA AVAILABILITY STATEMENT

Data are available for review from the manuscripts reviewed in this paper that have been published by our group.

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