Regulation of Respiration and Endothelial Gene Expression by S-Nitrosothiols in Health and Disease

Lisa A. Palmer

Department of Pediatrics and Anesthesiology, University of Virginia Health System, Charlottesville, Virginia

The effects of nitric oxide (NO) are mediated by cyclic guanosine monophosphate (cGMP)-dependent and cGMP-independent processes. Most cGMP-independent effects are mediated by the actions of S-nitrosothiols (SNOs). SNOs have been shown to play a role in health and in disease. In studies performed in the mouse and rat, the ventilatory response to hypoxia is regulated in the nucleus tractus solitarius by SNOs exported from red blood cells. This may affect the treatment of respiratory distress in newborns and sleep apnea in adults. Likewise, SNOs have been shown to alter the stability and abundance of the transcription factor hypoxia inducible factor-1, altering the expression of hypoxia-regulated genes. Identification of the proteins involved in these signaling events will lead to new therapeutic approaches in the treatment of diseases characterized by limited oxygen availability.

Keywords: γ glutamyl transpeptidase; hypoxia inducible factor-1; S-nitrosothiols

S-nitrosothiols (SNOs) are products of S-nitrosylation that can be processed, transported, or degraded (1, 2). The formation of SNOs is coupled to the stimulation of nitric oxide synthase (NOS) isoforms (3–5) and requires the presence of an electron acceptor (6). Red blood cells (RBCs) can be a vehicle for O₂-regulated delivery of NO signals in the form of SNOs. Hemoglobin binds NO at cysteine β 93 and converts it to bioactive SNO as a function of blood O₂ concentration (7, 8). The binding of O₂ changes the conformation of hemoglobin from the deoxygenated state to the oxygenated state (7, 8). This permits the transfer of nitrosonium (NO⁺) equivalents from the heme to the thiol. Upon deoxygenation, NO⁺ equivalents are transferred from the cysteine β 93 to a receptor cysteine in the cytosolic amino terminus of the anion exchange protein 1 (9, 10). The NO⁺ equivalents located on anion exchange protein 1 are exported from the RBCs by a mechanism that is not well defined. This transfer is mediated directly through cell–cell transfer or indirectly through the formation of S-nitrosoglutathione (GSNO) or S-nitrosocysteine (10). Entry of GSNO into cells can be mediated by γ glutamyl transpeptidase (γ GT) or other transporters (11). This transfer of NO can alter protein function and abundance resulting in physiologic and pathologic changes.

Respiration is an autonomic response regulated by a network of neurons in the hindbrain. Respiratory control centers are responsible for modulating ventilation according to oxygen availability and metabolic demand. Exposure to hypoxia increases ventilation. Yet, failure to increase ventilation in response to hypoxia can be fatal. This can lead to respiratory distress in

newborns and unfortunate consequences in adults with obstructive sleep apnea.

Several observations suggest that SNOs play a role in regulating respiration. First, $\dot{V}_E$ increases linearly with decreased oxyhemoglobin saturation. Second, afferents from peripheral hypoxia chemoreceptors project to NOS-rich areas of the nucleus tractus solitarius (NTS) in the brain (12). Third, activation of NOS isoforms has been linked to the production of SNOs (3, 4). Last, GSNO, an endogenously produced SNO, has been shown to be 7 μ M in the rat brainstem (13). These observations have led Lipton and coworkers (14) to examine the ability of SNO to modulate $\dot{V}_E$.

Using conscious rats and whole-body plethysmography, Lipton and coworkers (14) examined the effects of endogenously produced SNOs in the NTS on $\dot{V}_E$ in conscious rats. The results obtained were compared with changes in $\dot{V}_E$ on short-term exposure to hypoxia. Rapid increases in $\dot{V}_E$ followed by recovery to baseline were seen on injection of 1 nmol S-nitrosocysteinyl glycine (CGSNO), the bioactive bioproduct of GSNO (Figure 1A). A similar pattern of response was seen after a brief exposure to hypoxia (Figure 1B), suggesting that SNOs could mimic the ventilatory response to hypoxia. Moreover, the effects are stereospecific because only L—not D—isomers were able to elicit this response.

GSNO is an endogenously produced SNO formed during oxyhemoglobin desaturation. Having determined that SNOs can mimic the ventilatory effects of hypoxia, Lipton and colleagues (14) wanted to determine if SNOs, specifically GSNO, formed from deoxygenated blood could similarly increase $\dot{V}_E$. Mass spectrometry was used to demonstrate that GSNO is more efficiently generated in deoxygenated blood than in oxygenated blood and that the formation of GSNO is disrupted by ultraviolet light photolysis, which destroys the SNO by releasing NO (Figure 2A). Injection of GSNO isolated from the deoxygenated blood into the NTS reproduced the effects seen with endogenously produced SNO (Figure 2B). No effects were seen with SNOs produced in oxygenated blood. Thus, the ventilatory response to hypoxia is regulated in the nucleus tractus solitarius by SNO, specifically GSNO, exported from RBCs.

γ GT is required for bioactivation of GSNO to cell-permeable CGSNO (11). To determine if this enzyme was required for the effect of SNOs on $\dot{V}_E$, Lipton and colleagues (14) used pharmacologic and physiologic approaches. Pretreatment of the NTS with acivicin, an inhibitor of γ GT, before injection of GSNO was found to block the effects of GSNO but not CGSNO (Figure 3A). Moreover, γ GT-deficient mice were found to have an altered response to hypoxia (Figure 3B). Taken together, these findings suggest that SNOs require the activity of γ GT to duplicate the physiologic response to the exposure to and recovery from hypoxia.

In summary, the research by Lipton and colleagues (14) defines one mechanism by which hypoxia can alter $\dot{V}_E$. The data demonstrate that (1) SNOs can mimic the effects of hypoxia on $\dot{V}_E$ at the NTS, (2) the effects are stereospecific, and (3) the effects are related to O₂ saturation and the RBCs rather than Po₂. In addition, this effect of SNOs (particularly GSNO) is

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Correspondence and requests for reprints should be addressed to Lisa A. Palmer, Ph.D., Department of Pediatrics, P.O. Box 801366, University of Virginia, Charlottesville, VA 22908. E-mail: lap5w@virginia.edu

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Figure 1. S-nitrosothiols mimic the effects of hypoxia. (A) S-nitrosocysteiny glycine (CGSNO; 1 nmol) was injected into the nucleus tractus solitarius of conscious rats, or (B) rats were exposed to a brief period of hypoxia. Changes in $\dot{V}_E$ were measured by whole-body plethysmography. The effects of CGSNO on $\dot{V}_E$ were similar to those seen in hypoxia. Reprinted by permission from Reference 14.

Figure 2. S-nitrosoglutathione (GSNO) formed from blood deoxygenation mimics the effects of hypoxia. The formation of GSNO from deoxygenated (blue), oxygenated (red), and deoxygenated blood + ultraviolet light photolysis (purple) was determined by mass spectroscopy and reductive chemiluminescence. S-nitrosothiols isolated and purified from the peak fractions were injected into the nucleus tractus solitarius, and changes in $\dot{V}_E$ were determined as described in Figure 1. GSNO derived from deoxygenated blood altered $\dot{V}_E$. Reprinted by permission from Reference 14.

Figure 3. The effect of S-nitrosothiols on $\dot{V}_E$ requires γ glutamyl transpeptidase (γ GT). (A) Changes in $\dot{V}_E$ were determined after treatment with GSNO, GSNO after pretreatment with acivicin (an inhibitor of γ GT), and CGSNO (the bioactive component of GSNO after pretreatment with acivicin). GSNO-induced increases in $\dot{V}_E$ were abolished by acivicin. Acivicin had no effect on the ability of CGSNO to induce an increase in $\dot{V}_E$. (B) Changes in $\dot{V}_E$ in γ GT-deficient mice (+/+, wild type; +/- heterozygous; -/-, null) after exposure to hypoxia. Data are expressed as percent changes from prehypoxia baseline. Reprinted by permission from Reference 14.

Figure 4. Regulation of hypoxia inducible factor-1 (HIF-1). Schematic representation of the pathways regulating the expression of the transcription factor HIF-1. The HIF-1α gene generates HIF-1α mRNA, which is transcribed into HIF-1α protein. In normoxia, HIF-1α protein is hydroxylated and is targeted for ubiquitination and destruction. In hypoxia, HIF-1α protein is no longer hydroxylated and can combine with HIF-1β to regulate the activation of hypoxia-regulated genes. Activities altered by S-nitrosothiols are indicated in purple. E1 = ubiquitin-activating enzyme; E2 = ubiquitin-activating enzyme 2; E3 = ubiquitin protein ligase; VHL = von Hippel Lindau protein.

inhibited by γ GT. The identification of the role of SNOs in regulating $\dot{V}_E$ provides a starting point to identify and characterize components in this signaling pathway, which may lead to the identification of protein targets that may modulate the effects of SNOs on the hypoxic response and thus provide treatments for sleep apnea.

Hypoxia can cause pulmonary hypertension in humans and in animals. In addition, it has been shown to induce the expression of the transcription factor hypoxia inducible factor-1 (HIF-1). This transcription factor is a heterodimer composed of α and β subunits. The α subunit is constitutively transcribed and translated but is rapidly degraded, whereas the β subunit is constitutively expressed. In response to hypoxia, HIF-1 α is stabilized (16). Normoxia is sensed by proline hydroxylation at residues Pro564 and Pro402 of HIF-1 α , permitting interaction with von Hippel Lindau protein and subsequent ubiquitination and degradation of HIF-1 α by the proteasome (Figure 4) (17–20). However, in hypoxia, hydroxylation does not occur, and HIF-1 is stabilized, dimerizes with a β subunit, and alters the transcription of hypoxia-regulated genes.

SNOs have been shown to stabilize HIF-1 α in normoxia in cultured cells (Figure 5) (15). The activation of HIF-1 exhibits classical S-nitrosylation pharmacology: (1) It is not mimicked by 8-bromo cGMP; (2) it is not inhibited by oxyhemoglobin; (3) it is reversed by dithiothreitol; and (4) for GSNO, it is inhibited by the γ GT inhibitor acivicin (15). Two potential mechanisms have been described for HIF-1 stabilization by GSNO. GSNO has been shown to impair prolyl hydroxylase activity (21) and/or directly impair S-nitrosylate HIF-1 at Cys 800 (22),

with the former resulting in a decrease in HIF-1 α ubiquitination and the latter increasing the recruitment of the p300 coactivator proteins.

Hypoxia-induced pulmonary hypertension has been shown to be impaired in mice heterozygous for HIF-1 α (23) and is eliminated in mice heterozygous for HIF-2 α (24). Thus, aberrant expression of HIF-1 *in vivo* could lead to the development of pulmonary hypertension. The P_{O_2} values used to stabilize HIF *in vitro* are much less than the P_{O_2} values normally seen in the pulmonary vasculature (postnatal 40 mm Hg, prenatal 30 mm Hg). Because SNO formation has been found to be associated with hemoglobin deoxygenation, one could speculate that signaling through SNOs rather than changes in P_{O_2} could alter HIF-1 α expression and thus provide an alternative mechanism for the development of this disease.

In summary, many effects of NO are mediated by the actions of SNOs. SNOs have been found to duplicate the ventilatory response to the exposure to and recovery from hypoxia and to change the stability and DNA-binding activity of the transcription factor HIF-1. Identification of the physiologic processes that are altered by S-nitrosylation and the proteins involved in SNO signaling could lead to the development of new therapeutic approaches for the treatment of disease, especially in cases where limited supplies of oxygen are involved.

Conflict of Interest Statement: L.A.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Figure 5. Stabilization of HIF-1 α by GSNO. Proteins present in nuclear extracts made from bovine pulmonary artery endothelial cells treated with GSNO were separated by Western blot analysis. Stabilization of HIF-1 α is seen to occur with time of exposure. Reproduced by permission from Reference 15.

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