Hypoxia-Induced Changes in Protein S-Nitrosylation in Female Mouse Brainstem

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Abstract

Exposure to hypoxia elicits an increase in minute ventilation that diminishes during continued exposure (roll-off). Brainstem N-methyl-D-aspartate receptors (NMDARs) and neuronal nitric oxide synthase (nNOS) contribute to the initial hypoxia-induced increases in minute ventilation. Roll-off is regulated by platelet-derived growth factor receptor- β (PDGFR- β) and S-nitrosoglutathione (GSNO) reductase (GSNOR). S-nitrosylation inhibits activities of NMDAR and nNOS, but enhances GSNOR activity. The importance of S-nitrosylation in the hypoxic ventilatory response is unknown. This study confirms that ventilatory roll-off is virtually absent in female GSNOR^{+/-} and GSNOR^{-/-} mice, and evaluated the location of GSNOR in female mouse brainstem, and temporal changes in GSNOR activity, protein expression, and S-nitrosylation status of GSNOR, NMDAR (1, 2A, 2B), nNOS, and PDGFR- β during hypoxic challenge. GSNOR-positive neurons were present throughout the brainstem, including the nucleus tractus solitarius. Protein abundances for GSNOR, nNOS, all NMDAR subunits and PDGFR- β were not altered by hypoxia. GSNOR activity and S-nitrosylation status temporally increased with hypoxia. In addition, nNOS S-nitrosylation increased with 3 and 15 minutes of hypoxia. Changes in NMDAR S-nitrosylation were detected in NMDAR 2B at 15 minutes

of hypoxia. No hypoxia-induced changes in PDGFR- β S-nitrosylation were detected. However, PDGFR- β phosphorylation increased in the brainstems of wild-type mice during hypoxic exposure (consistent with roll-off), whereas it did not rise in GSNOR^{+/-} mice (consistent with lack of roll-off). These data suggest that: (1) S-nitrosylation events regulate hypoxic ventilatory response; (2) increases in S-nitrosylation of NMDAR 2B, nNOS, and GSNOR may contribute to ventilatory roll-off; and (3) GSNOR regulates PDGFR- β phosphorylation.

Keywords: hypoxic ventilatory response; neuronal nitric oxide synthase; N-methyl-D-aspartate receptor; platelet-derived growth factor receptor- β ; S-nitrosoglutathione reductase

Clinical Relevance

This article reports that the change in S-nitrosylation status of key brainstem proteins may underlie the roll-off phase of the hypoxic ventilatory response. This has potential clinical applications for understanding how mechanisms that affect S-nitrosothiol bioavailability (i.e., S-nitrosoglutathione reductase) play a role in central breathing disorders.

Hypoxia increases minute ventilation (V_M) by activation of carotid body chemoafferents and integration of these signals by brainstem structures, including the nucleus tractus solitarius (NTS) (1–4). The neurochemical mechanisms mediating the initial increase in V_M upon exposure to hypoxia include: (1) activation of

N-methyl-D-aspartate (NMDA) receptors (NMDARs); (2) increased activity of neuronal nitric oxide synthase (nNOS) via NMDAR-induced increases in intracellular Ca^{2+} ; and (3) activation of phospholipase C, protein kinase (PK) C, and tyrosine kinase phosphorylation cascades (3, 4). With sustained hypoxia,

V_M diminishes—a phenomenon known as ventilatory roll-off (1–4). The mechanisms involved in roll-off include production of neurotransmitters inhibitory to respiration, such as γ -amino-butyric acid and adenosine, as well as inhibition of NMDAR function via activation of platelet-derived growth factor

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(PDGF)-BB/PDGF receptor (PDGFR)- β signaling pathways (4–6).

The S-nitrosothiol, S-nitrosoglutathione (GSNO), exists in rat and mouse tissues, such as the brain (7, 8). Cell abundances of GSNO and S-nitrosylated proteins in transnitrosation equilibrium with GSNO are regulated by the reduced nicotinamide adenine dinucleotide (NADH)-dependent GSNO-catabolizing enzyme, GSNO reductase (GSNOR), and by thioredoxin/thioredoxin reductase and carboxyl reductase (7, 9). GSNOR is widely distributed in the body, including the brain (10). Mice deficient in GSNOR have elevated tissue levels of GSNO (7). S-nitrosothiols (SNOs) are involved in ventilatory control processes (11, 12), and microinjections of these compounds, including GSNO, into the NTS of conscious rats elicit marked increases in V_M (12). The finding that ventilatory roll-off does not occur in male and female mice deficient in GSNOR (11) suggests that mechanisms decreasing GSNO abundance and protein S-nitrosylation contribute to this phenomenon.

Chronic hypoxia increases protein expression and activity of GSNOR in lung (13). However, little is known about the mechanisms that regulate GSNOR activity in the brainstem in response to an acute hypoxic exposure. Under normoxic conditions, cell NADH/NAD⁺ is low (14) and therefore not conducive for GSNOR activity. Hypoxic exposure increases the NADH:NAD⁺ ratio in rabbit carotid body glomus cells (15) and NADH levels in respiratory centers in the brainstem of mice (16). Thus, hypoxia-induced changes in NADH levels may contribute to an increase in GSNOR activity. GSNOR activity is also enhanced by S-nitrosylation, in part, by an NOS-dependent mechanism (17). As such, temporal modulation of GSNOR activity during acute hypoxic exposure, through alterations of NADH abundance or S-nitrosylation status, may decrease GSNO abundance, contributing to ventilatory roll-off. In this report, the importance of proteins that modulate GSNO bioavailability within the brainstem of female C57BL6 mice was evaluated for their potential role in the expression of ventilatory roll-off.

Materials and Methods

Animals

Studies were performed in accordance with the National Institutes of Health Guide for

Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80–23) revised in 1996. Protocols were approved by the University of Virginia Animal Care and Use Committee. Female C57BL6 mice (10–12 wk old) were from Jackson Laboratories (Bar Harbor, ME). Female GSNOR^{+/-} and GSNOR^{-/-} mice (10–12 wk old) were from breeding colonies maintained at the University of Virginia. The status of the menstrual cycle was not taken into account, because its effects on hypoxic ventilatory response (HVR) in mice are minor, and range from an increased HVR in the luteal phase by 10–20% compared with the follicular phase, because of the lack of correlation between the ratio of progesterone and estradiol and the HVR response, and because sex differences in HVR in rats are due to mechanisms independent of ovarian hormones (11, 18).

Immunohistochemistry and Fluorescence Studies

Mice were perfused with phosphate-buffered formalin. Brainstems were removed and embedded in paraffin. Sequential coronal 3- μ m (brightfield) or 5- μ m (fluorescence) sections were cut with a an RM2155 microtome (Leica, Buffalo Grove, IL) and affixed to slides. Consecutive sections were stained with antibodies to GSNOR (1:750 dilution; Proteintech, Chicago, IL) and nNOS (1:200 dilution; Abcam, Cambridge, MA). Sections were treated with biotinylated secondary antibodies and developed using Vector Elite Standard Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine (BioGenex, Fremont, CA) as the chromogen. Sections were photographed with a QColor 3 digital camera (Olympus, Center Valley, PA) mounted on a Leica DMLS bright-field compound microscope. Fluorescence imaging was performed using a Leica TCS-SP5 confocal laser-scanning microscope coupled with a pulsed White Light Laser (19). Fluorescence excitation was 488 nm (Alexa goat anti mouse: nNOS) and 568 nm (Alexa donkey anti rabbit: GSNOR). Fluorescence emission was at 500 to 540 nm for green, 580 to 640 nm for red.

Hypoxic Challenge in Conscious Mice

Female C57BL6, GSNOR^{+/-}, and GSNOR^{-/-} mice were placed in whole-body plethysmographs. The ventilatory responses elicited during hypoxic challenge (10% O₂, 90% N₂ for 15 min) were

recorded as described previously (11, 18). These mice were taken randomly from the pool that also provided mice for the *in vitro* tissue studies described in this article.

Preparation of Brainstem

Homogenates and Measurements

Brainstems were homogenized in 750 μ l homogenation buffer (50 mM Tris HCl [pH 7.4], 0.25% Triton X 100, 100 mM EDTA, 0.1% NP40) containing 1 $\times$ HALT protease and 1 $\times$ phosphatase inhibitor (Thermo Scientific, Waltham, MA) cocktails using Lysing Matrix D (MP Biomedicals, Solon, OH). Samples were homogenized in a Fast Prep homogenizer (MP Biomedicals) for 45 seconds on setting 6. GSNOR activity was measured by the modified Saville Assay (17) using 100 μ g of brainstem homogenate and 100 μ M GSNO. The GSNOR activity assay was performed in the presence of 2 mM GSH and 300 μ M NADH, as previously described (17). Biotin switch was performed with brainstem homogenates (150 μ g) (17). Western blot was performed for total protein abundance and S-nitrosylation status (17). Proteins were detected using antibodies to nNOS, endothelial NOS (eNOS; BD Transduction Laboratories, San Jose, CA), GSNOR, phosphorylated PDGFR- β , PDGFR- β (Upstate, Lake Placid, NY), NMDAR 1 (NR1; Cell Signaling, Danvers, MA), NMDAR 2A and 2B (NR2A and NR2B; BD Transduction), and β -actin (Novus Biologicals, Littleton, CO) using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) or Immobilon (Millipore, Billerica, MA).

Statistical Analysis

Data are expressed as means ($\pm$ SEM). Data were analyzed by one-way ANOVA. Significance was determined using the Holm Sidak *post hoc* test with *P* less than 0.05 considered significant.

Results

GSNOR Is Present in Neurons within the Brainstem of Female Mice

To determine the expression and cellular location of GSNOR and its colocalization with nNOS, immunohistochemical and confocal analysis was performed on brainstems harvested from C57BL6 mice. Serial sections demonstrated the expression of GSNOR and nNOS in neurons within the brainstem (Figure 1A). Moreover, both

proteins were seen in neurons located in the NTS, a region of the brain known to be involved in respiratory control (2, 4). Immunofluorescence studies demonstrated that a subset of neurons contained both GSNOR and nNOS (Figure 1B).

Ventilatory Responses of Conscious Mice during Hypoxic Challenge

Resting V_M was equivalent in conscious female C57BL6, GSNOR^{+/-}, and GSNOR^{-/-} mice (Figure 2A). Exposure to hypoxic challenge (10% O₂, 90% N₂) elicited an increase in V_M in C57BL6 mice that was maximal at 3 minutes, but which had resolved by 15 minutes. Exposure of GSNOR^{+/-} and GSNOR^{-/-} mice to hypoxia elicited pronounced increases in V_M that were still evident at 3 and 15 minutes. As such, C57BL6 mice displayed ventilatory roll-off, whereas the GSNOR^{+/-} and GSNOR^{-/-} mice did not.

GSNOR Activity Temporally Increases in Brainstem Homogenates of Female C57BL6 Mice, but Not GSNOR^{+/-} or GSNOR^{-/-} Mice Exposed to Acute Hypoxia

To determine whether the decrease in V_M during acute exposure to hypoxia (roll-off)

is associated with changes in GSNOR activity in the brainstem, GSNOR activity was measured in homogenates of brainstems from C57BL6, GSNOR^{+/-}, and GSNOR^{-/-} mice exposed to normoxia (room-air), 3 minutes of hypoxia (time of peak increase in V_M), or 15 minutes of hypoxia (return to baseline levels) (Figure 2A). In C57BL6 mice, GSNOR activity was increased at 3 and 15 minutes of hypoxia (Figure 2B). As expected, baseline GSNOR activity was reduced in brainstem homogenates of GSNOR^{+/-} mice (Figure 2B). GSNOR activity did not increase in homogenates of GSNOR^{+/-} mice exposed to 3 or 15 minutes of hypoxia (Figure 2B). In contrast, brainstem homogenates from GSNOR^{-/-} mice showed a slight production of GSNO (negative GSNO consumption; Figure 2B). Production of GSNO in brainstem homogenates of GSNOR^{-/-} mice was not altered by exposure to 3 or 15 minutes of hypoxia (Figure 2B).

Changes in GSNOR Activity Are Associated with Changes in GSNOR S-Nitrosylation Status

Chronic exposure to hypoxia increases GSNOR protein expression (13). Western

blot analysis was performed to determine whether the changes in GSNOR activity in response to acute hypoxia are due to changes in GSNOR protein expression. As can be seen in Figure 3, acute exposure to hypoxia did not alter the protein levels of GSNOR or the other proteins under investigation. NADH is a critical cofactor for GSNOR activity. Hypoxia has been shown to increase the production of NADH (15). To determine if hypoxia-induced changes in NADH abundance contribute to the hypoxia-induced increases in GSNOR activity, the abundance of NADH was determined in brainstem homogenates from C57BL mice exposed to normoxia and 3 and 15 minutes of hypoxia (*see* Figure E1 in the online supplement). No significant changes in NADH abundance were detected with acute exposure to hypoxia. S-nitrosylation of GSNOR enhances GSNOR activity (17). In contrast to protein expression, brainstem homogenates from female C57BL6 mice exposed to either 3 or 15 minutes of hypoxia demonstrated an increase in the S-nitrosylation status of GSNOR at both 3 and 15 minutes (Figure 4).

Acute Hypoxia Increases the S-Nitrosylation of nNOS

NO is an important neuromodulator in chemotransduction of the HVR (1–4). The activities of both nNOS and eNOS are inhibited by S-nitrosylation (20, 21). Therefore, protein expression and S-nitrosylation status of nNOS and eNOS were evaluated in response to acute hypoxic exposure. There were no significant changes in nNOS or eNOS protein expression in brainstem homogenates generated from female C57BL6 mice exposed to 3 or 15 minutes hypoxia as compared with normoxic controls (Figure 3). nNOS S-nitrosylation was significantly elevated in brainstem homogenates from female C57BL6 mice exposed to 3 and 15 minutes of hypoxia (Figure 4).

Acute Hypoxia Increases S-Nitrosylation of the NR2B Subunit of the NMDAR

The initial increase in V_M in response to hypoxia is mediated via activation of NMDARs (4). Mechanisms that inhibit NMDAR function could contribute to roll-off. Because S-nitrosylation inhibits NMDAR activity (22, 23), the changes in

Figure 1. S-nitrosoglutathione (GSNO) reductase (GSNOR) and neuronal nitric oxide synthase (nNOS) are located in neurons within the nucleus tractus solitarius (NTS). (A) Immunohistochemical identification of GSNOR and nNOS in the NTS. *Upper panels* demonstrate the location of these proteins in neurons within the brainstem in a region corresponding to the NTS. *Lower panels* contain an enlargement of the indicated sections in the *upper panels*. Scale bars, 200 μ m (*upper panels*), 100 μ m (*lower panels*). (B) Colocalization of GSNOR and nNOS in neurons in the NTS by immunofluorescence. Neurons that contain both GSNOR and nNOS are indicated by the *thin white arrows*. Neurons that do not contain GSNOR are indicated by the *thick white arrows*. Scale bars, 50 μ m for all panels.

Figure 2. Minute ventilation (V_m) during hypoxic challenge does not show roll-off in GSNOR^{+/-} or GSNOR^{-/-} mice. (A) V_m in female C57BL6, GSNOR^{+/-}, and GSNOR^{-/-} mice under normoxia (N), and 3 and 15 minutes of exposure to hypoxia. The data are presented as means ($\pm$ SEM). There were 12 mice in each group. * $P < 0.05$, significant change from N values; † $P < 0.05$, GSNOR^{+/-} or GSNOR^{-/-} mice versus C57BL6 mice. (B) GSNOR activity increases temporally with exposure to hypoxia in female C57BL6 mice. GSNOR activity is significantly less in normoxia (N) in GSNOR^{+/-} and GSNOR^{-/-} mice compared with C57BL6 mice. In addition, there are no hypoxia-induced increases in GSNOR activity in GSNOR^{+/-} or GSNOR^{-/-} mice. The data are presented as means ($\pm$ SEM). There were six to eight samples in each group. * $P < 0.05$, significant change from N values; † $P < 0.05$, GSNOR^{+/-} or GSNOR^{-/-} mice versus C57BL6 mice; ‡ $P < 0.05$, GSNOR^{-/-} mice versus C57BL6 and GSNOR^{+/-} mice.

expression and S-nitrosylation status of three NMDAR subunits, NR1, NR2A, and NR2B, during hypoxic challenge were examined. Protein expression of NR1, NR2A, and NR2B in brainstem homogenates generated from female C57BL6 mice exposed to 3 or 15 minutes of hypoxia was not different from that seen in brainstem homogenates from female C57BL6 mice exposed to normoxia (Figure 3). All NMDAR subunits were basally S-nitrosylated. The S-nitrosylation status of NR1, NR2A, and NR2B did not change after 3 minutes of hypoxia (Figure 4). The S-nitrosylation status of NR1 was not different after 15 minutes of exposure to hypoxia and, although the S-nitrosylation status of NR2A tended to increase at this time, the change did not reach statistical significance (Figure 4). The

S-nitrosylation status of NR2B was substantially increased after exposure to 15 minutes of hypoxia.

Acute Hypoxia Does Not Enhance PDGFR- β Phosphorylation in the Brainstems of GSNOR^{+/-} Mice

Mice deficient in GSNOR display minimal ventilatory roll-off (11), as is the case with PDGFR- β ^{+/-} mice (5, 6). To assess hypoxia-induced changes in PDGFR- β expression and phosphorylation between C57BL6 and GSNOR^{+/-} mice, Western blots were performed. Basal PDGFR- β protein expression and phosphorylated PDGFR- β abundance were similar in brainstem homogenates from C57BL6 and GSNOR^{+/-} mice (Figure E2). Exposure to acute hypoxia significantly increased PDGFR- β phosphorylation in C57BL6

mice (Figure 5). The hypoxia-induced increases in PDGFR- β phosphorylation were not seen in the brainstems of GSNOR^{+/-} mice.

Discussion

Overview of Findings

The present study confirms that female mice deficient in or null for GSNOR have minimal ventilatory roll-off (11), and provides indirect evidence that S-nitrosylation events controlled by GSNOR in the brainstem may regulate ventilatory responses during hypoxic challenge. First, GSNOR was present in neurons in the NTS of female C57BL6 mice and colocalized with nNOS in many of these neurons. As such, GSNOR is well placed to modulate the levels of GSNO, resulting from nNOS activity. Second, acute exposure to hypoxia increased GSNOR activity in the brainstems of C57BL6 mice, but not in GSNOR^{+/-} mice, in which GSNOR activity was present, albeit in less amount. The hypoxia-induced changes in GSNOR activity in C57BL6 mice were associated with increases in S-nitrosylation status of GSNOR, which increases GSNOR activity (17). GSNO and related S-nitrosothiols, such as S-nitrosocysteinyglycine and S-nitrosocysteine, increase V_m upon microinjection into the NTS (12). The increase in GSNOR activity, which was greater at 15 than at 3 minutes of hypoxic challenge, would limit increases in GSNO and the abundance of S-nitrosylated proteins in transnitrosation equilibrium with GSNO generated during hypoxic challenge (12). This would be consistent with the occurrence of roll-off in C57BL6 mice and partially explain the absence of roll-off in GSNOR^{+/-} and GSNOR^{-/-} mice (11). Third, the hypoxic challenge enhanced the S-nitrosylation status of NMDAR subunit NR2B and nNOS, effects that were especially pronounced at 15 minutes in C57BL6 mice. S-nitrosylation of these proteins decreases their activity (20, 21). As such, diminished excitatory influence of glutamatergic neurotransmission via the down-regulation of NMDAR activity, coupled with diminished S-nitrosothiol formation, would certainly be consistent with the occurrence of ventilatory roll-off. Taken together, the data suggest that temporal changes in protein S-nitrosylation contribute to ventilatory roll-off.

Figure 3. Acute hypoxia does not alter protein abundance. (A) Western blots demonstrating the lack of change in protein abundance of nNOS, endothelial NOS (eNOS), GSNOR, N-methyl-D-aspartate (NMDA) receptor (NMDAR) subunits, NR1, NR2A, and NR2B, platelet-derived growth factor receptor (PDGFR)- β , or β -actin. (B) Graphical representation of the changes in protein expression upon exposure to acute hypoxia. Hypoxia-induced changes in protein expression are presented by comparing protein abundance in hypoxia to the normoxic controls for each protein examined. The data are not normalized to β -actin. The data are presented as means ($\pm$ SEM). There were eight samples in each group. There were no significant effects of hypoxic challenge on protein expression ($P > 0.05$, for all comparisons).

GSNOR Expression, Function, and S-Nitrosylation Status

GSNOR is an NADH-dependent enzyme involved in the catabolism of endogenous GSNO. GSNO is present in the brain in substantial concentrations (8), and is associated with increased V_M (12). This study confirms that nNOS and GSNOR are present in the neurons in the brainstem, including the NTS (Figure 1). The exact spatial and physical relationships between GSNOR and nNOS have not been established. Our immunofluorescence and immunohistochemical data show that GSNOR is present in brainstem neurons. Biochemically, it would make sense that nNOS and GSNOR colocalize, as nNOS activation could directly S-nitrosylate GSNOR, thereby increasing its activity. However, nNOS-containing neurons did not always appear to contain GSNOR. This may reflect the differential subcellular location of the two proteins in the neuron that, depending on the section, may not have been seen. GSNOR may be expressed in a subset of neurons that are involved in S-nitrosothiol homeostasis, whereas its presence in other neurons may reflect its

role as a formaldehyde-dependent alcohol dehydrogenase (7).

This study also demonstrates that temporal increases in GSNOR activity occur during exposure to hypoxia (Figure 2). More importantly, there appears to be an inverse relationship between V_M and GSNOR activity (V_M decreases while GSNOR activity increases) in C57BL6 female mice (Figure 2), consistent with the idea that reduced bioavailability of GSNO is associated with ventilatory roll-off. The changes in GSNOR activity were not due to altered GSNOR protein expression (Figure 3), which has been reported with chronic hypoxic exposure (13), nor to hypoxia-induced increases in NADH abundance (15, 16), as no changes in NADH levels were detected (Figure E1). On the other hand, hypoxia-induced increases in GSNOR activity correlated with increases in the S-nitrosylation status of GSNOR (Figure 4). GSNOR is a tonically S-nitrosylated protein, and enhanced S-nitrosylation has been shown to increase GSNOR activity in the lung (17). Thus, one component of ventilatory roll-off may involve a hypoxia-induced

increase in GSNOR activity (and associated decreases in GSNO bioavailability) mediated by enhanced S-nitrosylation of the enzyme.

NMDA Expression, Function, and S-Nitrosylation Status

Brain NMDARs play a pivotal role in the initial increase in V_M upon exposure to hypoxic challenge (4, 24, 25). Mechanisms that inhibit NMDAR function contribute to ventilatory roll-off. NMDAR are heterotetramers composed of two NR1 subunits and two NR2 or NR3 subunits. The NR1 subunit is required for NMDAR function, whereas the NR2 and NR3 subunits confer distinct characteristics of the NMDAR ion channel, expression patterns, and function (26). NMDARs are known to be S-nitrosylated (22). There are at least seven cysteine residues among the NMDAR subunits, five of which are involved in S-nitrosylation (Cys744 and Cys798 of NR1 and Cys87, Cys320, and Cys399 of NR2A) (34). In all cases, S-nitrosylation of NMDARs is inhibitory, with S-nitrosylation of Cys399 of NR2A mediating the primary effect (23). All NMDAR subunits examined showed basal levels of S-nitrosylation (Figure 4). Significant increases in NR2B S-nitrosylation were seen with a 15-minute exposure to hypoxia (increases in NR2A S-nitrosylation were also noted, but did not reach statistical significance), whereas no differences were seen in NR1 S-nitrosylation. In developing animals, ventilatory roll-off is greater than in adult animals. This correlates with the developmental expression of NR2B, which is expressed early and decreases with age (26). Thus, it is plausible that the activities of NR2B containing NMDARs are decreased by S-nitrosylation, which would contribute to ventilatory roll-off. As reviewed by Palmer and Gaston (27), the denitrosylation of proteins (i.e., removal of the NO^+ group from thiol residues) can be achieved by preincubation with mercuric chloride (HgCl_2), exposure to ultraviolet light, or performing the assay in the absence of sodium ascorbate. The abundance of S-nitrosylated NR2B in brainstem homogenates in the absence or presence of HgCl_2 (100 μM) was $806 (\pm 86; \times 10,000)$ versus $354 (\pm 68; \times 10,000)$ densitometry units, respectively, which was a $48 (\pm 8)\%$ reduction in apparent S-nitrosylation of NR2B. As such,

Figure 4. Hypoxia alters the S-nitrosylation status of GSNOR, nNOS, and NR2B. Changes in protein S-nitrosylation were determined by the biotin-switch assay followed by Western blot analysis. (A) Western blot of proteins after biotin switch. All proteins have some level of S-nitrosylation under normoxic (baseline) conditions. Three proteins, GSNOR, nNOS and NR2B showed increases in S-nitrosylation with exposure to acute hypoxia. (B) Graphical representation of the change in S-nitrosylation status of the selected proteins with exposure to hypoxia. Change in S-nitrosylation status is determined by the S-nitrosylated protein:total protein ratio. The data are presented as means ($\pm$ SEM). There were eight samples in each group. * $P < 0.05$, significant change from normoxic (N) values.

it is apparent that NR2B is subject to significant S-nitrosylation.

Efficient NMDAR channel activation requires binding to L-glutamine and glycine

(see Ref. 25). The binding domains for these coagonists are distinct: the glycine-binding domain is present on the NR1 subunit, whereas the glutamate binding domain is

located on the NR2 subunit. Studies in cultured hippocampal neurons and synaptic membranes from pig cerebral cortex suggest that GSNO may also be a ligand for the NMDARs (28, 29). GSNO has also been shown to potentiate the responses of relay neurons to sensory stimuli and excitatory amino acids like glutamate when iontophoretically applied (30). Moreover, the agonist effects of GSNO cannot be inhibited by N-ethyl maleimide, suggesting that the response is not mediated by protein S-nitrosylation (28). Currently, it is not clear whether the NMDAR has a specific GSNO-binding domain, as both specific GSNO binding sites, not displaceable by glutamate analogs (31), and the ability of GSNO to displace [3 H] glutamate have been reported (28, 30). Thus, if GSNO is a coagonist for NMDAR, a reduction in GSNO bioavailability via increased GSNOR activity would reduce NMDAR activation, contributing to ventilatory roll-off during hypoxic challenge.

NOS Expression, Function, and S-Nitrosylation Status

NO bioactivity generated by nNOS is an important component of the HVR. Both inhibitory (32, 33) and stimulatory (34–36) effects of NOS activation have been described that reflect the differing actions of NO and S-nitrosothiols, respectively. nNOS activity is dynamically regulated (inhibited) by S-nitrosylation at cysteine 331 (20). Moreover, activation of NMDAR is associated with an increase in denitrosylation and decrease in nNOS phosphorylation at serine 847 (20). In this study, S-nitrosylation of nNOS was increased after 3 and 15 minutes of exposure to hypoxia (Figure 4). This post-translational modification of nNOS may contribute to ventilatory roll-off by limiting L-glutamate release (and NMDAR activation) by presynaptic mechanisms (35) or cGMP-dependent protein phosphorylation cascades (4).

PDGFR- β Expression, Function, and S-Nitrosylation Status

GSNOR $^{+/-}$ and GSNOR $^{-/-}$ mice have minimal ventilatory roll-off (11), similar to that seen in mice deficient in PDGFR- β (5, 6). The relationship between PDGFR- β , GSNOR, and the attenuation of roll-off in response to diminished expression of these genes is not known. Hypoxic

Figure 5. PDGFR- β phosphorylation is not altered by hypoxia in GSNOR $^{+/-}$ mice. (A) PDGFR- β protein abundance and phosphorylation in brainstem homogenates from female C57BL/6 mice exposed to normoxia and 3 and 15 minutes of hypoxia. (B) PDGFR- β protein abundance and phosphorylation in brainstem homogenates from GSNOR $^{+/-}$ mice exposed to normoxia, and 3 and 15 minutes of hypoxia. (C) Graphical representation of the changes in phosphorylated/total PDGFR- β protein in C57BL/6 and GSNOR mice ($n = 3-4$). * $P < 0.024$, significant change from normoxia.

Figure 6. Schematic diagram of hypoxia-induced changes in S-nitrosylation during the hypoxic ventilatory response (HVR). (A) S-nitrosylation status during phase I of the HVR. NR2B, nNOS and GSNOR have a low level of basal S-nitrosylation. NMDAR channel is open in response to glutamate, glycine, and/or GSNO, allowing influx of Ca^{2+} , which activates nNOS, and production of GSNO and NO. The GSNO produced is not efficiently catabolized, as GSNOR activity is minimal. Release of NO enhances release of glutamate from presynaptic cells. (B) S-nitrosylation status during ventilatory roll-off. Continued exposure to hypoxia augments S-nitrosylation of NR2B, nNOS, and GSNOR. S-nitrosylation of the NR2B decreases NMDAR channel opening, thus decreasing Ca^{2+} influx. Decreased Ca^{2+} influx and increased S-nitrosylation status of nNOS decreases the production of GSNO and NO. GSNOR activity is elevated, resulting in the increased catabolism of GSNO. Low NO production results in a minimal release of glutamate from presynaptic cells. All processes would thereby promote ventilatory roll-off during hypoxic challenge. (C) Schematic diagram of the potential mechanisms and sites of action of S-nitrosothiols in the HVR. Hb, hemoglobin.

exposure did not alter PDGFR- β protein expression (Figure 3) or S-nitrosylation status in C57BL6 female mice (Figure 4). Likewise, there was no change in NR2B expression (Figure 3), even though PDGFR- β activation has been shown to decrease the abundance of NR2B containing NMDAR (37). Brainstem PDGFR- β protein expression in GSNOR $^{+/-}$ animals was not

different from C57BL6 mice (Figure 5A), demonstrating that loss of GSNOR had minimal effects on PDGFR- β protein expression. Hypoxia-induced release of PDGF-BB activates PDGFR- β to stimulate signaling pathways involving phospholipase C, PKC, intracellular Ca^{2+} signaling, and actin cytoskeleton rearrangement (4), which inhibit NMDA activity. Exposure

to acute hypoxia increased the phosphorylation status of PDGFR- β , confirming PDGFR- β activation in the brainstems of C57BL6 mice (Figure 5B). This hypoxia-induced increase in phosphorylation was not seen in the brainstems of GSNOR $^{+/-}$ mice. These observations suggest that a loss of GSNOR and/or the corresponding increase in

GSNO modulates PDGFR- β signaling, contributing to regulation of ventilatory roll-off. The mechanism responsible for the lack of PDGFR- β phosphorylation in GSNOR^{+/-} mice is unknown. Potential mechanisms include limited PDGF-BB release and augmented S-nitrosylation of PDGFR- β in the brainstems, which limits receptor phosphorylation or activation of PDGFR- β -specific phosphatases. Regardless of the mechanism, the lack of PDGFR- β activation abrogates downstream signaling of PDGFR- β , including PKC, which modulates NMDAR activity (34). More specifically, decreases in PKC activity temporally coincide with the expression of ventilatory roll-off (34). PDGFR- β activation inactivates PKC via activation of protein phosphatases type I or 2a, leading to NMDAR inactivation (38). In the absence of PDGFR- β activation, protein phosphatases are not activated, and NMDAR activity is elevated, limiting ventilatory roll-off.

Conclusions

The results of this study demonstrate temporal changes in S-nitrosylation status of GSNOR, nNOS, and the NMDAR subunit, NR2B, proteins known to be involved in the HVR, in the brainstems of female C57BL6 mice. The key conclusion is that changes in S-nitrosylation status of these proteins did not correlate with the initial increases in V_M , but rather ventilatory roll-off. Potential mechanisms by which changes in the S-nitrosylation of these proteins within the brainstem contribute to ventilatory roll-off are summarized in the legend of Figures 6A and 6B. Inhibition of the activities of the NMDAR subunit, NR2B, and nNOS through S-nitrosylation may serve to reduce: (1) Ca^{2+} influx; (2) production of NO/GSNO from nNOS; and (3) release of

neurotransmitter pools of glutamate. These events would contribute to terminating the initial increase in V_M during hypoxic exposure. These events may be coordinated with the temporal increase in GSNOR activity during exposure to hypoxic challenge, which would limit accumulation of the ventilatory stimulant, GSNO, thereby promoting roll-off during the challenge.

There is now considerable evidence that S-nitrosothiols play important roles in the expression of ventilatory responses during and after hypoxic challenge. The potential mechanisms and sites of action of S-nitrosothiols are depicted in Figure 6C. First, the expression of HVR and short-term potentiation (STP) are absent in conscious mice with bilateral carotid sinus nerve transection (39), supporting a role of carotid body primary glomus cells/chemoafferents in these responses. As such, the potential release of S-nitrosothiols from eNOS-positive glomus cells (11) and subsequent activation of chemoafferent terminals may contribute to the initiation of HVR, whereas S-nitrosothiols generated from red blood cell hemoglobin (*see subsequent text*) do not appear to be vital for initiation of the HVR (39). Second, ventilatory roll-off during the hypoxic challenge is absent in conscious GSNOR^{+/-} or GSNO^{-/-} mice (11), clearly suggesting that roll-off is due to enhanced catabolism of GSNO in the carotid body and/or central structures. Third, direct microinjections of GSNO, S-nitrosocysteinyglycine, or L-S-nitrosocysteine into the NTS of conscious rats elicits pronounced increases in V_M (12). This observation would be consistent with a role of S-nitrosothiols in the expression of the HVR and STP in conscious mice. Whether these S-nitrosothiols emanate from

chemosensory afferent terminals or intrinsic neurons within the NTS remains to be established. Finally, the absence of STP in conscious mice expressing human hemoglobin in which the cysteine residue at position 93 in the β chain is replaced with alanine at residue 93 suggests that hemoglobin-derived S-nitrosothiols are essential for the expression of STP (39). Whether circulating S-nitrosothiols can elicit effects on V_M via actions at brain sites devoid of a blood-brain barrier (e.g., area postrema) has not been examined.

The roles of sex hormones and sex differences with respect to ventilatory control mechanisms, lung function, and ventilatory responses to hypoxic or hypercapnic challenges have received substantial attention. As reviewed by Palmer and colleagues (11, 18), studies in humans found that HVRs are greater in females, equal in females and males, or that women have a lower HVR and hypercapnic drive than men. Disparate findings have also been generated in rats. HVR was reported to be greater in females than males via mechanisms independent of the activities of ovarian hormones, similar in females and males, or smaller in females. Studies in rats and mice have ascribed disparities in HVR between males and females to sex-related differences in response/signaling processes in the carotid bodies (11, 18). We are currently performing studies similar to those described in the present article on male wild-type, GSNOR^{+/-}, and GSNOR^{-/-} mice. ■

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