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Hypoxia-induced hypotension elicits adenosine-dependent phrenic long-term facilitation after carotid denervation

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Abstract

Moderate acute intermittent hypoxia (AIH) elicits a persistent, serotonin-dependent increase in phrenic amplitude, known as phrenic long-term facilitation (pLTF). Although pLTF was originally demonstrated by carotid sinus nerve stimulation, AIH still elicits residual pLTF in carotid denervated rats (CBX) via a distinct, but unknown mechanism. We hypothesized that exaggerated hypoxia-induced hypotension after CBX, leading to greater spinal tissue hypoxia and extracellular adenosine accumulation, thereby triggering adenosine 2A receptor (A_{2A})-dependent pLTF. Phrenic activity, arterial pressure and spinal tissue oxygen pressure were measured in anesthetized CBX rats. Exaggerated hypoxia-induced hypotension after CBX was prevented via intravenous phenylephrine; without the hypotension, spinal tissue hypoxia during AIH was normalized, and residual pLTF was no longer observed. Spinal A_{2A} (MSX-3), but not serotonin 2 receptor (5-HT₂) inhibition (ketanserin), abolished residual pLTF in CBX rats. Thus, pLTF regulation may be altered in conditions impairing sympathetic activity and arterial pressure regulation, such as spinal cord injury.

Keywords

Hypotension; Respiratory motor plasticity; Tissue hypoxia; Carotid body denervation; Acute intermittent hypoxia

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None.

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INTRODUCTION

The respiratory and cardiovascular systems are inextricably linked in the delivery of oxygen to tissues, including neural tissues such as the spinal cord. Whereas the respiratory system establishes arterial oxygen pressure (PaO₂) and concentration, the cardiovascular system transports oxygenated blood to tissues where oxygen is released and consumed.

Physiological processes dependent on prevailing levels of spinal tissue oxygen pressure (PtO₂) could be affected in profound ways by changes in either breathing or cardiovascular function. Here, we test the hypothesis that hypotension and impaired spinal oxygen delivery account for certain forms of respiratory motor plasticity.

One of the best studied models of plasticity in respiratory motor control is phrenic long-term facilitation (pLTF), a persistent increase in phrenic nerve activity lasting hours after acute intermittent hypoxia (AIH) (Mitchell et al., 2001; Mahamed & Mitchell, 2007; Baker-Herman & Mitchell, 2008; Dale-Nagle et al., 2010; Devinney et al., 2013). Two completely distinct mechanisms can account for pLTF, depending on the severity of hypoxic episodes during AIH (Nichols et al., 2012; Devinney et al., 2016). With **moderate** AIH (PaO₂: 35–55 mmHg), pLTF requires: 1) episodic carotid body chemoreceptor activation; 2) increased activity in brainstem neural networks, including raphe serotonergic neurons; 3) episodic serotonin release and post-synaptic serotonin 2 receptor (5-HT₂) activation in the phrenic motor nucleus; and 4) an intracellular signaling cascade within phrenic motor neurons known as the Q pathway to phrenic motor facilitation (Dale-Nagle et al., 2010; Devinney et al., 2013; Fields & Mitchell, 2015). In striking contrast, pLTF following AIH consisting of **severe** hypoxic episodes (PaO₂: 25–30 mmHg) requires: 1) spinal tissue hypoxia and extracellular adenosine accumulation; 2) adenosine 2A receptor (A_{2A}) activation on phrenic motor neurons; and 3) a distinct intracellular signaling cascade known as the S pathway of phrenic motor facilitation (Golder et al., 2008; Nichols et al., 2012; Seven et al., 2018; Perim et al., 2019). There is no known obligatory role for carotid chemoreceptors in severe AIH-induced, adenosine-dependent pLTF.

Although serotonin-dependent pLTF was originally demonstrated with intermittent electrical stimulation of the carotid sinus nerve (Millhorn et al., 1980a), phenotypically and mechanistically similar pLTF is observed following moderate AIH (Baker-Herman & Mitchell, 2002). Surprisingly, moderate AIH-induced pLTF is blunted, but not eliminated by carotid denervation (*i.e.* residual pLTF) (Bavis & Mitchell, 2003; Sibigtroth & Mitchell, 2011). Mechanisms giving rise to residual pLTF are not known.

During hypoxia, carotid body- and baroreceptor-induced sympathetic activation minimizes decreases in arterial blood pressure in rats. Consequently, hypoxia-induced hypotension is exaggerated by carotid denervation (Bavis & Mitchell, 2003; Sibigtroth & Mitchell, 2011). Hypotension can impair spinal blood flow and tissue oxygen delivery, leading to greater

tissue hypoxia and extracellular adenosine accumulation (Takahashi et al., 2010; Yamashiro & Morita, 2017).

The fundamental objective of the present study was to understand mechanisms giving rise to residual pLTF following moderate AIH in rats with carotid denervation. Our central hypothesis is that carotid denervation enables greater hypotension during the same moderate AIH protocol, thereby impairing spinal oxygen delivery (which favors tissue adenosine accumulation), eliciting residual pLTF by an A_{2A} receptor-dependent mechanism. We tested 3 specific hypotheses to accomplish this goal: 1) greater hypotension during moderate hypoxia in the absence of intact carotid bodies compromises spinal oxygenation levels; 2) preventing hypotension mitigates spinal tissue hypoxia during moderate AIH and abolishes residual pLTF; and 3) spinal A_{2A}, but not 5-HT₂ receptor activation is necessary for residual pLTF after carotid denervation (*i.e.* similar to severe AIH-induced pLTF in intact rats).

Spinal PtO₂ and phrenic nerve activity were measured throughout our moderate AIH protocol in anesthetized, paralyzed, vagotomized and ventilated rats. Consistent with our hypotheses, we report an alternate, carotid body-independent pathway to phrenic motor plasticity that requires exaggerated hypotension, spinal tissue hypoxia and A_{2A} receptor activation. Our findings implicate that cardiovascular regulation can influence expression and mechanisms of respiratory motor plasticity. This finding may be of importance for conditions that impair sympathetic outflow and blood pressure regulation (*e.g.* spinal injury), or that compromise tissue oxygen delivery in other ways (*e.g.* anemia).

METHODS

Animals

All experiments were approved by the University of Florida Institutional Animal Care and Use Committee (protocol #201408657). Experiments were performed on 50 adult (300–500 gram) male Sprague Dawley rats (208A Colony, Envigo; IN, USA) housed in pairs at 24 °C with a 12/12 light cycle and access to food and water *ad libitum*. Sample size estimation was based on our extensive experience with this experimental preparation.

Neurophysiological experiments

Terminal neurophysiological experiments were performed as previously described (Baker-Herman et al., 2004; Perim et al., 2018b, 2019). Rats were induced with 3.0% isoflurane in oxygen in a plexiglass chamber, and transferred to a heated surgical table where anesthesia was continued (3.0% isoflurane; 60% oxygen, balance nitrogen). Body temperature was kept at ~ 37.5 °C throughout the experiment using a temperature controller (TC-1000; Ardmore, PA, USA). A polyethylene catheter (I.D., 1.67 mm) was inserted into the trachea through a mid-line neck incision, and rats were mechanically ventilated (2.5 mL/breath, 70 breaths/minute; VentElite small animal ventilator; Harvard Apparatus, Holliston, MA, USA). The inspired CO₂ fraction was adjusted as needed to keep end-tidal CO₂ within physiological limits.

Anesthesia was then slowly converted to urethane (2.1 g/kg at 6mL/hour through a tail vein catheter); while progressively decreasing the inspired isoflurane concentration over ~20

minutes. At least 1 hour was allowed after conversion to start experimental protocols. Throughout experiments, adequate anesthetic depth was confirmed by absence of withdrawal reflex or blood pressure response to toe pinch. Both vagus nerves were exposed and cut at the mid-cervical level to avoid respiratory entrainment with the ventilator. The right femoral artery was exposed and cannulated with a polyethylene catheter (inner diameter 0.58) to monitor blood pressure and blood gases.

Rats were placed on a stereotaxic frame and cervical muscle layers dissected and separated to allow C3 to C5 laminectomy. During some experiments, spinal PtO_2 was measured using a 50 μ m optical sensor (Optode, MicroOptode, Unisense; Aarhus, Denmark) positioned 1 mm lateral to midline between C3 and C4 (~ 1.5 mm deep). Raw signals acquired at 1 Hz were converted to mmHg using a two-point calibration in saline at ~ 37°C. This solution was equilibrated with 21% O_2 for > 40 minutes (i.e. normoxia), while sodium hydroxide and sodium ascorbate (0.1 M each) were dissolved in warmed saline to form an anoxic calibration solution (0% O_2). Calibration was confirmed at the end of experiments by comparing oxygen pressure in anoxic solution *versus* spinal PtO_2 measured several minutes after death from urethane overdose (data not shown).

A_{2A} receptor (MSX-3, 10 μ M, 12 μ L; Sigma-Aldrich, St. Louis, MO, USA) or serotonergic 2A/2C receptor antagonists (ketanserin, 500 μ M, 15 μ L; Tocris Biosciences, Minneapolis, MN, USA) were initially diluted in 100% dimethyl sulfoxide (DMSO), aliquoted into vials and stored at -20 °C. On the experimental day, stock solutions were diluted in aCSF (in mM: 125 NaCl, 3 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 1.25 $MgSO_4 \cdot 7H_2O$, 1.25 KH_2PO_4 , 25 $NaHCO_3$, bubbled with 95% O_2 -5% CO_2) to the final concentration. Prior to AIH, antagonists were administered via an intrathecal silicone catheter (2 French, 0.6 mm OD; Access Technologies; Skokie, IL, USA) attached to a 50 μ L Hamilton syringe. The catheter tip was advanced caudally through a small hole in the dura and stabilized on the dorsal side of ~ C4. Ten minutes before AIH, drugs (final DMSO concentration < 5%) were administered slowly from the catheter over a 2-minute period.

The left phrenic nerve was isolated and cut distally. Epineurium was retracted to expose the nerve tip, and electrical nerve signals were acquired using suction electrodes connected to a differential amplifier (A-M Systems, Model 1700; Sequim, WA, USA). Nerve signals were amplified (10,000x), band-pass filtered (0.3 to 5 kHz), and digitized (CED 1401; Cambridge Electronic Design; Cambridge, England, UK) using Spike2 software (version 8.08; Cambridge Electronic Design; Cambridge, England, UK). Rats were paralyzed with pancuronium bromide (3 mg/kg i.v.; Sigma-Aldrich; Saint Louis, MO).

Experimental Design

After establishing stable nerve activity, the inspired CO_2 fraction was slowly decreased until the apneic threshold was reached, defined as the end-tidal CO_2 causing sustained apnea (> than 1 min). Inspired CO_2 fraction was then progressively increased until phrenic nerve activity resumed (i.e. recruitment threshold). Baseline end-tidal CO_2 was then set at 2–3 mmHg above the recruitment threshold. Baseline nerve activity was characterized over a 15-minute period in which two stable blood gas values were obtained. Inspired O_2 fraction was reduced to ~ 0.13 for 5 minutes, targeting a PaO_2 between 35–55 mmHg (blood sample ~

2.5 minutes after hypoxic episode onset). This 5-minute hypoxia cycle was repeated 3 times, separated by 5-minute 60% O₂ intervals. Phrenic nerve activity and spinal PtO₂ were recorded up to 90 minutes after AIH in 3 experimental groups: 1) carotid-denervated (CBX) (Perim et al., 2018a); 2) CBX-Phenyl: in CBX rats, systemic phenylephrine was administered during AIH to counteract hypoxia-induced hypotension; and 3) Sham, in which the carotid bifurcation was exposed, but the carotid sinus nerve left intact. Spinal A_{2A} or serotonin 2A/2C receptors were inhibited in additional groups of CBX rats to further investigate mechanisms of residual pLTF. Carotid denervation was confirmed by an absence of increase in phrenic nerve burst amplitude following low dose intravenous injections of a sodium cyanide solution (25 µg in 0.2 mL saline), as in previous reports (Bavis & Mitchell, 2003; Amorim et al., 2016; Perim et al., 2018a). Experiments were excluded only if: 1) not possible to establish baseline (see above); 2) substantial hypotension developed post-AIH, affecting stability of the preparation and ability to regulate blood gases.

Data analyses

For quantitative analyses, raw nerve signals were smoothed (0.05 sec time constant) and rectified for off-line analysis. Integrated phrenic nerve burst amplitude 90 minutes post-AIH was compared with baseline values. One-way ANOVA was used to compare response means (mean arterial pressure during hypoxia, hypoxic ventilatory response, spinal PaO₂ during baseline, spinal PaO₂ during hypoxia, mean arterial pressure during hypoxia, and spinal PaO₂ during hypoxia, both from baseline to 90 minutes post-AIH) among the 3 main experimental groups (Sham, CBX, CBX-phenyl; Schabenberger & Pierce, 2002). Analysis of covariance (ANCOVA) was used to estimate group-specific trend line intercepts and slopes of a single predictor regressed on a response variable, and to compare slopes among the 3 experimental groups (Schabenberger & Pierce, 2002). A linear mixed effect random coefficients model was used to estimate group-specific mean trend line intercepts and slopes from animal-specific trend line intercepts and slopes of time regressed on spinal PaO₂ (Brown & Prescott, 2015). In the random coefficients model, animal-specific intercepts and slopes within an experimental group are assumed to follow a multivariate normal distribution with mean intercept and slope parameters and associated covariance parameters. Mean slopes were compared among the 3 experimental groups within the framework of the random coefficients model. A linear mixed effect model with time modeled as a 4-level within-animal fixed effect (0, 30, 60, 90 minutes), experimental group modeled as a 3-level between-animal fixed effect, and animal modeled as a random effect, was used to assess change in group-specific mean response over time (integrated phrenic amplitude, respiratory frequency) and to compare patterns of change in mean response among the 3 experimental groups (Brown & Prescott, 2015). A linear mixed effect model was also used to compare patterns of mean response over time (integrated phrenic amplitude, respiratory frequency) between CBX-5-HT₂ and CBX-A_{2A} antagonist or between time control and phenyl groups. Normal distribution of residual errors was confirmed by visual inspection of histograms and normal probability plots. Pre-planned pairwise comparisons of mean response within the framework of each type of analysis model were carried out using appropriately weighted contrasts based on the t statistic (Brown & Prescott, 2015). Values are expressed as mean ± 1 standard error of the mean. Means are also displayed in some figures with 95% confidence intervals. An alpha-level of 0.05 was used to assess statistical significance for all pre-

planned comparisons. All analyses were carried out using SAS Version 9.4 (SAS Institute, Cary NC) or R Version 3.4.2 (R Core Team, Vienna, Austria).

RESULTS

CBX rats presented more severe hypoxia-induced hypotension (52 ± 6 mmHg) versus Sham rats (101 ± 10 mmHg; $p < 0.05$) during the last minute of hypoxic episodes, as in previous reports (Bavis & Mitchell, 2003; Sibigtroth & Mitchell, 2011). Hypoxia-induced hypotension was successfully eliminated in CBX-Phenyl rats (121 ± 13 mmHg; $p < 0.05$ vs. CBX; Figure 1, panels A, B and C). PaO_2 during hypoxic episodes was similar among groups, but a longer time was needed for blood sample withdrawal during hypoxic episodes in CBX rats, consistent with systemic blood flow impairment (Figure 1, panel D). Short-term hypoxic phrenic response was blunted/abolished in CBX ($: 13 \pm 3\%$) and CBX-Phenyl rats ($: 12 \pm 10\%$) versus Sham rats ($: 63 \pm 13\%$; $p < 0.05$; Figure 1, panel E), which is consistent with previous studies from our laboratory using this preparation (Bavis & Mitchell, 2003; Sibigtroth & Mitchell, 2011).

Figure 2 shows direct spinal PtO_2 measurement near/in the phrenic motor nucleus during AIH (panels A and B). More severely impaired spinal tissue oxygenation developed in CBX rats during the last minute of hypoxic episodes. However, the severity of spinal tissue hypoxia was lessened by systemic phenylephrine (Figure 2, panels B, C, and D). Spinal PtO_2 was similar among groups during baseline ($p > 0.05$; Figure 2, panel E), but increased from baseline to 90 minutes post-AIH in CBX rats ($: 17 \pm 3$ mmHg) vs. CBX-Phenyl ($: -7 \pm 5$ mmHg) and Sham rats ($: -5 \pm 8$ mmHg; $p < 0.05$; Figure 2, panel F).

A steep spinal PtO_2 drop took place soon after initiating each hypoxic episode during AIH in all experimental groups. In the last 3 minutes of the first AIH episode, spinal PtO_2 slope did not differ from 0 in Sham (0.7499 ± 0.6825 mmHg/minute; $p = 0.284$) or CBX-Phenyl rats (0.770 ± 0.638 mmHg/minute; $p = 0.241$), as suggested by linear trend model fit to individual curves. On the other hand, a slightly negative spinal PtO_2 slope was estimated for CBX rats (-1.31 ± 0.60 mmHg/minute; $p = 0.042$), supporting our hypothesis that this group experiences more severe spinal tissue hypoxia during AIH. Comparing slope estimates among groups, CBX rats had a significantly lower spinal PtO_2 slope than CBX-Phenyl (-2.08 ± 0.88 mmHg/minute; $p = 0.028$) and Sham rats (-2.06 ± 0.91 mmHg/minute; $p = 0.035$; Figure 3, panels A, B and C; Figure supplement 1).

Integrated phrenic burst amplitude increased in both CBX ($: 0.023 \pm 0.007$ a.u.; $p = 0.002$) and Sham rats ($: 0.035 \pm 0.009$ V; $p = 0.0001$) 90 minutes post-AIH (i.e. pLTF). However, residual pLTF was no longer present in CBX-Phenyl rats ($: -0.005 \pm 0.003$ a.u.; $p = 0.76$), indicating that blood pressure regulation greatly affects the expression of phrenic motor plasticity. The pLTF magnitude was also higher in Sham ($p = 0.0001$) and CBX rats ($p = 0.017$) vs. CBX-Phenyl rats 90 minutes post-AIH. Sham rats had the highest pLTF magnitude 90 minutes post-AIH, but were not significantly different from CBX rats in this study ($p = 0.077$; Figure 4, panels A, B, and D). Baseline integrated phrenic burst amplitude was similar between Sham, CBX & CBX-Phenyl rats ($p > 0.05$). No changes in respiratory frequency were observed among groups ($p > 0.05$; Figure 4, panel C and E).

Spinal A_{2A} receptor inhibition blunted/abolished residual pLTF in CBX rats ($p = 0.55$ vs. baseline and $p = 0.015$ vs. CBX rats; Figure 5, panels A, B, and D). Preliminary data ($n = 2$, not shown) suggested that residual pLTF in CBX rats was serotonin-independent since its expression was still present after spinal 5-HT_{2A} receptor inhibition ($p = 0.014$). CBX rats pre-treated with intrathecal injections of A_{2A} receptor antagonist had stable respiratory frequencies throughout experimental protocols (Figure 5, panel C and E).

Because spinal adrenergic receptor activation elicits a form of phrenic motor plasticity (Huxtable et al., 2014), we tested whether phrenic motor output is affected by intravenous phenylephrine, which does not cross the blood-brain barrier. Approximately 1–2 mL of 100 μ M phenylephrine was slowly delivered i.v. for 3 $\times$ 5-minute episodes (without hypoxia), separated by 5-minute intervals. Integrated phrenic burst amplitude did not change from baseline to 90 minutes post-phenylephrine ($: 0.002 \pm 0.003$ a.u.; $p > 0.05$); and integrated phrenic burst amplitude changes were not different between Phenyl- and Time-control rats ($: -0.004 \pm 0.003$ a.u.; $p > 0.05$; Figure 6, panels A, B, and D). Changes in respiratory frequency were not observed (Figure 6, panel C and E; $p > 0.05$). The outcome of these control experiments rules out the possibility that systemic phenylephrine administration per se would have any long-term effect on phrenic burst amplitude, which supports our conclusions that mitigating hypoxia-induced hypotension is the relevant factor to prevent residual pLTF after carotid denervation.

As in previous studies, variables known to affect phrenic burst amplitude were tightly regulated during experimental protocol, aiming: 1) PaO₂ > 150 mmHg (except during hypoxic episodes); 2) PaCO₂ within ± 1.5 mmHg of baseline values; 3) standard base excess within ± 4 mmol/L; and 4) body temperature within 37.5 ± 1 °C. Average values for each group are reported in Tables 1, 2 and 3. No statistical differences among groups or relative to baseline were observed in PaCO₂, standard base excess or body temperature. Although a progressive drop in blood pressure and PaO₂ normally develops in this experimental preparation over time (Bach & Mitchell, 1996), statistical differences between baseline and 90 minutes post-AIH were detected only in CBX-Phenyl rats for blood pressure ($: -33 \pm 8$ mmHg) and PaO₂ ($: -77 \pm 22$ mmHg). Blood pressure and PaO₂ did not differ from baseline in CBX ($: -11 \pm 8$ mmHg and $: -36 \pm 8$ mmHg, respectively) or Sham rats ($: -12 \pm 11$ mmHg and $: -43 \pm 10$ mmHg, respectively; $p > 0.05$). Hematocrit and oxygen saturation remained stable throughout experimental protocols (data not shown).

DISCUSSION

We conclude that exaggerated hypotension within (AIH) hypoxic episodes following carotid denervation elicits residual moderate AIH-induced pLTF by an adenosine-dependent mechanism. Within hypoxic episodes, greater systemic hypotension lowers spinal PtO₂ (despite the same arterial PO₂), increasing adenosine accumulation and triggering A_{2A} receptor-induced phrenic motor plasticity. When hypoxic hypotension was prevented by phenylephrine infusion, PtO₂ was less hypoxic, undermining residual pLTF. We conclude that exaggerated hypotension during moderate hypoxic episodes results from loss of carotid chemoreflexes and baroreflexes, decreasing tissue PO₂ more than in intact rats. After CBX, the neural network giving rise to serotonin-dependent pLTF is silenced, and greater

extracellular spinal adenosine accumulation reaches a threshold necessary to elicit A_{2A} receptor-induced phrenic motor plasticity (*i.e.* the S pathway). Thus, circulatory control and spinal oxygen delivery indirectly regulate AIH-induced pLTF, with hypotension shifting the balance towards adenosine dependent mechanisms of plasticity.

Adenosine versus serotonin dependent phrenic motor facilitation

Phrenic motor facilitation can be elicited by either spinal 5-HT₂ or A_{2A} receptor activation (Golder et al., 2008; Perim et al., 2018b; Seven et al., 2018; Perim et al., 2019). While 5-HT₂-dependent phrenic motor facilitation requires intact carotid bodies and serotonergic raphe neuron activity, A_{2A} receptor activation is triggered by spinal hypoxia, and does not require carotid chemoreflex activation. CBX is predicted to reduce serotonergic neuron activation, thereby preventing serotonin-dependent phrenic motor plasticity. In its absence, hypoxemia and levels of PtO₂ characteristic of our normal moderate AIH protocol would not be sufficient to trigger adenosine dependent plasticity. However, with loss of carotid chemo- and baroreflexes, greater systemic hypotension despite the same arterial PO₂ decreases spinal oxygen delivery, causing greater tissue hypoxia, triggering greater ATP and/or adenosine release from glia and raising adenosine to levels sufficient to trigger the S pathway to phrenic motor facilitation (Fuller et al., 2001; Baker-Herman & Mitchell, 2002). Thus, with our standard moderate AIH protocol (35–55 mmHg PaO₂), the threshold of spinal tissue hypoxia and adenosine accumulation necessary to elicit adenosine-dependent pLTF is achieved through the combined effects of hypoxemia and hypotension following CBX.

Brainstem neural network vs spinal hypoxia in regulating phrenic motor facilitation

In intact rats, spinal tissue hypoxia is not required for moderate AIH-induced pLTF since electrical stimulation of the carotid sinus nerve (without hypoxia) elicits similar serotonin-dependent phrenic motor plasticity (Millhorn et al., 1980b, a). Carotid sinus nerve stimulation activates brainstem neural networks, including serotonergic raphe neurons that project to the spinal cord and release serotonin on/near phrenic motor neurons (Millhorn et al., 1980b; Holtman et al., 1984a; Holtman et al., 1984b; Holtman et al., 1986a; Holtman et al., 1986b; Morris et al., 1996; Kinkead et al., 1998; Kinkead et al., 2001). Spinal serotonin receptor activation triggers an intracellular signaling cascade known as the Q pathway, giving rise to pLTF (Baker-Herman et al., 2004; Nichols et al., 2012; Perim et al., 2018b, 2019; Perim & Mitchell, 2019). On the other hand, extracellular adenosine accumulation occurs during hypoxia, particularly with greater severity and/or duration of tissue hypoxia. With moderate AIH, adenosine accumulation is limited in intact rats, constraining, but not replacing moderate AIH-induced pLTF due to inhibitory cross-talk interactions. However, it is not sufficient to elicit adenosine-dependent phrenic motor facilitation in this case (Nichols et al., 2012).

With greater hypoxia-induced hypotension characteristic of CBX rats, spinal PtO₂ dropped to lower levels versus intact rats during our standard AIH protocol, despite matched levels of arterial PO₂ (Figure 3, panel B; Figure 4, panels C-F). For example, PtO₂ levels dropped below 5 mmHg during hypoxic episodes in CBX rats due to severe hypotension and impaired spinal blood flow. Blood flow auto-regulatory mechanisms are inefficient at arterial

pressures < 60 mmHg (MacKenzie et al., 1979); blood pressure often fell below this level after CBX during hypoxic episodes due to the combined loss of the pressor chemoreflex and baroreflexes. Pericytes can also regulate local blood flow, particularly after ischemic stroke (Rennels & Nelson, 1975; Peppiatt et al., 2006; Dalkara et al., 2011; Burdyga & Borysova, 2014; Hall et al., 2014). These contractile cells extend processes around capillaries and contract in response to severe tissue hypoxia (Attwell et al., 2016). Indeed, blood flow can be impaired, or even interrupted by pericyte-mediated capillary constriction, despite normal blood flow in upstream arterioles (O'Farrell et al., 2017). Thus, multiple factors could limit local blood flow and exaggerate spinal tissue hypoxia during the same moderate AIH protocol in CBX rats.

Extracellular adenosine accumulates during severe tissue hypoxia throughout the central nervous system, including the spinal cord (Takahashi et al., 2010; Yamashiro & Morita, 2017). At least two mechanisms of local adenosine accumulation have been described: 1) adenosine release by spinal astrocytes (Takahashi et al., 2010); and 2) extracellular ATP release and conversion to adenosine by ecto-enzymes (Van Wylen et al., 1986; Yamashiro & Morita, 2017). pLTF is expressed following both moderate (PaO_2 between 35–45 mmHg) and severe AIH (PaO_2 below 30 mmHg), although it arises from distinct mechanisms (serotonergic vs adenosinergic). pLTF is not expressed with moderate sustained hypoxia (25 minutes), at least in part due to opposing influences of competing adenosine- and serotonin-dependent mechanisms (Devinney et al., 2016). Similarly, pLTF is not expressed in a “window” of intermediate AIH (PaO_2 30–35 mmHg vs. 35–55 mmHg in moderate AIH and 25–30 in severe AIH; Perim & Mitchell, 2019), likely due to balanced, cross talk interactions. We suggest that spinal adenosine accumulation increases progressively from moderate to intermediate to severe AIH (see Figure supplement 2), accounting for the transition from adenosine constraint (moderate AIH; Hoffman et al., 2010), and adenosine cancellation (intermediate AIH; Perim & Mitchell, 2019) to adenosine dominance (severe AIH) (Nichols et al., 2012). Thus, spinal cord adenosine accumulation is a key regulator of AIH-induced phrenic motor plasticity, initially acting as a brake and then transitioning to dominance. Pharmacological activation of phrenic motor neuron $\text{A}_{2\text{A}}$ receptors in the absence of hypoxia is sufficient to elicit phrenic motor facilitation (Golder et al., 2008; Agosto-Marlin et al., 2017; Seven et al., 2018). Without carotid chemoreceptors, serotonergic inputs are reduced/eliminated, and spinal tissue hypoxia reaches levels sufficiently low to trigger adenosine-dependent pLTF, even with the same levels of arterial PO_2 achieved during our moderate AIH protocol.

Mechanisms of reduced spinal PtO_2 during moderate AIH in CBX rats were not directly investigated in this study. However, critically low arterial pressure levels were observed in CBX rats during moderate AIH, potentially leading to capillary collapse and greatly reduced tissue O_2 delivery. Sham and CBX-Phenyl rats exhibited less hypoxia-induced hypotension and, accordingly, spinal tissue hypoxia was less severe and residual pLTF was no longer observed.

Since CBX rats had the most severe spinal tissue hypoxia during moderate AIH, greater spinal adenosine concentrations would be achieved. Adenosine at high levels is expected both to elicit phrenic motor facilitation, and mitigate the decrease in blood flow and O_2

availability for > 1 hour post-AIH. Whether or not spinal adenosine accumulation is sufficient to cause a long-lasting increase in spinal PtO₂ observed following the AIH protocol requires further investigation.

Systemic phenylephrine activates smooth muscle cells via α_1 adrenergic receptors, evoking short-term vasoconstriction and increased arterial pressure. However, systemic phenylephrine does not elicit phrenic motor plasticity, consistent with the inability of phenylephrine to cross the blood brain barrier (Olesen, 1972). On the other hand, when delivered to the cervical intrathecal space, phenylephrine elicits phrenic motor facilitation *via* spinal α_1 receptors (Huxtable et al., 2014). Thus, although intravenous phenylephrine was an efficient way to minimize hypoxia-induced hypotension after carotid denervation, it was not expected to have independent effects on phrenic motor plasticity due to the route of administration.

Physiological and Translational Significance

AIH has been used in animal models and in humans to improve respiratory and nonrespiratory function in clinical conditions that compromise breathing, including cervical spinal cord injury and motor neuron disease (Dale et al., 2014; Hayes et al., 2014; Gonzalez-Rothi et al., 2015). Thus, our findings have important translational implications, suggesting that patients with systemic hypotension, as often observed after spinal cord injury, may have compromised spinal tissue oxygenation at any given arterial PO₂ level established during therapeutic AIH treatments (Karlsson, 2006; Phillips & Krassioukov, 2015). Under those conditions, milder hypoxic episodes may be appropriate to minimize spinal tissue hypoxia, optimizing the serotonin-dependent therapeutic benefits of repetitive AIH (Figure 7). For example, robust (and even enhanced) pLTF is observed following mild AIH (versus moderate) protocols (Mahamed & Mitchell, 2008) expected to minimize spinal tissue hypoxia, adenosine accumulation and cross-talk inhibition of dominant serotonin-dependent facilitation (Perim & Mitchell, 2019).

CONCLUSION

Although moderate AIH normally elicits pLTF via carotid body-dependent neural network activation, a distinct, local mechanism assumes dominance when the carotid bodies are no longer intact. CBX exacerbates systemic hypotension during moderate hypoxia, decreasing spinal oxygen levels during the same AIH protocol. Greater tissue hypoxia increases adenosine accumulation, eventually reaching levels sufficient for phrenic motor facilitation via spinal A_{2A} receptor activation. Based on our results, we suggest that case-specific adjustments in AIH protocols should be considered when experimental animals (or human subjects) have impaired blood pressure regulation or other forms of compromised oxygen delivery (e.g. anemia). Impaired arterial pressure regulation may affect the capacity and/or mechanism of respiratory motor plasticity elicited by the same AIH protocol. With hypotension, milder AIH protocols may be appropriate to optimize the therapeutic benefits of AIH by minimizing spinal tissue hypoxia and, consequently, adenosinergic constraints to respiratory motor plasticity (Hoffman et al., 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms:

pLTF	phrenic long-term facilitation
AIH	acute intermittent hypoxia
A_{2A}	adenosine 2A receptor
5-HT₂	serotonin 2 receptor
PaO₂	arterial oxygen pressure
PtO₂	spinal tissue oxygen pressure
DMSO	dimethyl sulfoxide
CBX	carotid-denervated

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Figure 1.

A pronounced hypoxia-induced hypotension was observed in carotid-denervated rats (CBX). A: representative traces of pulsatile arterial pressure from a Sham rat, a carotid-denervated rat (CBX), and a CBX rat treated with phenylephrine during AIH episodes (i.v.; CBX-Phenyl). Red line indicates mean arterial pressure (MAP, 1 second smooth time-constant) and grey dashed line indicates 50 mmHg. Recordings were briefly interrupted for blood sample withdrawal (arrows). B: MAP at the end of 1st AIH episode (the red * represent baseline MAP for respective group); and C: MAP drop from baseline to hypoxia. D:

schematic showing period when blood samples were taken during first hypoxia episode. Blood sample withdrawal time is indicated by horizontal bars, with corresponding PaO₂ for each rat represented on the left portion of the graph by an asterisk (highlighted in grey). This panel indicate that the time required to withdraw a blood sample during first AIH episode tended to be longer in CBX rats and that PaO₂ was similar among groups. E: hypoxic phrenic response (HPR) expressed as percent change in integrated phrenic burst amplitude relative to baseline, demonstrating effective carotid denervation. * and #, $p < 0.05$ vs. Sham and CBX-Phenyl rats, respectively. Data are means and 95% confidence intervals.

Figure 2.

A; Carotid-denervated rats (CBX) present more pronounced spinal tissue hypoxia despite the same level of PaO_2 . Greater tissue hypoxia was counteracted by systemic phenylephrine.

A: Illustration of the neurophysiological experiment with simultaneous recording of integrated phrenic nerve activity and PtO_2 near phrenic motor nucleus in vivo. Inset shows electrode location in the cervical spinal cord in one rat. B: Group average of spinal tissue oxygenation from a Sham rat, a carotid-denervated rat (CBX) and a CBX rat treated with phenylephrine during AIH (i.v.; CBX-Phenyl) during moderate AIH protocol. Dashed line

indicates 10 mmHg spinal PtO₂. Inset shows the spinal tissue oxygenation average traces from Sham (gray line), CBX (gray line) and CBX-Phenyl group (black line), which highlights the further drop in PtO₂ during first hypoxic episode in CBX relative to Sham and CBX-Phenyl groups. Red arrow indicates the nadir spinal PtO₂ during first hypoxia episode in CBX group. C: spinal PtO₂ at the end of 1st hypoxic episode. D: spinal PtO₂ average during hypoxia from all AIH episodes. E: spinal PtO₂ during baseline. F: spinal PtO₂ change from baseline to 90 minutes post-AIH. * and #, $p < 0.05$ vs. Sham and CBX-Phenyl rats, respectively. Data presented as means and 95% confidence intervals.

Figure 3.

Spinal PtO₂ slope during acute intermittent hypoxia (AIH) is frequently negative in carotid-denervated rats (CBX), suggesting ineffective tissue blood flow autoregulation. Transparent lines represent individual slopes from Sham (black), CBX (red) and CBX-Phenyl rats, while solid lines are corresponding group slope averages. Because spinal PtO₂ linearity took longer to achieve in some cases, only the last 3 hypoxia (Hx) minutes were considered for analysis.

Figure 4.

Preventing greater hypoxia-induced hypotension after carotid denervation abolishes AIH-induced residual phrenic long-term facilitation (pLTF). A: representative traces of compressed integrated phrenic activity from a Sham rat, a carotid-denervated rat (CBX) and a CBX rat treated with phenylephrine during AIH episodes (i.v.; CBX-Phenyl) during moderate AIH protocol. Dashed gray line indicates integrated phrenic nerve burst amplitude at baseline. B and C: integrated phrenic burst amplitude and respiratory frequency, respectively, at baseline, 30, 60 and 90 minutes post-AIH. D: change in raw phrenic burst

amplitude from baseline to 90 minutes post-AIH. E; change in respiratory frequency (RF) from baseline to 90 minutes post-AIH. #, $p < 0.05$ vs. CBX-Phenyl rats. Data presented as mean and standard error of the mean (panel B and C) or 95% confidence intervals (panels D and E).

Figure 5.

Spinal adenosine 2A (A_{2A}) receptor blockade blunts/abolishes AIH-induced residual phrenic long-term facilitation (pLTF). A: representative trace of compressed integrated phrenic activity from a carotid-denervated rat (CBX) that was treated with A_{2A} antagonist. Dashed gray line indicates integrated phrenic burst amplitude at baseline. B and C: integrated phrenic burst amplitude and respiratory frequency, respectively, at baseline, 30, 60 and 90 minutes post-AIH. D: change in raw phrenic burst amplitude from baseline to 90 minutes post-AIH. E: change in respiratory frequency (RF) from baseline to 90 minutes post-AIH. *,

$p < 0.05$ vs. CBX rats treated with A_{2A} antagonist. Data presented as mean and standard error of the mean (panel B and C) or 95% confidence intervals (panels D and E).

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Figure 6.

Intermittent increases in arterial pressure induced by systemic phenylephrine administration do not affect integrated phrenic burst output. A: representative traces of compressed integrated phrenic activity from a time control rat and a phenylephrine-treated rat (Phenyl; 3 × 5 minutes of 100 μM intravenous phenylephrine administration sufficient to increase arterial pressure by 20 mmHg) throughout experimental protocol. Dashed gray line indicates integrated phrenic burst amplitude at baseline. Inset shows pulsatile arterial pressure from a Phenyl-treated rat during episodic phenylephrine administration. Red line indicates mean

arterial pressure (MAP, 1 second smooth time-constant). B and C: integrated phrenic burst amplitude and respiratory frequency, respectively, at baseline, 30, 60 and 90 minutes post-AIH. D: change in raw phrenic burst amplitude from baseline to 90 minutes post-AIH. E: change in respiratory frequency (RF) from baseline to 90 minutes post-AIH. Data presented as mean and standard error of the mean (panel B and C) or 95% confidence intervals (panels D and E).

Figure 7.

Schematic representation depicting the mechanisms of residual pLTF in carotid-denervated rats (CBX). Modest arterial hypoxemia activates carotid body (CB) chemoreceptors, which increase brainstem respiratory neuron activity including raphe serotonergic neurons. Raphe neurons project to the phrenic motor nucleus where they release serotonin and elicit serotonin-dependent pLTF (green). Hypotension leads to more severe tissue hypoxia, resulting in adenosine (ADO) accumulation in the phrenic motor nucleus, initiating adenosine-dependent pLTF (red) that is preserved in the absence of carotid bodies.

Table 1.

Stability of blood gases and MAP throughout our standard moderate AIH protocol in Sham, carotid-denervated (CBX) and CBX treated with and CBX rats treated with phenylephrine during AIH episodes (i.v.; CBX-Phenyl).

VARIABLES	TIME, min	EXPERIMENTAL GROUPS		
PaCO ₂ , mmHg		Sham (n = 8)	CBX (n = 9)	CBX-Phenyl (n = 9)
	Baseline	43.0 ± 1.0	43.3 ± 1.0	44.0 ± 1.1
	Hx	42.4 ± 1.1	42.5 ± 1.1	44.8 ± 1.4
	30	43.6 ± 1.1	45.0 ± 0.9	45.2 ± 1.3
	60	43.8 ± 1.0	45.2 ± 0.9	45.0 ± 0.9
	90	42.4 ± 1.0	43.9 ± 1.0	43.7 ± 1.3
PaO ₂ , mmHg				
	Baseline	316 ± 8	313 ± 9	316 ± 6
	Hx	45 ± 2	48 ± 2	42 ± 1
	30	254 ± 20	267 ± 22	180 ± 24
	60	382 ± 12	264 ± 17	214 ± 19
	90	272 ± 14	278 ± 10	230 ± 19
sBE, mmol/L				
	Baseline	1.7 ± 0.6	1.1 ± 1.3	2.5 ± 0.6
	Hx	-0.4 ± 0.9	-0.6 ± 1.4	-1.1 ± 1.4
	30	1.3 ± 0.6	0.2 ± 1.5	0.4 ± 0.9
	60	0.7 ± 0.6	0.2 ± 1.7	2.5 ± 1.1
	90	-0.5 ± 0.17	-0.8 ± 1.5	0.4 ± 1.0
pH				
	Baseline	7.40 ± 0.01	7.39 ± 0.02	7.40 ± 0.01
	Hx	7.37 ± 0.01	7.37 ± 0.02	7.34 ± 0.02
	30	7.39 ± 0.01	7.36 ± 0.03	7.37 ± 0.02
	60	7.38 ± 0.01	7.36 ± 0.03	7.39 ± 0.02
	90	7.37 ± 0.00	7.36 ± 0.03	7.38 ± 0.02
MAP, mmHg				
	Baseline	121 ± 6	110 ± 9	139 ± 6
	Hx	101 ± 10	52 ± 6	121 ± 13
	30	120 ± 3	111 ± 8	144 ± 6
	60	115 ± 4	107 ± 9	123 ± 6
	90	109 ± 7	98 ± 9	106 ± 8

Table 2.

Stability of blood gases and MAP throughout our standard moderate AIH protocol in time control and Phenylephrine-treated rats.

VARIABLES	TIME, min	EXPERIMENTAL GROUPS	
PaCO ₂ , mmHg		Time-control (n = 5)	Phenyl (n = 6)
	Baseline	40.8 ± 0.7	44.6 ± 1.4
	30	41.3 ± 1.0	44.3 ± 1.2
	60	41.0 ± 0.9	44.9 ± 1.4
	90	41.9 ± 0.7	45.1 ± 1.5
PaO ₂ , mmHg			
	Baseline	307 ± 10	293 ± 16
	30	304 ± 7	281 ± 14
	60	300 ± 5	278 ± 15
	90	307 ± 5	290 ± 11
sBE, mmol/L			
	Baseline	0.5 ± 0.6	1.7 ± 0.6
	30	1.2 ± 1.0	0.5 ± 0.5
	60	0.7 ± 0.1	1.5 ± 0.3
	90	2.0 ± 0.5	1.6 ± 0.4
pH			
	Baseline	7.40 ± 0.01	7.39 ± 0.01
	30	7.41 ± 0.01	7.37 ± 0.01
	60	7.40 ± 0.01	7.38 ± 0.01
	90	7.41 ± 0.01	7.38 ± 0.01
MAP, mmHg			
	Baseline	130 ± 6	139 ± 8
	30	132 ± 10	140 ± 10
	60	135 ± 10	139 ± 10
	90	139 ± 10	136 ± 10

Table 3.

Stability of blood gases and MAP throughout our standard moderate AIH protocol in rats treated with either adenosine 2A (A_{2A}) or serotonin 2 receptor (5-HT₂) antagonists.

VARIABLES	TIME, min	EXPERIMENTAL GROUPS	
PaCO ₂ , mmHg		A _{2A} ant. (n = 4)	5-HT ₂ ant. (n = 2)
	Baseline	42.4 ± 1.7	44.3 ± 3.2
	Hx	41.0 ± 2.0	43.9 ± 2.3
	30	42.5 ± 3.0	43.1 ± 1.5
	60	42.5 ± 1.7	45.1 ± 4.0
	90	41.8 ± 1.9	43.5 ± 2.9
PaO ₂ , mmHg			
	Baseline	274 ± 46	312 ± 3
	Hx	42 ± 2	35 ± 0
	30	262 ± 15	195 ± 25
	60	280 ± 15	253 ± 4
	90	282 ± 15	249 ± 15
sBE, mmol/L			
	Baseline	2.1 ± 0.7	3.6 ± 0.2
	Hx	0.6 ± 0.4	2.5 ± 3.5
	30	1.1 ± 0.4	1.2 ± 1.9
	60	2.5 ± 0.9	1.4 ± 2.3
	90	1.5 ± 0.7	1.5 ± 1.7
pH			
	Baseline	7.41 ± 0.02	7.41 ± 0.03
	Hx	7.40 ± 0.02	7.40 ± 0.06
	30	7.40 ± 0.02	7.39 ± 0.04
	60	7.41 ± 0.02	7.38 ± 0.05
	90	7.41 ± 0.02	7.39 ± 0.05
MAP, mmHg			
	Baseline	113 ± 12	109 ± 13
	Hx	38 ± 5	38 ± 13
	30	107 ± 12	102 ± 17
	60	105 ± 7	100 ± 18
	90	105 ± 5	92 ± 21