Influence of vagal afferents on supraspinal and spinal respiratory activity following cervical spinal cord injury in rats

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Influence of vagal afferents on supraspinal and spinal respiratory activity following cervical spinal cord injury in rats. J Appl Physiol 109: 377–387, 2010. First published May 27, 2010; doi:10.1152/japplphysiol.01429.2009.—C2 spinal hemisection (C2HS) interrupts ipsilateral bulbospinal pathways and induces compensatory increases in contralateral spinal and possibly supraspinal respiratory output. Our first purpose was to test the hypothesis that after C2HS contralateral respiratory motor outputs become resistant to vagal inhibitory inputs associated with lung inflation. Bilateral phrenic and contralateral hypoglossal (XII) neurograms were recorded in anesthetized and ventilated rats. In uninjured (control) rats, lung inflation induced by positive end-expired pressure (PEEP; 3–9 cmH2O) robustly inhibited both phrenic and XII bursting. At 2 wk after C2HS, PEEP evoked a complex response associated with phrenic bursts of both reduced and augmented amplitude, but with no overall change in the mean burst amplitude. PEEP-induced inhibition of XII bursting was still present but was attenuated relative to controls. However, by 8 wk post-C2HS PEEP-induced inhibition of both phrenic and XII output were similar to that in controls. Our second purpose was to test the hypothesis that vagal afferents inhibit ipsilateral phrenic bursting, thereby limiting the incidence of the spontaneous crossed phrenic phenomenon in vagal-intact rats. Bilateral vagotomy greatly enhanced ipsilateral phrenic bursting, which was either weak or absent in vagal-intact rats at both 2 and 8 wk post-C2HS. We conclude that 1) compensatory increases in contralateral phrenic and XII output after C2HS blunt the inhibitory influence of vagal afferents during lung inflation and 2) vagal afferents robustly inhibit ipsilateral phrenic bursting. These vagotomy data appear to explain the variability in the literature regarding the onset of the spontaneous crossed phrenic phenomenon in spontaneously breathing (vagal intact) vs. ventilated (vagotomized) preparations.

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Inspiratory bursting in the ipsilateral phrenic neurogram and/or diaphragm electromyogram (EMG) can be induced after C2HS via physiological stimulation (e.g., hypoxia) as well as various pharmacological treatments (16, 19, 27, 57). The removal of spinal sensory inputs via cervical dorsal rhizotomy can also reveal or enhance ipsilateral phrenic output after C2HS (15, 24). This induced recovery of ipsilateral phrenic output has been termed the crossed phrenic phenomenon (23, 44). In addition to the induced crossed phrenic phenomenon, a gradual and spontaneous return of ipsilateral diaphragm EMG activity can be observed over weeks to months after injury (41, 43). The relative degree of phrenic motoneuron activation associated with the spontaneous appearance of the crossed phrenic phenomenon is minimal and thus does not approximate “normal” phrenic output (14, 32).

Lung inflation is associated with activation of slowly adapting receptors (SARs), which are located primarily in the intrapulmonary airways (48). In spinal-intact animals, lung inflation and SAR activation via positive end-expired pressure (PEEP) inhibit phrenic inspiratory motor output (37). However, it is not known whether chronic cervical spinal cord injury can alter these vagally mediated respiratory reflexes. For example, the compensatory increase in contralateral phrenic motor output after C2HS (41, 45) may attenuate or “over ride” the normal vagally mediated inhibition that occurs during lung inflation. Changes in lung volume and/or compliance (49) after cervical spinal cord injury also have the potential to alter vagal reflexes. Since compensatory respiratory control mechanisms following C2HS appear to occur at both supraspinal (20, 58) and spinal (20, 42, 45) levels, our first purpose was to test the hypothesis that contralateral phrenic and hypoglossal (XII) motor output become more resistant to vagal inhibitory inputs associated with lung inflation following chronic C2HS.

The postinjury time interval required for the spontaneous crossed phrenic phenomenon to become evident is highly variable across published reports (see Refs. 41, 47 for commentary). Most spontaneously breathing rats do not have inspiratory ipsilateral diaphragm EMG activity during euapneic breathing as late as 4 wk post-C2HS (1, 41, 43). On the other hand, under normoxic, normocapnic conditions [i.e., arterial partial pressure of O2 (PaO2) ~100 mmHg, arterial partial pressure of CO2 (PaCO2) ~35–40 mmHg], ipsilateral phrenic inspiratory bursting is readily apparent in >60% of mechanically ventilated, vagotomized rats by 14 days after injury (11, 14, 16, 47). By 1 mo post-C2HS, close to 100% of vagotomized, ventilated rats show ipsilateral phrenic inspiratory bursting (11, 14, 16, 18). A common finding is that the incidence of the spontaneous crossed phrenic phenomenon and the magnitude of ipsilateral bursting can be increased by raising the overall level of respiratory drive (10, 11, 14, 16, 18). Therefore, processes that actively inhibit ipsilateral phrenic motoneurons during spontaneous breathing may “mask” the recovery of ipsilateral phrenic bursting. There is one report from 1948 in which the potential vagal influence on the crossed phrenic phenomenon was investigated by observing diaphragm movements in rabbits following acute C2–C3 hemisection (7). It was noted that section of the vagus nerves evoked movement of the previously paralyzed hemidiaphragm.
(7). Accordingly, we reasoned that the discrepancy in the literature regarding the onset of the spontaneous crossed phrenic phenomenon in rats with chronic C2HS could be explained by the integrity of the vagus nerves. Our second purpose was to test the hypothesis that vagal afferents attenuate expression of ipsilateral phrenic bursting after chronic C2HS, and therefore acute vagotomy can reveal or enhance ipsilateral phrenic motor output.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats obtained from Harlan (Indianapolis, IN) were divided into the following groups: control (uninjured): n = 7, 373 ± 28 g, age 123 ± 18 days; sham operated (laminaectomy only): n = 2, 425 and 440 g, age of both animals 144 days; 2 wk post-C2HS: n = 9, 321 ± 22 g, age 107 ± 5 days; and 8 wk post-C2HS: n = 7, 380 ± 39 g, age 145 ± 7 days (data are expressed as means ± SD). There were no significant differences in respiratory or cardiovascular responses between the uninjured animals and those that received sham surgery. The data from these animals were therefore combined as a single control group. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Spinal Cord Injury

Rats were injured at 90 ± 1 days (mean ± SE) of age by previously described methods (10, 11, 14, 47). Briefly, the animals were anesthetized by injection of xylazine (10 mg/kg sq) and ketamine (140 mg/kg ip; Fort Dodge Animal Health). A dorsal cervical incision was made from the base of the skull to the C3 segment, followed by C2 laminectomy and left C2 dural section. A left C2HS was performed by microscalpel incision and aspiration. The dura and overlying muscles were sutured with 9-0 sutures (Ethicon) and 4-0 polyglycolic acid sutures (Webster Veterinary), respectively. The skin was subsequently closed with stainless steel wound clips (Stoelting), and the animal received an injection of yohimbine (1.2 mg/kg sq; Lloyd) to reverse the effect of xylazine. After surgery, animals were given an analgesic (butapironephine, 0.03 mg/kg sq; Hospira, Lake Forest, IL) and sterile lactated Ringer solution (5 ml sq). The postsurgical care protocol included injection of lactated Ringer solution (5 ml/day sq) and oral supply of Nutri-cal supplements (1–3 ml, Webster Veterinary), which were given until adequate volitional drinking and eating resumed.

Neurophysiology Preparation

Many aspects of these procedures have been published previously (10, 11, 14, 47). Isoflurane anesthesia (3–4%) was initially induced in a closed chamber and then maintained via nose cone (2–3%). After tracheotomy rats were mechanically ventilated (model 683, Harvard Apparatus, South Natick, MA) with an oxygen-nitrogen mixture [inspired O2 fraction (FiO2) = 0.5–0.6; volume = 7 ml/kg; frequency = 60–65/min] throughout the experiment. The femoral vein was catheterized (PE-50) to permit conversion to urethane anesthesia (1.6 g/kg iv; Sigma, St. Louis, MO) and injection of a paralytic drug (pancuronium bromide, 2.5 mg/kg iv; Hospira). Another catheter was inserted into the femoral artery and connected to a pressure transducer (Statham P-10EZ pressure transducer, CP122 AC/DC strain gauge amplifier, Grass Instruments, West Warwick, RI) for pressure measurements. The tracheal pressure was monitored by a pressure transducer (Statham P-10EZ pressure transducer, CP122 AC/DC strain gauge amplifier, Grass Instruments) connected to the inspired line of the ventilator. The partial pressure of end-tidal CO2 (PtCO2) was monitored with a Capnogard neonatal CO2 monitor placed on the expiratory line of the ventilator circuit (Novametrix Medical Systems, Wallingford, CT) (10, 11, 47) and was maintained at 50 mmHg by adjusting inspired CO2. Rectal temperature was monitored by an electrical thermometer and maintained at 37.5 ± 1°C by a servo-controlled heating pad (model TC-1000, CWE, Ardmore, PA). The phrenic nerves were isolated in the cervical region via a ventral approach and sectioned distally. The right XII nerve was exposed and cut peripherally after removal of the diaphragm muscle (34, 36, 37). Nerve activity was recorded with monopolar or bipolar hook silver electrodes and then amplified (1,000×, model 1700, A-M Systems, Carlsborg, WA), band-pass filtered (0.3–10 kHz), and integrated (time constant 100 ms; model MA-1000, CWE). The raw and integrated neural signals were digitized by the CED Power 1401 data acquisition interface and recorded on a PC with Spike2 software (Cambridge Electronic Design, Cambridge, UK). The sampling frequency of all signals was 100 Hz, with the exception of the raw neurograms, which were sampled at 10 kHz.

Experimental Protocols

Two separate protocols were performed in each animal. In the first protocol, lung inflation was induced by changing the PEEP under vagal-intact status. The purpose of this protocol was to examine whether phrenic and/or XII efferent bursting during lung inflation were altered after C2HS. Baseline was established with PEEP at 0 cmH2O. After stable nerve recordings were obtained, different levels of PEEP (3, 6, and 9 cmH2O) were randomly applied once by inserting the outlet tubing of the ventilator into a graduated cylinder of water. Each condition was maintained for 10 s, and then PEEP was returned to 0 cmH2O until nerve activity returned to baseline values. In the second protocol, rats were bilaterally vagotomized in the midcervical region after a prolonged (5 min) period of baseline recordings. This protocol was designed to test whether ipsilateral phrenic inspiratory bursting would be revealed after removal of vagal afferent inputs. During the baseline period, PtCO2 was maintained at 50 mmHg and no PEEP was applied. Different levels of PEEP (3, 6, and 9 cmH2O) were subsequently applied again to confirm that the effects of PEEP in the first protocol were mediated by the vagus nerves.

Spinal Cord Histology

Our procedures have been published along with detailed histological examples of the C2HS lesion (33, 47). At the conclusion of the neurophysiology experiments, rats, which were already deeply anesthetized with urethane (see above), were euthanized by systemic saline perfusion followed by 4% paraformaldehyde (Sigma). The cervical spinal cord was removed, cryoprotected, and sectioned at 40 μm via vibrotome. The spinal cord tissue sections were serially mounted on glass slides (Fisher Scientific, Pittsburgh, PA), stained with cresyl violet, and then evaluated by light microscopy. Two rats were removed from the analysis after their hemilesions were assessed to be incomplete.

Data Analyses

Neurograms, blood pressure, and tracheal pressure were analyzed with Spike 2 software. The integrated phrenic neurogram (∫Phr) was used to calculate inspiratory (Ti) and expiratory (Te) duration as previously described (34, 38). Briefly, the Ti was defined as the period between inspiratory phrenic burst onset and the time when ∫Phr was greater than 110% of the baseline value. The characteristics of these bursts did not match the previously described (34, 38). Briefly, the Ti was defined as the period between inspiratory phrenic burst onset and the time when ∫Phr amplitude declined by 50% of the peak value. The overall respiratory frequency was calculated as 60/(Ti + Te). Phrenic and XII inspiratory activity were defined as the peak height of the integrated neurograms. Although phrenic motor output was generally inhibited during application of PEEP, the peak ∫Phr signal was also sporadically augmented during PEEP (e.g., Figs. 1 and 2). These augmented discharge patterns were identified when the peak ∫Phr was greater than 110% of the baseline value. The characteristics of these bursts did not match previously published descriptions of “augmented breaths” in rats (14, 17). Augmented breaths are characterized by a distinct two-phase pattern. The first phase is similar to the preceding breath pattern.
second phase is characterized by a steeper rate of rise in the inspiratory flow trace or diaphragm EMG pattern (8). The augmented discharges observed during PEEP in our study (Fig. 2) did not show the characteristic two-phase pattern, but rather consisted of a single phase in which the rate of rise in the peak \( \text{Phr} \) signal was greater than the previous breath.

In the first protocol, data were averaged over 10 s at each level of PEEP. In the second protocol, data were averaged over 30 s before and after bilateral cervical vagotomy. The peak amplitude of the \( \text{Phr} \) and \( \text{XII} \) neurograms was expressed relative to the maximum PEEP response (% max) in the first protocol. In the second protocol, changes in efferent bursting following vagotomy were represented as a percentage of peak response (% peak) after vagotomy. A two-way repeated measurement analysis of variance (ANOVA) and Student-Newman-Keuls post hoc test (Sigma Stat 2.03, Jandel Scientific, St. Louis, MO) were used to compare differences across conditions (e.g., control vs. injured group at different levels of PEEP; ipsilateral vs. contralateral phrenic amplitude before and after vagotomy). All data are presented as means \( \pm \) SE. A \( P \) value \( < 0.05 \) was considered statistically significant for all analyses.

**RESULTS**

**Effects of PEEP in Vagal-Intact Rats**

Phrenic and XII output. Representative examples depicting phrenic and XII activity during different levels of PEEP in control and C2HS rats are shown in Fig. 1. Both phrenic and...
XII activity were reduced by increasing PEEP in control animals. In all C2HS rats, both the contralateral phrenic and XII nerves showed clear rhythmic inspiratory activity during baseline conditions (0 cmH₂O PEEP) with intact vagus nerves (see bursting before PEEP application, Fig. 1). However, inspiratory bursting in the ipsilateral phrenic nerve during baseline was either very weak or absent in vagal-intact C2HS rats at both 2 and 8 wk after injury. There was an indication of a time-dependent recovery of spontaneous ipsilateral phrenic activity in vagal-intact rats (43). Specifically, ipsilateral phrenic bursting was observed in 33% (3/9) of rats at 2 wk post-C2HS and 71% (5/7) of rats at 8 wk post-C2HS. Of note, the 2 wk data are remarkably similar to those in a recent report showing a 30% incidence of ipsilateral diaphragm EMG activity in spontaneously breathing and unanesthetized rats at 2 wk post-C2HS (41).

PEEP evoked two distinct responses in the contralateral peak fPhr neurogram (or the right fPhr signal of control rats): 1) suppression of phasic inspiratory bursts and 2) augmented discharges associated with enhanced fPhr amplitude (141 ± 4% baseline) and reduced Ti (75 ± 4% baseline; P < 0.01, t-test). In both control and 8 wk post-C2HS rats, the appearance of augmented phrenic discharges was relatively rare (Table 1). Accordingly, in these groups increasing PEEP caused a reduction of the contralateral peak fPhr burst (injured rats) or bilateral fPhr bursts (controls) (P < 0.01, Figs. 1 and 3A). In contrast, the augmented phrenic discharges occurred more frequently in 2 wk post-C2HS animals (Table 1), and therefore the contralateral peak fPhr amplitude was maintained as PEEP was increased in these rats (P > 0.05, Fig. 3A). When augmented discharges were excluded from the analyses, the inhibitory effects of PEEP on contralateral activity were still attenuated in 2 wk post-C2HS animals (data not shown). Interestingly, the augmented discharges observed in the contralateral phrenic nerve were usually associated with activation of crossed phrenic pathways. Thus ipsilateral phrenic bursting could be observed during augmented contralateral phrenic discharges evoked by PEEP (Fig. 2). The onset of augmented discharges in the ipsilateral phrenic nerve always occurred later than for contralateral augmented discharges in C2HS animals (Fig. 2). Specifically, the onset difference between ipsilateral and contralateral augmented phrenic discharges was 78 ± 10 ms and 50 ± 36 ms at 2 and 8 wk post-C2HS, respectively.

The responses of the contralateral XII neurogram during PEEP were similar to what was observed in the contralateral phrenic neurogram. The extent of XII inhibition during PEEP was indistinguishable between control and 8 wk postinjury rats (P > 0.05, Fig. 3B), whereas the inhibitory effects of PEEP on XII activity were attenuated 2 wk post-C2HS (P < 0.01, Fig. 3B). Occasionally, XII motor output demonstrated a similarly augmented burst coinciding with the augmented phrenic discharges evoked by PEEP (Fig. 2A). On average, the increase in peak fXII discharge (121 ± 11%) associated with augmented bursting was lower than observed in the fPhr output (141 ± 4% baseline; P < 0.01, t-test on ranks). The augmented XII response was observed in 18 of 36 cases of augmented phrenic discharges.

Table 1. Augmented discharges in phrenic nerve during PEEP application

<table>
<thead>
<tr>
<th></th>
<th>3 cmH₂O</th>
<th>6 cmH₂O</th>
<th>9 cmH₂O</th>
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<tbody>
<tr>
<td>2 wk</td>
<td>Animals</td>
<td>44%</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td>Breaths</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>8 wk</td>
<td>Animals</td>
<td>29%</td>
<td>29%</td>
</tr>
<tr>
<td></td>
<td>Breaths</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>33%</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>Animals</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

The percentage of rats showing augmented discharges in each experimental group (e.g., 2 wk, 8 wk, or control) at each level of positive end-expired pressure (PEEP) is shown. In addition, the total number of augmented discharges at each level of PEEP (breaths) is shown.
recorded in all animals. In the remaining cases, the XII burst was either unchanged or reduced during augmented phrenic discharges (Fig. 2B). Thus the increased respiratory drive associated with augmented phrenic discharges was not always transmitted to inspiratory upper airway motor output.

After bilateral vagotomy, PEEP no longer induced changes in phrenic or XII bursting in control or C2HS rats (data not shown). Accordingly, the inhibitory effects of PEEP on inspiratory motor output were mediated by vagal afferent neurons.

Respiratory cycle and frequency. Increasing PEEP caused a gradual decrease in TI in all groups (P < 0.01, Fig. 4A). On the other hand, TE was significantly increased when PEEP was set at 6–9 cmH2O (P < 0.01, Fig. 4B), and this resulted in a reduction in respiratory frequency (P < 0.01, Fig. 4C). Interestingly, PEEP-induced changes in respiratory cycle duration and frequency were significantly less in the 2 wk post-C2HS animals compared with control and 8 wk post-C2HS animals (P < 0.05, Fig. 4).

Tracheal pressure. As PEEP increased from 0 to 9 cmH2O, the peak tracheal pressure associated with lung inflation increased as expected (P < 0.01, Table 2). There were no significant differences in tracheal pressure between C2HS and control rats (P > 0.01, Table 2). This finding indicates that the degree of lung inflation induced by PEEP was similar in all groups.

Mean arterial blood pressure and heart rate. Similar to prior reports (37), mean arterial blood pressure declined when PEEP was elevated to 6 and 9 cmH2O in control animals (P < 0.05, Table 3). Application of PEEP caused a slight reduction in mean arterial pressure in control and 2 wk post-C2HS rats. The mean arterial pressure tended to be lower in 8 wk post-C2HS rats at baseline (P = 0.059 vs. control), and PEEP did not

Fig. 3. Effects of PEEP on contralateral phrenic (A) and XII (B) burst amplitude. PEEP of 6–9 cmH2O reduced peak contralateral ∫Phr in both control and 8 wk post-C2HS animals. In contrast, this response was not observed at 2 wk after injury. PEEP reduced peak ∫XII in all groups; however, the effects of PEEP were attenuated at 2 wk after injury. **Different from baseline condition of PEEP = 0 cmH2O (P < 0.01); #P < 0.05, ##P < 0.01, significant difference between the 2 wk postinjury group and both the 8 wk postinjury and control groups.

Fig. 4. Effects of PEEP on inspiratory (TI, A) and expiratory (TE, B) duration and respiratory frequency (C) in control and C2HS rats. Elevation of PEEP induced a typical lung inflation reflex resulting in reduction of TI, elongation of TE, and a decline in respiratory frequency (bursts/min) in all groups. However, the extent of inhibition during PEEP was attenuated in the 2 wk post-C2HS animals. *P < 0.05, **P < 0.01 vs. baseline value; #P < 0.05, ##P < 0.01 significant difference between the 2 wk postinjury group and both the 8 wk postinjury and control groups.
cause a further reduction in mean arterial pressure in these rats (Table 3). With one exception, heart rate (HR) was unchanged during PEEP: the 8 wk postinjury group showed a small but significant decrease in HR during 9 cmH2O PEEP (P < 0.05, Table 3).

Phrenic and XII Activity Following Bilateral Vagotomy

Vagotomy induced similar increases in peak bilateral \( \Delta \text{Phr} \) in the control group (P > 0.05, Figs. 5 and 6). In contrast, the increase in peak ipsilateral \( \Delta \text{Phr} \) amplitude was greater than the increase in contralateral amplitude in C2HS animals at both 2 and 8 wk after injury (P < 0.01, Figs. 5 and 6). Additionally, the number of C2HS rats showing spontaneous ipsilateral phrenic activity increased after vagotomy. Specifically, after vagotomy rhythmic ipsilateral phrenic activity occurred in 78% (7/9) of rats at 2 wk post-C2HS and in all rats at 8 wk post-C2HS. Moreover, evidence of time-dependent (i.e., postinjury) recovery of ipsilateral bursting was more robust after vagotomy (Fig. 7). After vagotomy, peak ipsilateral \( \Delta \text{Phr} \) burst amplitude was greater at 8 wk vs. 2 wk post-C2HS when data were expressed either as arbitrary units (P < 0.05) or relative to the contralateral peak \( \Delta \text{Phr} \) amplitude (% contralateral, P < 0.01) (Fig. 7). Although rhythmic bursting in the ipsilateral phrenic nerve was enhanced after vagotomy, the onset of the ipsilateral phrenic burst was delayed with respect to the contralateral phrenic nerve by 82 ± 11 ms and 72 ± 8 ms at 2 wk and 8 wk post-C2HS, respectively. Vagotomy caused a robust increase in XII activity in control rats as previously reported (38). The increase in contralateral peak \( \Delta \text{XII} \) amplitude following vagotomy was similar in control and C2HS rats (Fig. 6).

Respiratory frequency (min\(^{-1}\)) was similar in vagal-intact control and C2HS rats because of the entrainment effects of the mechanical ventilator; however, some differences in timing were noted between groups (Table 4). Most notably, TI was significantly less 2 wk after injury compared with control rats. After vagotomy, respiratory frequency was significantly reduced in control and 2 wk post-C2HS rats. TI was significantly lower in 2 wk post-C2HS versus control rats (P < 0.01, Table 4).

### DISCUSSION

This study provides the first comprehensive analyses of the impact of vagal afferents on phrenic and XII motor output following chronic cervical spinal cord injury. These data demonstrate that the PEEP-induced inhibition of contralateral phrenic and XII burst amplitude and timing normally observed in neurologically intact animals is attenuated at 2 wk post-C2HS. However, this attenuated inhibition is no longer evident at 8 wk post-C2HS, indicating that vagally mediated lung inflation reflexes are altered only transiently after unilateral high cervical spinal cord injury. Additionally, these data indicate that ipsilateral phrenic burst amplitude becomes considerably more robust after bilateral vagotomy at both 2 and 8 wk post-C2HS. In contrast, rhythmic inspiratory activity in the ipsilateral phrenic nerve was weak or absent entirely in vagal-intact animals at both time points. Thus vagal afferents have persistent inhibitory effects on ipsilateral phrenic motor output after C2HS. We suggest that vagally mediated inhibition of phrenic output contributes to the delayed appearance of the crossed phrenic phenomenon in spontaneously breathing, vagal-intact animals compared with ventilated and vagotomized animals.

### Critique of Methods

Our data indicate that the functional impact of activating vagal afferent neurons during PEEP is transiently changed after C2HS. This interpretation, however, is subject to caveats. Lung volume was manipulated by applying PEEP to the expiratory line of the mechanical ventilator (9, 13, 35, 37, 46), and it is possible that the relative degree of lung inflation differed between experimental groups. For example, decreased lung compliance has been reported after cervical spinal cord injury in humans (reviewed in Ref. 49), and altered compliance may influence the extent of lung inflation during PEEP. However, the tracheal pressure during PEEP application was similar between C2HS and control animals (Table 2), suggesting similar lung compliance between groups. Additionally, animals in the present study were paralyzed, and therefore end-expiratory lung volume could not change due to recruitment of expiratory muscles during PEEP application. Accordingly, we suggest that the changes in PEEP-induced reflexes following C2HS do not reflect differential lung inflation compared with control animals.

Application of PEEP will activate different populations of pulmonary vagal receptors including SARs, rapidly adapting receptors (RARs), and bronchial C-fiber receptors (40, 56). Consequently, the vagally mediated reflexes evoked in this study cannot be specifically attributed to activation of a single type of vagal afferent neuron. Another prospect not addressed in this study is that the sensitivity of pulmonary vagal receptors may change after chronic spinal cord injury. We are unaware of any data directly addressing this possibility, and this will be an interesting topic for future studies. Finally, the \( \text{PETCO}_2 \) in the present study (50 mmHg) was maintained ~10 mmHg above baseline.

### Table 2. Effects of PEEP on tracheal pressure

<table>
<thead>
<tr>
<th>PEEP</th>
<th>0 cmH2O</th>
<th>3 cmH2O</th>
<th>6 cmH2O</th>
<th>9 cmH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk</td>
<td>11 ± 1</td>
<td>12 ± 1</td>
<td>15 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>8 wk</td>
<td>10 ± 1</td>
<td>12 ± 1</td>
<td>15 ± 1</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Control</td>
<td>10 ± 1</td>
<td>11 ± 1</td>
<td>14 ± 1</td>
<td>19 ± 1</td>
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</table>

Tracheal pressure values (in cmH2O) are means ± SE.

### Table 3. Effect of PEEP on mean arterial pressure and heart rate

<table>
<thead>
<tr>
<th>PEEP</th>
<th>0 cmH2O</th>
<th>3 cmH2O</th>
<th>6 cmH2O</th>
<th>9 cmH2O</th>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 wk</td>
<td>135 ± 5</td>
<td>133 ± 4</td>
<td>128 ± 4*</td>
<td>126 ± 4*</td>
</tr>
<tr>
<td>8 wk</td>
<td>116 ± 7</td>
<td>115 ± 7</td>
<td>112 ± 7</td>
<td>112 ± 7</td>
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<tr>
<td>Control</td>
<td>135 ± 6</td>
<td>132 ± 5</td>
<td>129 ± 5*</td>
<td>125 ± 5*</td>
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<td>HR, beats/min</td>
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<tr>
<td>2 wk</td>
<td>456 ± 9</td>
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<tr>
<td>8 wk</td>
<td>437 ± 8</td>
<td>436 ± 8</td>
<td>433 ± 8</td>
<td>430 ± 11†</td>
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<td>445 ± 9</td>
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<td>443 ± 8</td>
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</table>

Values are means ± SE. MAP, mean arterial pressure; HR, heart rate. *P < 0.05; †P < 0.01 compared with values during baseline (PEEP = 0 cmH2O).
the CO₂ recruitment threshold for inspiratory phrenic activity as reported in prior studies (10, 11, 14, 16, 19). We felt it was important to maintain \( P_{ETCO₂} \) well above the recruitment threshold to ensure robust contralateral bursting, and to confirm that the weak ipsilateral phrenic bursting in vagal intact rats was not due to low CO₂ values. The potential interaction between arterial CO₂ values and vagal afferents in modulating phrenic output after C2HS represents another interesting topic for future studies.

**Influence of PEEP on Contralateral Phrenic and XII Activity**

Both phrenic and XII responses indicated that the classic Hering-Breuer inflation reflex was evoked by PEEP in control rats as previously reported (37). In contrast, PEEP-induced phrenic and XII reflexes were transiently attenuated after C2HS. The attenuated inhibition of respiratory motor output evident at 2 wk after injury appears to reflect the sum of two potentially independent processes. Specifically, PEEP evoked a decrease in the amplitude of the typical “eupneic” bursts but also induced the occurrence of augmented phrenic bursts. As a result, the overall contralateral phrenic burst amplitude was not reduced during PEEP. The augmented phrenic bursts during PEEP do not appear to be identical to the previously described “augmented breaths” that occur during spontaneous breathing (8, 17). First, the pattern of the augmented discharges observed in the contralateral phrenic nerve did not match the two-phase pattern of the typical augmented breath (8). Second, two prior studies have demonstrated that while augmented breaths occur...
more frequently after C2HS, this effect is not evident until ~3 mo after injury (14, 17). The occurrence of the augmented discharges may counteract the inhibitory impact of PEEP on “eupneic activity,” thereby enabling C2HS animals to maintain ventilation during activation of inhibitory vagal inputs.

Several mechanisms could underlie the altered contralateral phrenic output during lung inflation after C2HS. We initially hypothesized that compensatory increases in contralateral respiratory output would blunt vagally mediated inhibition. However, if that were the case, we would have expected the attenuated inhibitory effects of PEEP to persist at 8 wk after injury because ipsilateral phrenic motor output remained very low in vagal-intact rats (i.e., compensatory contralateral output was presumably still required). The augmented phrenic discharges during PEEP were also observed in ipsilateral output. Thus the mechanisms driving this response are not unique to contralateral compensation per se, and this response likely reflects plasticity within the brain stem neurons and/or networks activated by vagal afferent neurons. Pulmonary vagal afferents terminate primarily in the nucleus tractus solitarii (NTS), which integrates peripheral sensory inputs and initiates reflexes to modulate ventilation (3, 4, 31). The rapid shallow breathing pattern induced by C2HS (21) will likely be associated with persistent changes in vagal inputs to the NTS, and this in turn may trigger changes in neural connectivity within brain stem respiratory neural circuits. Prior work indicates that both short-term and long-term plasticity in NTS neurons can be induced under experimental or pathophysiological conditions (4, 29).

Ipsilateral Phrenic Motor Output and Vagotomy

A time-dependent, spontaneous recovery of ipsilateral phrenic activity occurred over 2–8 wk post-C2HS (e.g., Fig. 7). This observation is consistent with the results of many previous investigations (1, 10, 11, 14, 16, 19, 20, 41, 42). Evaluation of these prior studies indicates that the appearance of ipsilateral diaphragm EMG activity in spontaneously breathing, vagal-intact rats after C2HS usually occurs later than the return of ipsilateral phrenic bursting recorded in ventilated, vagotomized rats (14, 16, 43, 54). For example, Nantwi et al. (43) and Vinit et al. (54) reported that inspiratory EMG activity of the ipsilateral diaphragm was not observed until 1–3 mo post-C2HS. In contrast, inspiratory bursting in the ipsilateral phrenic nerve of vagotomized, ventilated rats occurs as early as 2 wk post-C2HS (14, 16). We observed robust increases in ipsilateral phrenic bursting after vagotomy at both 2 and 8 wk post-C2HS. In light of these results, we suggest that the status of the vagus nerves contributes to differences in the time course of ipsilateral phrenic versus diaphragm recovery across prior studies.

The increase in ipsilateral phrenic bursting after vagotomy most likely reflects a decrease in vagally mediated inhibitory inputs to brain stem respiratory centers with a resultant increase in depolarizing inputs to ipsilateral phrenic motoneurons/premotor neurons. Indeed it is well established that the overall level of “respiratory drive” can increase crossed phrenic activity (16, 19, 27, 58). Since tracheal pressure was held constant before and after vagotomy, it is unlikely that changes in lung mechanics contributed to the changes in phrenic bursting. Interestingly, a previous report showed that recovery of ipsilateral phrenic bursting was accelerated in C2HS rats with chronic carotid body excision (2). Accordingly, both mechanical and/or chemical-sensitive vagal afferent inputs may inhibit ipsilateral phrenic motor output following

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**Fig. 6. Average changes in peak integrated phrenic and XII burst amplitude following bilateral vagotomy.** Peak contralateral (Phr) and XII burst amplitudes showed similar increases after vagotomy in control and C2HS rats. However, vagotomy induced a relatively greater increase in the ipsilateral Phr burst amplitude after C2HS. In addition, the increase in the ipsilateral Phr burst amplitude after vagotomy was greater at 8 compared with 2 wk post-C2HS. *P* < 0.05 vs. contralateral phrenic amplitude; **#P** < 0.01 vs. control group; *#P* < 0.05, significant difference between 2 and 8 wk post-C2HS groups.

**Fig. 7. Mean ipsilateral phrenic inspiratory burst amplitude at 2 and 8 wk post-C2HS.** Peak ipsilateral [Phr] burst amplitude was quantified as an absolute voltage [i.e., arbitrary units (a.u.), A] and as % of the burst amplitude in the contralateral phrenic nerve (% CL, B). Both analyses suggest a progressive increase in ipsilateral phrenic burst amplitude over 2–8 wk after C2HS. Differences between 2 and 8 wk post-C2HS rats were statistically significant only after vagotomy. *P* < 0.05, **#P** < 0.01 vagal-intact vs. vagotomized; **##P** < 0.01, significant difference between 2 and 8 wk post-C2HS groups.
Table 4. Effects of vagotomy on respiratory cycle and frequency

<table>
<thead>
<tr>
<th>Vagal-intact</th>
<th>Vagotomized</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ti, s</strong></td>
<td><strong>Ti, s</strong></td>
</tr>
<tr>
<td>2 wk</td>
<td>0.29 ± 0.01†</td>
</tr>
<tr>
<td>8 wk</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>Control</td>
<td>0.34 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. Ti, inspiratory duration; Te, expiratory duration. *P < 0.01 compared with values under vagal-intact condition; †P < 0.05, ‡P < 0.01 compared with control group.

C2HS and partially mask the onset of the crossed phrenic phenomenon.

There are a few caveats related to our suggestion that vagal inhibition at least partially explains the discrepancy in the onset of the spontaneous crossed phrenic phenomenon among prior studies. First, ipsilateral phrenic neurograms may not necessarily predict EMG activity in the ipsilateral diaphragm. However, since both are strongly influenced by phrenic motoneuron activity, it is reasonable to speculate that a strong relationship will exist between these two variables. Second, differences in arterial blood gases across published reports make it difficult to compare the results directly. While a wide range of arterial CO2 values has been reported for spontaneously breathing, anesthetized, spinal-intact rats [~33–49 mmHg (21, 25, 28)], there is considerably less information about blood gases after chronic C2HS in unventilated animals. Golder et al. (21) found that anesthetized, spontaneously breathing rats studied at 2 mo post-C2HS are slightly hypoxic (PaO2 ~ 82 mmHg) with PaCO2 values of ~49 mmHg. These blood gases would seem to favor crossed phrenic activity compared with values typically maintained in ventilated preparations [e.g., PaO2 > 100 mmHg, PaCO2 = 35–40 mmHg (10, 11, 14, 16, 19)]. In any case, here we document that when CO2 is held constant at a level associated with robust contralateral phrenic activity, activation of vagal afferents will dramatically inhibit the crossed phrenic phenomenon.

**Ipsilateral vs. Contralateral Phrenic Motor Output**

The vagotomy-induced enhancement of burst amplitude was considerably greater for the ipsilateral phrenic nerve compared with the contralateral nerve. This result could reflect the minimal output of the ipsilateral phrenic nerve before vagotomy. Assuming a similar change in motoneuron discharge frequencies, an increase from 5 to 15 active ipsilateral phrenic motoneurons (200% increase) following vagotomy may manifest as a much larger response compared with an increase from 75 to 100 contralateral phrenic motoneurons (33% increase). Another possibility is that the intrinsic cellular properties of ipsilateral motoneurons are altered after injury. For example, ipsilateral phrenic motoneuron soma size decreases after C2HS (41), and changes in synaptic morphology have also been described (51). Ipsilateral phrenic motoneurons may therefore be more excitable after chronic C2HS (23, 41), and this adaptation might affect their responses to vagotomy. It also appears that ipsilateral and contralateral phrenic motoneurons have different bursting patterns after C2HS. Studies in animals indicate that inspiratory phrenic activity is generated by cells that burst at the onset of inspiration [early-inspiratory (early-I)] and during the middle of the inspiration [late-inspiratory (late-I)] (30, 38, 52). A previous study demonstrated that most phrenic motoneurons burst with a late-I pattern immediately following C2HS (12). Similarly, we have observed that the majority of active ipsilateral phrenic motoneurons discharge as the late-I type following chronic C2HS (K.-Z. Lee and D. D. Fuller, unpublished results). At least one prior report (26) indicates that late-I phrenic motoneuron discharge is more strongly modulated by lung inflation compared with early-I bursting. Hence, the enhancement of ipsilateral versus contralateral phrenic motor output following vagotomy may reflect a shift in the distribution of ipsilateral phrenic motoneurons toward the late-I phenotype. This alteration of phrenic motoneuron distribution may also explain why bursting in the ipsilateral phrenic nerve after C2HS is usually initiated slightly after the inspiratory burst in the contralateral phrenic nerve (Fig. 5; Ref. 14). Finally, the responses of ipsilateral versus contralateral phrenic motoneurons to vagotomy may reflect a difference in descending inputs to these cells. Phrenic premotor neurons are primarily located in the rostral ventral respiratory group (rVRG) and the Bötzinger complex. These brain stem regions project primarily excitatory (rVRG) and inhibitory (Bötzinger) inputs to phrenic motoneurons (50, 53). However, in contrast to the bilateral nature of rVRG projections, Bötzinger complex neurons primarily innervate the ipsilateral phrenic nucleus (5). Hence, ipsilateral phrenic motoneurons may receive fewer descending inhibitory inputs after C2HS and may therefore be more easily excited after vagotomy.

**Functional Implications**

There are several implications of our data for respiratory control following high cervical spinal cord injury. First, we suggest that the blunted inhibition of phrenic burst frequency during PEEP and the increase in the number of augmented phrenic discharges are both compensatory responses that enable ventilation to be maintained in the face of inhibitory inputs in the initial days and weeks following injury. Second, these results confirm that ipsilateral phrenic motor output is relatively weak or even absent in vagal-intact rats over 2–8 wk post-C2HS (1, 43). These results are consistent with the notion that ipsilateral phrenic bursting does not make a substantial contribution to tidal volume during “eupneic” breathing after C2HS (14, 18). Third, both the PEEP and vagotomy data support the idea that magnitude of crossed phrenic activity correlates with the overall intensity of central respiratory drive (16, 39) and indicate that vagal afferents have a particularly strong inhibitory influence on ipsilateral phrenic motor output. The potential influence of vagal afferents on assessment of functional respiratory outcomes should be considered in studies of rehabilitation or other interventions after C2HS (see, e.g., Refs. 16, 19, 55). Finally, persons with cervical spinal cord injury experiencing respiratory failure often require mechani-
cal ventilator support involving relatively large tidal volumes and PEEP (6). These ventilator settings help to maintain alveolar ventilation, but they may limit activation of spinal (phrenic) motoneurons and any associated activity-dependent plasticity that could otherwise facilitate phrenic motor recovery.

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