Plasticity in Respiratory Motor Control
Selected Contribution: Intermittent hypoxia induces phrenic long-term facilitation in carotid-denervated rats

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Bavis, Ryan W., and Gordon S. Mitchell. Selected Contribution: Intermittent hypoxia induces phrenic long-term facilitation in carotid-denervated rats. J Appl Physiol 94: 399–409, 2003. First published July 12, 2002; 10.1152/japplphysiol.00374.2002.—Episodic hypoxia elicits a long-lasting augmentation of phrenic inspiratory activity known as long-term facilitation (LTF). We investigated the respective contributions of carotid chemoafferent neuron activation and hypoxia to the expression of LTF in urethane-anesthetized, vagotomized, paralyzed, and ventilated Sprague-Dawley rats. One hour after three 5-min isocapnic hypoxic episodes [arterial PO2 (PaO2) = 40 ± 5 Torr], integrated phrenic burst amplitude was greater than baseline in both carotid-denervated (n = 8) and sham-operated (n = 7) rats (P < 0.05), indicating LTF. LTF was reduced in carotid-denervated rats relative to sham (P < 0.05). In this and previous studies, rats were ventilated with hyperoxic gas mixtures (inspired oxygen fraction = 0.5) under baseline conditions. To determine whether episodic hypoxia induces LTF, phrenic activity was recorded under normoxic (PaO2 = 90–100 Torr) conditions before and after three 5-min episodes of isocapnic hypoxia (PaO2 = 40 ± 5 Torr; n = 6) or hyperoxia (PaO2 > 470 Torr; n = 6). Phrenic burst amplitude was greater than baseline 1 h after episodic hypoxia (P < 0.05), but episodic hypoxia had no detectable effect. These data suggest that hypoxia per se initiates LTF independently from carotid chemoafferent neuron activation, perhaps through direct central nervous system effects.

EPISTODIC HYPOXIA OR ELECTRICAL stimulation of carotid chemoafferent neurons elicits a long-lasting augmentation of respiratory motor output known as long-term facilitation (LTF; 44, 54). When repeated carotid sinus nerve (CSN) stimulation is used to elicit LTF in cats (40, 41) and rats (23, 33), thus bypassing chemotransduction in the carotid body, central neural mechanisms must be involved. LTF is also observed after episodic hypoxia in anesthetized rats (23; additional references in Ref. 44), sleeping humans (2), and awake dogs (10), goats (60), ducks (43), rats (52), and mice (31); the pattern of hypoxia is critical because episodic hypoxia elicits LTF whereas a single bout of continuous hypoxia does not (5, 6, 14, 52, 60). It has been proposed that hypoxia stimulates carotid body chemoreceptors and that the associated intermittent chemoafferent neuron activation elicits LTF by central neural mechanisms without the need for hypoxia per se (3). Although this model is intuitively appealing because it links LTF induced by intermittent hypoxia and electrical CSN stimulation, there is no clear evidence that the same neural mechanisms underlie both forms of LTF.

LTF induced by episodic hypoxia and electrical CSN stimulation both require serotonin receptor activation (3, 7, 18, 28, 39, 41). Moreover, in anesthetized rats, LTF in phrenic nerve motor output after episodic hypoxia requires serotonin receptor activation and protein synthesis in the cervical spinal cord (7), confirming that hypoxia-induced LTF results from central nervous system (CNS) mechanisms (44, 53). On the other hand, there are indications that LTF involves at least two distinct mechanisms, one associated with carotid chemoafferent neuron activation and the other involving direct effects of hypoxia on the CNS. For example, LTF expression in anesthetized rats differs qualitatively after CSN stimulation and episodic hypoxia (44). CSN stimulation elicits decrementing phrenic LTF of relatively short duration (<1 h; 23, 33). In contrast, hypoxia-induced LTF starts small immediately after hypoxia but increases progressively over the next hour (3, 44). These contrasting manifestations of LTF could...
arise from differences in chemoafferent activation by electrical vs. hypoxic stimulation or from differences in the balance of inhibitory and facilitatory mechanisms that they evoke (6, 29). Alternatively, hypoxia-induced LTF may arise from a fundamentally different mechanism, perhaps through direct effects of hypoxia on CNS neurons. Consistent with this hypothesis, a form of LTF has been reported after brief hypoxic exposures in peripherally chemodenervated, anesthetized cats (20). Thus hypoxia may contribute to LTF by mechanisms that do not involve carotid chemoafferent neuron activation.

There is also some question as to the relative roles of hypoxia and hyperoxia in the initiation of LTF by episodic hypoxia. In previous studies on hypoxia-induced LTF in anesthetized rats (e.g., 19, 23) and cats (20), animals were ventilated with hyperoxic gas mixtures [inspired oxygen fraction (FiO2) = 0.5–1.0] before and after hypoxia, ostensibly to improve preparation stability. Consequently, each hypoxic episode ended with a rapid oxygen increase to hyperoxic levels. The repeated oxygen rise after the hypoxic bouts may alter cell signaling and gene expression, perhaps through repeated oxygen rise after the hypoxic bouts may alter cell signaling and gene expression, perhaps through repeated application.

In this study, we investigated the role of hypoxia in phrenic LTF in anesthetized rats. In one set of experiments, we tested the hypothesis that episodic hypoxia, and not episodic hyperoxia, is a proximate stimulus for LTF. Specifically, we compared the effects of episodic hypoxia vs. hyperoxia in otherwise normoxic rats. After confirming that episodic hypoxia was the relevant stimulus, we studied carotid-denervated (CSNX) rats to test the hypothesis that episodic hypoxia elicits LTF independently from carotid chemoafferent neuron activation.

METHODS

Experiments were conducted on 3- to 5-mo-old male Sprague-Dawley rats (311–414 g; Charles River Laboratories, Wilmington, MA; rat colony K-62, Kingston, NY). The general experimental preparation and protocol have been described previously (3, 19). All experimental procedures were approved by the Animal Care and Use Committee of the School of Veterinary Medicine at the University of Wisconsin-Madison.

Experimental Preparation

Anesthesia was induced with isoflurane and maintained (2.5% isoflurane, FIO2 = 0.2 or 0.5, balance N2) first through a nose cone and then via a tracheal cannula placed to allow pump ventilation (Rodent Respirator, model 683, Harvard Apparatus, Holliston, MA). A catheter was placed in the femoral vein, and the rats were slowly converted to urethane anesthesia (1.6 g/kg iv). Adequacy of anesthesia was monitored throughout the experiment by testing blood pressure responses to toe pinch; supplemental urethane was given if a response was observed. A catheter was placed in the femoral artery to monitor blood pressure (P23ID pressure transducer and P122 amplifier, Gould) and to withdraw 0.2-ml blood samples to determine blood gases and pH with a blood analysis system (ABL-500, Radiometer, Copenhagen, Denmark). Arterial blood values were corrected to rectal temperature, which was maintained at 37–38°C with a heated table. Animals received a continuous intravenous infusion (2.5 ml/h) of a 1:1 (vol/vol) solution of sodium bicarbonate (5%) and lactated Ringer solution to maintain fluid and acid-base balance, beginning shortly after urethane administration. Animals were bilaterally vagotomized in the midcervical region and paralyzed with pancuronium bromide (3.25–3.5 mg/kg) to prevent spontaneous breathing movements and entrainment of neurograms with the ventilator. End-tidal carbon dioxide partial pressures (PETCO2) were measured with a rapidly responding flow-through carbon dioxide analyzer (Capnogard, Novametrix, Wallingford, CT) placed in the expired line of the ventilator circuit.

In this study, we compared the effects of episodic hypoxia vs. hyperoxia in otherwise normoxic rats. After baseline phrenic activity was established (30 min), an arterial blood sample (0.2–0.3 ml saline) was drawn; subsequent blood samples were compared with this baseline value.

Series 1: episodic hypoxia vs. episodic hyperoxia, normoxic background. Baseline phrenic nerve activity was standardized among preparations by maintaining PETCO2 3 Torr above the PETCO2 at which activity resumed. After baseline phrenic activity was established (30 min), an arterial blood sample (0.2–0.3 ml saline) was drawn; subsequent blood samples were compared with this baseline value.

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threshold]. Rats were then exposed to 1) three 5-min bouts of hypoxia (episodic hypoxia; \(P_{\text{O}_2} = 40 \pm 5\) Torr; \(n = 6\), 2) three 5-min bouts of hyperoxia (episodic hyperoxia; \(P_{\text{O}_2} \sim 1.0, P_{\text{O}_2} > 470\) Torr; \(n = 6\), or 3) maintained baseline conditions (time control; \(n = 6\); \(P_{\text{ACO}_2}\) was maintained within 2 Torr of baseline during hypoxic and hyperoxic episodes. Blood samples were collected during the final 30 s of the first treatment bout, and, if blood gases were not within the acceptable range, \(P_{\text{O}_2}\) and/or inspired carbon dioxide fraction were adjusted during the next bout. Rats were returned to normoxia for 5 min between bouts of hypoxia or hyperoxia. After the last bout of hypoxia or hyperoxia, or the equivalent period of normoxia, phrenic nerve activity was monitored for 1 h under baseline conditions. Blood samples were collected at 15, 30, and 60 min to ensure that blood gases remained isocapnic (\(P_{\text{ACO}_2}\) within 1 Torr of baseline). At the conclusion of experiments, rats were killed via urethane overdose.

**Series 2: episodic hypoxia in CSNX rats.** Baseline phrenic nerve activity was established under hyperoxic and normoxic conditions (\(F_{\text{IO}_2} = 0.5, P_{\text{O}_2} > 190\) Torr; \(P_{\text{ACO}_2} \sim 3\) Torr above apneic threshold). CSNX or sham-operated rats were then exposed to three 5-min bouts of hypoxia (episodic hypoxia; \(P_{\text{O}_2} = 40 \pm 5\) Torr; \(n = 8\) CSN, 7 sham) or maintained under baseline conditions (time control; \(n = 8\) CSN, 8 sham; \(P_{\text{ACO}_2}\) was maintained within 2 Torr of baseline during hypoxic episodes. Blood samples were collected during the final 30 s of the first hypoxic bout, and, if blood gases were not in the acceptable range, \(P_{\text{O}_2}\) and/or inspired carbon dioxide fraction were adjusted during the next bout. Rats were returned to hyperoxia for 5 min between bouts of hypoxia. After the last bout of hypoxia or the equivalent period of hyperoxia, phrenic nerve activity was monitored for 1 h under baseline conditions. Blood samples were collected at 15, 30, and 60 min to ensure that blood gases remained isocapnic (\(P_{\text{ACO}_2}\) within 1.5 Torr of baseline). At the conclusion of experiments, rats were killed via urethane overdose.

**Data Analysis**

Phrenic activity was averaged in 30-s bins (immediately preceding blood sampling) under baseline conditions; during the fifth minute of hypoxia, hyperoxia, or normoxia; and 15, 30, and 60 min after episodic treatment. Variables measured included peak amplitude of integrated phrenic activity, phrenic burst frequency, and their product, the minute phrenic activity. Changes from baseline in burst amplitude and minute activity were normalized as a percentage of baseline values (% baseline).

Apneic thresholds were compared among treatment groups by use of one-way ANOVA. Acute hypoxic and hyperoxic responses and time-dependent changes in phrenic activity, blood gases, and blood pressure were compared among treatment groups by use of two-way repeated-measures ANOVA followed by Student-Newman-Keuls post hoc tests. In the series 2 experiments, acute hypoxic responses were reanalyzed by using one-sample \(t\)-tests to improve statistical power. Linear regression was used to investigate the potential influence of arterial blood pressure on the expression of LTF. Effects were considered statistically significant at \(P \leq 0.05\). Unless otherwise noted, statistical tests were conducted using SigmaStat 2.03, SPSS, Chicago, IL. All data are presented as means ± SE.

**RESULTS**

**Series 1: Episodic Hypoxia Vs. Episodic Hyperoxia**

**Blood gases and mean arterial blood pressure.** Baseline \(P_{\text{O}_2}\), \(P_{\text{ACO}_2}\), and mean arterial pressure (MAP) were similar among the three treatment groups used in these experiments, and rats remained isocapnic (\(P_{\text{ACO}_2}\) within 1 Torr of baseline) throughout the protocol (Table 1). Acute hypoxic challenges (mean \(P_{\text{O}_2} = 40\) Torr) decreased MAP (\(\Delta = -49 \pm 5\) mmHg; \(P < 0.001\)), but MAP returned to baseline in normoxia and did not change significantly throughout the remainder of the protocol in the episodic hypoxia rats. Increasing \(F_{\text{IO}_2}\) to nearly 1.0 (mean \(P_{\text{O}_2} > 495\) Torr) caused a small but statistically significant increase in MAP (\(\Delta = 16 \pm 3\) mmHg; \(P = 0.011\)) that disappeared on return to normoxia. \(P_{\text{O}_2}\), \(P_{\text{ACO}_2}\) and MAP were stable throughout the protocol in rats maintained under normoxic conditions.

**Responses to acute hypoxia and hyperoxia.** Acute hypoxia elicited a brisk increase in phrenic activity.

**Table 1. \(P_{\text{O}_2}\), \(P_{\text{ACO}_2}\), and mean arterial pressure during baseline conditions, during isocapnic treatment (normoxic time control, hypoxia, or hyperoxia), and 15, 30, and 60 min after episodic treatment (series 1)**

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<th>Baseline</th>
<th>Treatment</th>
<th>After Episodic Treatment</th>
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<tr>
<td></td>
<td>(P_{\text{O}_2})</td>
<td>15 min</td>
<td>30 min</td>
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<tr>
<td>(P_{\text{O}_2}), Torr</td>
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<tr>
<td>Normoxia</td>
<td>96 ± 2</td>
<td>96 ± 1</td>
<td>95 ± 1</td>
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<tr>
<td>Hypoxia</td>
<td>94 ± 2</td>
<td>40 ± 1\†</td>
<td>84 ± 3\†</td>
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<tr>
<td>Hyperoxia</td>
<td>97 ± 2</td>
<td>495 ± 11\†</td>
<td>96 ± 2</td>
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<tr>
<td>(P_{\text{ACO}_2}), Torr</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normoxia</td>
<td>44.8 ± 1.1</td>
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<tr>
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<tr>
<td>Hyperoxia</td>
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<td>Mean arterial pressure, mmHg</td>
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<tr>
<td>Hypoxia</td>
<td>101 ± 10</td>
<td>52 ± 6\†</td>
<td>106 ± 5</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>111 ± 4</td>
<td>127 ± 5\†</td>
<td>107 ± 6</td>
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</tbody>
</table>

Values are means ± SE for 6 rats per group. \(P_{\text{O}_2}\), arterial \(P_{\text{O}_2}\); \(P_{\text{ACO}_2}\), arterial \(P_{\text{ACO}_2}\). *P < 0.05 vs. baseline; †P < 0.05 vs. normoxic time control group at same time point.
Peak burst amplitude increased significantly (87 ± 11% baseline; \( P < 0.001 \)) with no change in burst frequency (\( \Delta = 1 \pm 3 \text{ bursts/min}; P = 0.769 \)) during the fifth minute of hypoxia; consequently, minute phrenic activity also increased significantly during hypoxia (93 ± 22% baseline; \( P = 0.008 \)). Acute hyperoxia caused no change in burst amplitude (8 ± 5% baseline; \( P = 0.154 \)) or minute phrenic activity (0 ± 7% baseline; \( P = 0.980 \)), although there was a small decrease in burst frequency (\( \Delta = -4 \pm 1 \text{ bursts/min}; P = 0.048 \)).

**LTF of phrenic activity.** Episodic hypoxia elicited significant LTF in the peak amplitude of integrated phrenic bursts and in phrenic minute activity but not in burst frequency (Fig. 1). LTF was evident as an increase in phrenic amplitude by 30 min posthypoxia (30 ± 9% increase from baseline; \( P = 0.001 \)) that remained elevated 60 min posthypoxia (44 ± 10%; \( P < 0.001 \)). Burst frequency was somewhat reduced 15 min posthypoxia (\( \Delta = -4 \pm 2 \text{ bursts/min}; P = 0.008 \)) but returned to baseline by 30 min posthypoxia. As a result, minute phrenic activity was elevated above baseline at the 30 (32 ± 12%; \( P = 0.005 \)) and 60 (47 ± 11%; \( P < 0.001 \))-min time points. The progressive facilitation of phrenic amplitude and minute activity between 15 and 30 min posthypoxia was statistically significant (\( P = 0.006 \) and 0.002, respectively). Although phrenic activity continued increasing between 30 and 60 min posthypoxia, the changes in amplitude and minute activity were not statistically significant during this time (\( P = 0.088 \) and 0.117, respectively). There was no relationship between the magnitude of LTF and the blood pressure (MAP and/or \( \Delta \text{ MAP} \)) at baseline, during hypoxia, or 60 min posthypoxia (all \( P > 0.05 \)).

There were no significant effects of episodic hyperoxia on phrenic burst amplitude, burst frequency, or minute phrenic activity during the 60-min period after the final episode (Fig. 1; all \( P > 0.05 \)). Similarly, there were no time-dependent changes in phrenic burst amplitude, burst frequency, or minute phrenic activity in rats maintained in normoxia throughout the protocol (i.e., time controls; Fig. 1; all \( P > 0.05 \)), confirming the stability of the preparation.

**Series 2: Episodic Hypoxia in CSNX rats**

**Apneic threshold, blood gases, and MAP.** Acute CSN section produced a marginally significant increase in carbon dioxide apneic threshold \( (\overline{P}_{\text{ETCO}_2} = 42 \pm 1 \text{ vs. } 40 \pm 1 \text{ Torr in CSNX (\( n = 16 \)) and sham-operated (\( n = 15 \)) rats, respectively; \( P = 0.051 \), but baseline \( \overline{P}_{\text{ACO}_2} \) did not differ between groups (45.1 ± 0.7 vs. 44.2 ± 0.7; \( P = 0.345 \)). Consequently, baseline \( \overline{P}_{\text{ACO}_2} \) as well as baseline \( \overline{P}_{\text{AO}_2} \) and MAP, were similar among the four treatment groups (Table 2). Rats remained isocapnic (average \( \overline{P}_{\text{ACO}_2} \) within 1 Torr of baseline) throughout the protocol (Table 2).

Levels of hypoxia were similar in CSNX and sham-operated rats (\( \overline{P}_{\text{AO}_2} = 40 \pm 1 \text{ vs. } 38 \pm 1 \), respectively; \( P = 0.886 \)) and both groups decreased MAP during hypoxia (\( P < 0.001 \) vs. baseline in both groups). However, hypoxic hypotension was significantly greater in CSNX rats (\( \Delta \text{ MAP} = -72 \pm 6 \text{ vs. } -32 \pm 6 \text{ mmHg in sham-operated rats; } P < 0.001 \)); MAP returned to baseline after episodic hypoxia in both groups. Although MAP tended to fall (5–15 mmHg) over the course of the protocol in all four treatment groups, this drop reached statistical significance only in CSNX rats 60 min after episodic hypoxia (\( \Delta \text{ MAP} = -14 \pm 5 \); \( P = 0.003 \) vs. baseline). \( \overline{P}_{\text{AO}_2} \), \( \overline{P}_{\text{ACO}_2} \), and MAP were stable in CSNX and sham-operated rats maintained under hyperoxic conditions throughout the protocol.

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Phrenic responses to acute hypoxia in CSNX and sham-operated rats. Acute CSN section had no effect on peak amplitude of integrated phrenic bursts or phrenic burst frequency under baseline, hypoxic conditions [for CSNX (n = 16) vs. sham-operated (n = 15) rats: amplitude 2.4 ± 0.3 vs. 2.4 ± 0.2 volts, P = 0.651; frequency 47 ± 1 vs. 46 ± 2 bursts/min, P = 0.624]. Acute hypoxia (P_{O_2} ~ 40 Torr) elicited a brisk increase in minute phrenic activity in sham-operated rats, but this response was greatly reduced in CSNX rats (Figs. 2 and 3; P < 0.001). In the fifth minute of hypoxia, sham-operated rats exhibited significantly elevated minute phrenic activity (128 ± 24% increase from baseline; P < 0.001), resulting from an increase in burst amplitude (111 ± 24% increase from baseline; P < 0.001) without change in burst frequency (Δ = 3 ± 2 bursts/min; P = 0.353). Despite apparent increases in phrenic amplitude and minute activity during acute hypoxia, changes in phrenic amplitude, burst frequency, and minute activity were not statistically significant for CSNX rats when analyzed with sham-operated rats by two-way repeated-measures ANOVA (Δ = 15 ± 4% baseline, 1 ± 1 bursts/min, and 18 ± 7% baseline, respectively; all P > 0.05). This lack of significance is likely the result of unequal variance between groups; indeed, when hypoxic phrenic responses are considered for CSNX rats alone (one sample t-test; SPSS 10.1, SPSS), the small increases in phrenic amplitude and minute activity reach statistical significance (both P < 0.05). Thus CSNX rats typically experienced some residual increase in phrenic activity during hypoxia, although the increase was greatly reduced and considerably delayed (20–40 s) relative to sham-operated rats (Fig. 2).

**LTF of phrenic activity.** Phrenic activity did not change over the course of the experiment in either sham-operated or CSNX rats that were not exposed to episodic hypoxia (i.e., time controls; Fig. 4; all P > 0.05 vs. baseline).

Episodic hypoxia elicited LTF of phrenic nerve activity in sham-operated rats (Fig. 4). LTF was expressed as a progressive increase in phrenic burst amplitude after episodic hypoxia such that phrenic burst amplitude was significantly greater than baseline at 30 (27 ± 12%; P = 0.002) and 60 min (55 ± 10%, range 24–104%; P < 0.001) posthypoxia. Burst frequency was somewhat reduced at 15 min posthypoxia (~4 ± 2 bursts/min; P = 0.003 vs. baseline) but returned to baseline for the remainder of the experiment (P > 0.05). Consequently, minute phrenic activity was significantly greater than baseline at 30 (28 ± 14%; P = 0.003) and 60 min (60 ± 8%; P < 0.001) posthypoxia. Increases in phrenic amplitude and minute activity were significantly greater at 60 min posthypoxia than at 30 min and greater at 30 min than at 15 min (all P < 0.05). Moreover, the time-dependent changes in phrenic activity discussed above (vs. baseline) were significant compared with sham-operated rats that were not exposed to episodic hypoxia (Fig. 4; all P < 0.05). An eighth sham-operated rat was originally studied with this group; however, this rat responded to episodic hypoxia with a long-lasting depression of phrenic activity (34 and 38% decrease from baseline in phrenic amplitude and minute activity, respectively). Using the same protocol, our laboratory has never observed a long-lasting respiratory depression (decrease from baseline > 15%) in comparable rats after episodic hypoxia (n > 100 healthy, male rats); for example, in a recent meta-analysis from our laboratory (19), only 2 of 63 rats exhibited phrenic amplitudes less than baseline at 60 min (~1 and ~13%). Consequently, this anomalous rat was excluded from the analyses.

### Table 2. \(P_{O_2}, P_{CO_2},\) and mean arterial pressure during baseline conditions, during isocapnic hypoxia, and 15, 30, and 60 min after episodic hypoxia, or time-matched hyperoxic controls, in carotid body-denervated and sham-operated rats (series 2)

<table>
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<th>Baseline</th>
<th>Treatment</th>
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<tr>
<td></td>
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<td>30 min</td>
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<tr>
<td>(P_{O_2}, ) Torr</td>
<td></td>
<td></td>
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<tr>
<td>Sham control</td>
<td>229 ± 10</td>
<td>245 ± 9</td>
<td>220 ± 12</td>
</tr>
<tr>
<td>Sham + hypoxia</td>
<td>219 ± 8</td>
<td>38 ± 1(\dag)</td>
<td>191 ± 13(\dag)</td>
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<tr>
<td>CSNX control</td>
<td>220 ± 7</td>
<td>243 ± 4(\dag)</td>
<td>222 ± 9</td>
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<tr>
<td>CSNX + hypoxia</td>
<td>228 ± 9</td>
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<tr>
<td>(P_{CO_2}, ) Torr</td>
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<tr>
<td>Sham control</td>
<td>44.8 ± 1.3</td>
<td>44.7 ± 1.4</td>
<td>44.6 ± 1.1</td>
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<td>Sham + hypoxia</td>
<td>43.4 ± 0.6</td>
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<td>CSNX control</td>
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<td>CSNX + hypoxia</td>
<td>45.5 ± 1.1</td>
<td>45.2 ± 1.0</td>
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<td>Mean arterial pressure, mmHg</td>
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<tr>
<td>Sham control</td>
<td>118 ± 6</td>
<td>118 ± 7</td>
<td>113 ± 7</td>
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<td>Sham + hypoxia</td>
<td>132 ± 7</td>
<td>99 ± 9(\dag)</td>
<td>131 ± 6</td>
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<td>CSNX control</td>
<td>124 ± 5</td>
<td>125 ± 5</td>
<td>119 ± 5</td>
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<td>CSNX + hypoxia</td>
<td>128 ± 9</td>
<td>56 ± 5(\dag)</td>
<td>126 ± 8</td>
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Values are means ± SE for 8 rats per group, 7 rats per group for sham + hypoxia. CSNX, carotid denervated. \(\dag P < 0.05\) vs. baseline; \(\dag\) \(P < 0.05\) vs. corresponding hyperoxic control group (i.e., same surgery) at same time point; \(\dag\) \(P < 0.05\) vs. corresponding sham-operated group (i.e., same treatment, hypoxia or control) at same time point.
Although including this rat lowers the mean value for LTF at 60 min posthypoxia (to 44 ± 14 and 47 ± 14% increase from baseline in phrenic amplitude and minute activity, respectively), LTF in both phrenic amplitude and minute activity would remain statistically significant in sham-operated rats (both \( P < 0.001 \)).

Episodic hypoxia also elicited LTF in CSNX rats, although the expression of LTF differed somewhat from sham-operated rats (Fig. 4). As in sham-operated rats, the magnitude of integrated phrenic burst amplitude increased after episodic hypoxia in CSNX rats and was significantly greater than baseline at 30 (26 ± 6%; \( P = 0.002 \)) and 60 min (27 ± 7%, range 1–50%; \( P = 0.002 \)) posthypoxia; these increases in phrenic amplitude were also significant compared with CSNX rats that were not exposed to episodic hypoxia (\( P = 0.002 \) and \( P < 0.001 \) at 30 and 60 min, respectively). However, rather than having reduced burst frequencies 15 min posthypoxia, CSNX rats exhibited a small (3 ± 1 bursts/min), but statistically significant, increase in burst frequency relative to baseline at all posthypoxia time points (all \( P < 0.05 \)). Compared with CSNX rats that were not exposed to episodic hypoxia, this frequency LTF was significant at 30 min posthypoxia (\( P = 0.018 \)) and marginally significant at 60 min (\( P = 0.054 \)). As a result of increases in phrenic amplitude and burst frequency, minute phrenic activity was significantly greater than baseline at 15 (20 ± 10%; \( P = 0.021 \)), 30 (37 ± 8%; \( P < 0.001 \)), and 60 min (38 ± 9%; \( P < 0.001 \)) posthypoxia. Likewise, minute phrenic activity was significantly greater at 30 and 60 min compared with CSNX rats that were not exposed to episodic hypoxia (both \( P < 0.001 \)). Overall, phrenic activity tended to increase between 15 and 30 min after episodic hypoxia in CSNX rats due to a gradual increase in phrenic amplitude, although this change did not reach statistical significance. There were no changes in phrenic amplitude, burst frequency, or minute activity between 30 and 60 min posthypoxia.

Fig. 2. Phrenic motor output (upper trace) and arterial blood pressure (ABP; lower trace) before, during, and 15, 30, and 60 min after episodic hypoxia. The integrated phrenic neurogram and raw blood pressure traces are presented for representative sham-operated (Sham; A) and carotid-denervated (CSNX; B) rats. In the Sham rat, the increase in phrenic activity during hypoxia coincides with the initial drop in ABP. In contrast, the smaller increase in phrenic activity during hypoxia in the CSNX rat occurs after blood pressure drops. Long-term facilitation is evident in both rats as a progressive increase in phrenic activity 15, 30, and 60 min posthypoxia.
Although phrenic amplitude and minute activity were similar between sham-operated and CSNX rats at 15 and 30 min posthypoxia (P < 0.05), the leveling off of phrenic amplitude between 30 and 60 min in CSNX rats (vs. progressive increase in phrenic amplitude in sham-operated rats) resulted in significantly less LTF of phrenic amplitude (P = 0.002) and minute activity (P = 0.043) at 60 min posthypoxia compared with sham-operated rats.

There was no statistically significant relationship between the magnitude of LTF and the blood pressure (MAP and/or Δ MAP) at baseline, during hypoxia, or 60 min posthypoxia in CSNX or sham-operated rats (all P > 0.05). However, there was a nonsignificant trend toward diminished LTF with lower blood pressures during hypoxia within the CSNX group (r² = 0.46, P = 0.064).

DISCUSSION

This study demonstrates that LTF is initiated by episodic hypoxia in otherwise normoxic rats but not by episodic hyperoxia. Moreover, the LTF observed in CSNX rats indicates that carotid chemoafferent neuron activation is not essential for hypoxia-induced LTF in anesthetized rats. However, the magnitude of LTF was somewhat reduced in CSNX rats, providing suggestive evidence that chemoafferent neuron activation and hypoxia per se both contribute to the expression LTF. Together, these results suggest a specific role for CNS hypoxia in hypoxia-induced LTF.

Hyperoxia and LTF

Time-dependent effects of episodic hypoxia on respiratory motor output have been studied in anesthetized rats and cats with hyperoxic baseline conditions (e.g., 19, 20, 23); in these studies, the animals were ventilated with hyperoxic gas mixtures (F₁O₃ > 0.5) to prolong viability of the experimental preparation. Even in the absence of overt hyperoxic toxicity, hyperoxia has the potential to influence cellular processes through the generation of reactive oxygen species (ROS; Refs. 11, 13, 26, 56). Previous studies have shown that hyperoxia can induce plasticity in the control of breathing in humans and other mammals (e.g., 21, 22, 24, 32). However, the results of the present study suggest that hyperoxia does not influence the expression of LTF in anesthetized rats. When superimposed on normoxic arterial blood, episodic hypoxia elicited an LTF of phrenic nerve activity that was qualitatively similar to the LTF reported for hyperoxic rats (19, 44, present study): the amplitude of integrated phrenic bursts re-

Fig. 3. Acute phrenic response to isocapnic hypoxia in Sham (n = 7) and CSNX (n = 8) rats as a percentage increase from baseline. Values are means ± SE. *Different from Sham, P < 0.05. The increase in phrenic minute activity is significantly different from baseline for both groups when analyzed separately (P < 0.05; see text).

Fig. 4. Changes in phrenic activity from baseline after episodic hypoxia in Sham (○, n = 7) and CSNX (●, n = 8) rats. Time-matched hyperoxic time control rats (Sham Control, ◯, n = 8; CSNX Control, □, n = 8) were treated the same but were maintained in hyperoxia throughout the protocol. Values are means ± SE. *Different from baseline, P < 0.05; †different from corresponding sham-operated group (i.e., same treatment, hypoxia or control) at same time point, P < 0.05; #different from corresponding sham-operated group (i.e., same treatment, hypoxia or control) at same time point, P < 0.05. A: amplitude; B: frequency; C: minute activity.
turned toward baseline after the third hypoxic episode and then gradually increased over the ensuing 1 h (Fig. 1). The magnitude of LTF in these normoxic rats was somewhat lower than typically reported for anesthetized rats (19), but considerable interstudy variation makes quantitative comparisons among studies difficult. Recent studies in awake rats (52) and mice (31) also revealed LTF after episodic hypoxia in otherwise normoxic, awake animals. Our findings are consistent with hypoxia serving as the proximate stimulus for LTF in anesthetized rats.

One consequence of using a background of hyperoxia in episodic hypoxia studies is that, in addition to hypoxic episodes, animals are exposed to rapid rises in oxygen to hyperoxic levels at the end of each hypoxic bout. Brief hyperoxia has been shown to induce plasticity in respiratory control (22, 24). For example, 10-min preexposure to hyperoxia leads to a nitric oxide-dependent augmentation of the hypoxic ventilatory response in rats (22). To our knowledge, the effects of repeated, brief exposures to hyperoxia on resting respiratory motor output have not been investigated. In contrast to episodic hypoxia, three 5-min bouts of hyperoxia failed to elicit LTF of phrenic activity (Fig. 1), thus providing additional evidence that the expression of LTF is unique to hypoxia. Indeed, there was no evidence for any time-dependent changes in phrenic activity after episodic hyperoxia, at least in the time domain studied here, although this does not preclude the possibility that episodic hyperoxia and/or ROS exposures initiate other forms of plasticity (56). We produced hyperoxia by raising $F_{O_2}$ from normoxia to nearly 100% oxygen (supplemental carbon dioxide was used to maintain isocapnia), and it is not known how the ROS produced by our protocol compare with ROS generated during episodic hypoxia. Therefore, we cannot rule out causative roles of ROS produced during hyperoxia or during arterial reoxygenation (i.e., hypoxia to normoxia).

**Carotid Denervation and the Short-Term Hypoxic Response**

In adult rats, the brisk increase in respiratory motor output during hypoxia is normally mediated through the activation of carotid body chemoreceptors, although other oxygen sensitive tissues contribute to a lesser extent (37). As expected, carotid denervation caused a substantial reduction in the phrenic response to hypoxia ($P_{A_2} = 40$ Torr) in the present study. However, residual hypoxic sensitivity remained after bilateral CSN section, as evidenced by a small increase in phrenic amplitude during the fifth minute of hypoxia (Fig. 3; see also Fig. 2). Residual hypoxic responses have been reported previously in awake (37, 51) and anesthetized rats (34) after carotid denervation and most likely reflect extracarotid chemosensitivity or barosensitivity (see below). Although extracarotid peripheral chemoreceptors (e.g., aortic and abdominal chemoreceptors) contribute to hypoxic responses in rats (9, 37), these inputs were eliminated in the present study by bilateral cervical vagotomy. It is also possible that additional oxygen-sensitive chemoreceptors in the cervical region remain intact after CSN transection (37, 38) or that regions of the carotid chemoafferent pathway downstream of the carotid body possess innate hypoxic sensitivity (e.g., neurons of the petrosal ganglion or nucleus tractus solitarius; Refs. 36, 45, 59); however, the existence and functional significance of such residual hypoxic sensitivity are questionable (1, 42, 55). The residual hypoxic response in the present study more likely resides in the CNS.

Central hypoxic sensitivity has been implicated in the hypoxic responses in rats (37) and other mammals (e.g., Ref. 12). Many areas of the brain known to modulate respiratory activity have been shown to possess hypoxic sensitivity and could stimulate breathing during central hypoxia (15, 25, 57, 58). Systemic hypoxia has also been shown to produce acidosis in the CNS, including the ventral medulla (50, 63), although this acidosis may not have a stimulatory effect on breathing (49). Alternatively, changes in phrenic activity during hypoxia could be secondary to changes in blood pressure in CSNX rats, either directly through a respiratory baroreflex (61) or through CNS hypoxia, hypercapnia, and acidosis resulting from inadequate perfusion. Consistent with the latter hypothesis, the increase in phrenic amplitude during hypoxia was delayed (20–40 s) in CSNX rats relative to sham-operated rats and consistently followed a drop in arterial blood pressure (see Fig. 2). Moreover, as previously reported for awake rats (8), hypoxic hypotension was more pronounced in CSNX rats than in sham-operated rats. Because brain blood flow regulation is compromised at the low arterial blood pressures achieved in CSNX rats (27), brain $P_{O_2}$ and acid-base homeostasis may be compromised in CSNX rats during hypoxia and ultimately stimulate respiratory activity. The present study was not designed to differentiate between these possibilities (i.e., direct or secondary effects of hypoxia on CNS neurons).

### Carotid Denervation and LTF

Episodic hypoxia induced a long-lasting (>1 h) facilitation in phrenic motor output in CSNX rats, demonstrating that the carotid body itself is not necessary for the expression of LTF. This result is consistent with previous evidence indicating that LTF is primarily a CNS mechanism (44). Indeed, Peng and colleagues (53) found no evidence of LTF in carotid chemoafferent activity after episodic hypoxia in the rat, unless the rat was pretreated with 10 days of intermittent hypoxia (8 h/day). Chronic intermittent hypoxia enhances the expression of phrenic LTF in anesthetized rats (35), apparently evoking additional central and peripheral plasticity (35, 44, 53, 56).

In carotid body-intact rats (series 1 and 2), phrenic motor output increased progressively throughout the hour after episodic hypoxia, and this facilitation was entirely attributable to changes in integrated phrenic burst amplitude. In contrast, phrenic LTF in CSNX rats involved augmentation of both amplitude and fre-
quency of respiratory bursts. Frequency LTF has been observed in anesthetized (3, 5, 7) and awake (52) rats with functional carotid bodies after episodic hypoxia, although this finding has not been consistent among studies (17, 18, 65, 66, this study). Accordingly, it is difficult to interpret the differential expression of frequency LTF in CSNX and intact rats. A progressive increase in phrenic amplitude for at least 1 h after episodic hypoxia is typical in anesthetized-rat studies (44). Over the first 30 min, this “winding up” pattern was observed in CSNX rats (Fig. 4) as opposed to the decrementing pattern of LTF observed in CSN stimulation experiments (23, 33), suggesting that LTF induced by hypoxia is similar in CSNX and intact rats; additional studies are needed to verify that this LTF has the same underlying mechanisms. However, phrenic amplitude failed to continue increasing between 30 and 60 min in CSNX rats, and CSNX rats ultimately exhibited only one-half to two-thirds as much LTF as sham-operated rats (Fig. 4). It is possible, therefore, that chemoeffluent neuron activation is necessary for the full expression of LTF.

Expression of LTF after episodic hypoxia appears to be localized to regions near respiratory motor nuclei (44). For example, phrenic LTF is abolished after intrathecal injection of serotonin receptor antagonists or protein synthesis inhibitors in the cervical spinal cord (7), suggesting that LTF results from plasticity within the phrenic motor nucleus. Recent models of phrenic LTF (6, 19, 29, 44) propose that episodic hypoxia leads to episodic release of serotonin in the region of respiratory motoneurons from projections of the caudal raphe nuclei. Repeated activation of serotonergic 5-HT2 receptors on phrenic motor neurons is expected to initiate an intracellular signaling cascade that ultimately potentiates glutamate-dependent activation of phrenic motor neurons in response to bulboespinal respiratory drive. In this model, as in earlier incarnations (e.g., Ref. 3), it has been hypothesized that hypoxia initiates the release of serotonin at the phrenic motor nucleus through the activation of carotid chemoeffluent pathways. Consistent with this hypothesis, both electrical stimulation of the CSN and hypoxia elicit c-fos expression in serotonergic raphe neurons (16); activation of carotid chemoeffluent pathways has also been shown to influence raphe discharge in rats (46–48, 64). Assuming that hypoxia-induced LTF has a similar serotonin dependence in CSNX and intact rats, the results of the present study suggest that hypoxia may activate serotonergic cells through multiple, potentially overlapping, pathways. For example, central hypoxia could directly activate raphe cells leading to the release of serotonin near phrenic motor neurons; serotonergic cells from the rat medullary raphe are stimulated by changes in PCO2 and pH in vitro (62), but we are unaware of any studies on the hypoxic sensitivity of these cells. Alternatively, hypoxia could modulate the activity of raphe neurons through direct or indirect interactions of raphe cells with peripheral and/or central hypoxia sensitive tissues remaining after CSN transection and vagotomy (see Carotid Denervation and the Short-Term Hypoxic Response).

Our laboratory recently reported a positive, linear relationship between the short-term hypoxic phrenic response and posthypoxic LTF in anesthetized rats (19); rats exhibiting greater short-term hypoxic responses had significantly greater LTF at 60 min posthypoxia. This relationship is not a simple function of increased respiratory drive during hypoxia because increasing respiratory activity with nonhypoxic chemical (e.g., hypercapnia) and nonchemical stimuli does not initiate LTF (4, 6, 40). One possible explanation for the correlation is that rats stimulated more by hypoxia may also experience greater activation of serotonergic raphe neurons and greater serotonin release near phrenic motoneurons, thereby promoting greater LTF (29, 30). We observed a similar relationship between the short-term hypoxic response and LTF in sham-operated rats in the present study (linear regression, phrenic burst amplitude: \( r^2 = 0.93, P < 0.001; \) Fig. 5), but no such relationship was detected in CSNX rats \( (r^2 < 0.01, P = 0.841) \). Although the residual short-term hypoxic response does not appear to explain the variation in LTF in CSNX rats (several CSNX rats had much greater LTF than expected on the basis of their hypoxic responses), the average value for CSNX rats falls within the 95% confidence limits for the LTF-hypoxic response relationship constructed for sham-operated rats (Fig. 5). Thus it is possible that the mechanisms underlying the correlation of LTF with short-term hypoxic responses also apply to whatever responsiveness persists after peripheral chemodenervation.

Alternatively, hypoxia could initiate phrenic LTF independent of increases in respiratory motor output during hypoxia. For example, hypoxia could alter intracellular signaling or gene expression directly at the level of the phrenic motor nucleus or perhaps at higher levels of the phrenic motor nucleus or perhaps at higher levels of the phrenic motor nucleus or perhaps at higher

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**Fig. 5.** Relationship between long-term facilitation at 60 min posthypoxia and the acute hypoxic response in sham-operated rats (●) exposed to episodic hypoxia \( (r^2 = 0.93, P < 0.001) \). Each data point represents 1 rat, and the dashed lines indicate 95% confidence limits for the linear regression. Average data for CSNX rats \( (\bullet, n = 8) \) exposed to episodic hypoxia have been plotted for comparison; horizontal and vertical error bars are ±SE. \( \text{Pao}_2 \), arterial PO2.
brain centers. Gallman and Millhorn (20) investigated the effects of hypoxic challenges of varying severity in anesthetized, vagotomized, and carotid body-denervated cats. They found that a single 10-min bout of hypoxia (PaO₂ = 36–65 Torr) produced a LTF of phrenic motor output that lasted more than 1 h post-hypoxia. Phrenic activity was diminished (often to apnea) during hypoxia in these peripherally chemodenervated cats, but phrenic activity returned to baseline by 15 min posthypoxia and increased progressively throughout the remainder of the protocol; this winding up of phrenic activity posthypoxia is qualitatively similar to what is seen in the anesthetized rat after three episodes of hypoxia (44, present study). The hypoxia-induced LTF observed by Gallman and Millhorn (20) was abolished in decerebrate cats, suggesting involvement of supraspinal mechanisms. In contrast, episodic CSN stimulation is equally or more effective at inducing LTF in unanesthetized, decerebrate vs. CNS-intact cats (40). These data suggest that, at least in cats, hypoxia can initiate a long-lasting facilitation of respiratory motor output through direct CNS effects, apparently by mechanisms distinct from carotid chemosensitive neuron activation. Therefore, it is plausible that CNS hypoxia, independent from hypoxic respiratory responsiveness, contributes to LTF induction in rats. Whether these effects of hypoxia contribute to LTF in carotid body intact animals, or whether they are revealed only in the absence of carotid chemosensitive inputs, is unknown.

Although additional studies are clearly needed to investigate their respective roles in the initiation of LTF, the present study suggests that hypoxia may have an important role in respiratory plasticity apart from the activation of chemosensitive neurons.

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