Specific carotid body chemostimulation is sufficient to elicit phrenic poststimulus frequency decline in a novel in situ dual-perfused rat preparation

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Day, Trevor A., and Richard J. A. Wilson. Specific carotid body chemostimulation is sufficient to elicit phrenic poststimulus frequency decline in a novel in situ dual-perfused rat preparation. Am J Physiol Regul Integr Comp Physiol 289: R532–R544, 2005. First published March 31, 2005; doi:10.1152/ajpregu.00812.2004.—Time-dependent ventilatory responses to hypoxic and hypercapnic challenges, such as posthypoxic frequency decline (PHxFD) and posthypercapnic frequency decline (PHcFD), could profoundly affect breathing stability. However, little is known about the mechanisms that mediate these phenomena. To determine the contribution of specific carotid body chemostimuli to PHxFD and PHcFD, we developed a novel in situ arterially perfused, vagotomized, deacrebrate rat preparation in which central and peripheral chemoreceptors are perfused separately (i.e., a nonanesthetized in situ dual perfused preparation). We confirmed that 1) the perfusion of central and peripheral chemoreceptor compartments was independent by applying specific carotid body hypoxia and hypercapnia before and after carotid sinus nerve transection, 2) the PCO2 chemoresponse of the dual perfused preparation was similar to other deacrebrate preparations, and 3) the phrenic output was stable enough to allow investigation of time-dependent phenomena. We then applied four 5-min bouts (separated by 5 min) of specific carotid body hypoxia (40 Torr PO2 and 40 Torr PCO2) or hypercapnia (100 Torr PO2 and 60 Torr PCO2) while holding the brain stem PO2 and PCO2 constant. We report the novel finding that specific carotid body chemostimuli were sufficient to elicit several phrenic time-dependent phenomena in the rat. Hypoxic challenges elicited PHxFD that increased with bout, leading to progressive augmentation of the phrenic response. Conversely, hypercapnia elicited short-term depression and PHcFD, neither of which was bout dependent. These results, placed in the context of previous findings, suggest multiple physiological mechanisms are responsible for PHxFD and PHcFD, a redundancy that may illustrate that these phenomena have significant adaptive advantages.

sleep apnea; respiratory chemosensitivity; working heart-brain stem preparation; control of breathing

NORMALLY, BREATHING MAINTAINS precise control over blood gasses through the interaction of central (brain stem) and peripheral (carotid body) respiratory chemoreceptors (e.g., Refs. 41, 65). However, under certain conditions, including sleep and neonatal apnea, individuals may suffer bouts of hypoxia and hypercapnia (19, 53, 73). Intermittent chemostimuli, in turn, result in ventilatory time-dependent responses, which may influence breathing stability (67). Despite recent experimental interest in time-dependent ventilatory phenomena and their likely importance to breathing disorders, their sites of origin, underlying mechanisms, and clinical implications remain to be fully determined.

Various time-dependent ventilatory responses to brief bouts of systemic hypoxia have been described (see Ref. 57 for review and nomenclature used herein). For a single bout, these can include 1) an acute response consisting of an initial, rapid augmentation of ventilatory rate and/or tidal volume (e.g., Refs. 10, 24), 2) short-term potentiation (24), a further progressive increase in tidal volume, 3) short-term depression, a slight fall in frequency (35), 4) a “roll off” or hypoxic ventilatory decline in tidal volume, if the bout is sustained (10, 58), 5) an afterdischarge in which ventilation frequency and/or amplitude decays to baseline after termination of hypoxia (23, 25, 26, 29), and 6) posthypoxic frequency decline (PHxFD), defined as a fall in frequency after the termination of hypoxia, below that preceding the bout (13, 20). Additional phenomena occur with repeated bouts of hypoxia, including a progressive augmentation of each ventilatory response (30, 45, 66), and, after a series of bouts, a prolonged increase in baseline ventilation above that preceding the first challenge (i.e., ventilatory long-term facilitation; e.g., Refs. 5, 7, 31, 44, 47, 51). Although many of the time-dependent effects described above are present in the rat, it is important to note that the effects of hypoxia on the ventilatory response appear to be both species and strain dependent (31, 57).

In some respects, the response to a systemic hypercapnic perturbation mirrors the response to hypoxia with the manifestation of both short-term depression and a poststimulus fall in frequency, which we will refer to here as posthypercapnic frequency decline (PHcFD; e.g., Refs. 4, 15, 16). However, in other respects, the responses to hypoxia and hypercapnia differ. With hypercapnia, long-term depression occurs in place of long-term facilitation, and no progressive augmentation has been reported (4).

In the present study, we determine whether specific carotid body chemostimulation in the rat is sufficient to elicit time-dependent ventilatory responses. We focus mainly on poststimulus frequency decline because, in a recent study (17), our group found that carotid body afferent activity declines below baseline after a bout of hypoxia (i.e., sensory posthypoxic decline). This observation is consistent with a direct role for carotid body activity dynamics in time-dependent ventilatory responses.

A number of mechanisms have been proposed to account for poststimulus frequency decline. One is that the decline in frequency is caused indirectly, by progressive hypocapnia resulting from stimulus-induced hyperpnea (60). In the awake goat, specific carotid body hypoxia triggered PHxFD during systemic poikilocapnia but not during systemic isocapnia (25).
However, in other preparations that produce PHxFD, a critical role for hypocapnia has been excluded. For example, termination of systemic hypoxia produces PHxFD in anesthetized artificially ventilated rats in which systemic isocapnia is maintained (2, 14, 35). Furthermore, a mechanism based on hyperpnea-induced hypocapnia cannot easily be adapted to explain PHcFD. Other explanations for poststimulus frequency decline include 1) central time-dependent effects of hypoxia on neuronal tissue, 2) activation of central respiratory circuits resulting from afferent input from peripheral chemoreceptors, and 3) time-dependent changes in afferent peripheral chemoreceptor activity.

Several lines of evidence suggest that chemostimuli may act centrally to elicit some time-dependent ventilatory phenomena. For example, carotid body denervated, but otherwise intact conscious rats are still capable of producing poststimulus frequency decline after bouts of hypoxia and hypercapnia (15). Similarly, multiple groups report hypoxia elicits PHxFD in the anesthetized, vagotomized, artificially ventilated rat, despite carotid body denervation (38, 40). However, others have found the opposite result, that carotid body denervation abolishes PHxFD (7). Thus, at least in some instances, the direct effects of hypoxia and hypercapnia acting on the carotid body may be sufficient to elicit poststimulus frequency decline.

Demonstrations of central effects, however, do not exclude the possibility of other mechanisms acting in parallel. Recently, we (17) found that chemostimulation of an isolated, perfused carotid body preparation with bouts of hypoxia, but not hypercapnia, elicited afferent CSN activity, having a time-dependent pattern consistent with a role in shaping short-term depression and PHxFD. Peng et al. (56) also demonstrated time-dependent responses of the carotid body. Furthermore, Hayashi et al. (35) showed that square-wave electrical stimulation of the carotid sinus nerve (CSN) in anesthetized, artificially ventilated rats produces short-term depression, poststimulus frequency decline, and long-term facilitation.

Together, these data indicate that time-dependent ventilatory responses may, in part, originate either from within the carotid body or from the central integration of peripheral chemosensory inputs. However, three caveats must be considered: 1) in Hayashi et al. (35), stimulating the CSN is likely to activate afferents coding for sensory modalities not limited to respiratory chemosensitivity [e.g., blood pressure (64), blood glucose, or another index of metabolism (9, 52)], 2) the results of Hayashi et al. (35) have yet to be confirmed in a nonanesthetized preparation, and 3) demonstration of time-dependent sensory responses to hypoxia, but not hypercapnia, provides only correlated evidence of involvement of carotid body dynamics in phrenic PHxFD and does not address whether the carotid body has a role in PHcFD. Thus whether specific carotid body chemostimuli are sufficient to elicit poststimulus frequency decline in the rat remains to be determined.

To address this issue and to facilitate the study of chemoreceptor interaction, we developed a novel in situ dual-perfused rat preparation (DPP) based on the working heart-brain stem preparation (WHBP) (54). The WHBP has a well-oxygenated brain stem (>200 Torr PO₂), a pH that matches the perfusate, intact vascular responses to PO₂ and PCO₂, a eupneic-like ramping phrenic discharge, and phrenic PHxFD (21, 71). In the DPP, central (brain stem) and peripheral (carotid body) compartments are perfused separately, allowing compartment-specific chemochallenges with defined medium containing precisely controlled gas concentrations. Here, we provide data to confirm that 1) perfusion of the brain stem and carotid bodies are independent in the DPP, 2) the systemic PCO₂ chemoreponse of the DPP is similar to that of other decerebrate preparations (i.e., the apneic threshold and peak response are hypocapnic relative to nondecerebrate rats), and 3) the stability of the DPP is adequate to study time-dependent phenomena. We then use this preparation to address the main aim of this paper, demonstrating that specific carotid body intermittent hypoxia (IHF) and hypercapnia (IHC) are sufficient to produce phrenic time-dependent responses.

**EXPERIMENTAL PROCEDURES**

**Preparation Modifications**

We have made several modifications to the WHBP (54), making it appropriate for studies of respiratory chemoreceptor interaction. We will refer to our preparation as the in situ DPP. Our modifications include 1) separate perfusion of central and peripheral chemoreceptor compartments, 2) clamping of descending aorta and carotid artery perfusion pressures at ~90 mmHg, 3) vagotomy, and 4) normokalemic perfusate.

**Dissection and Perfusion System**

Experiments were conducted with 34 juvenile male Sprague-Dawley albino rats (Charles River, Quebec, Canada) (120–150 g, ~4–6 wk old). Experimental procedures were approved by the University of Calgary Animal Care Committee and were in accordance with national guidelines. Rats were heparinized (1,500 U ip) 30 min before they were anesthetized for dissection. Deep anesthesia was induced with halothane via inhalation. Adequate anesthesia was assessed by testing for absence of response to noxious tail pinch. In rapid succession, rats were transected subdiaphragmatically and decerebrated at the midcollicular level (approximate level of lambda). All tissue rostral to the decerebration and all remaining cortex dorsal to the colliculi were removed. This decerebration transects the circle of Willis. Transection and decerebration were performed in cold perfusate (~5–8°C, volume: 500 ml) containing (in mM) 115 NaCl, 24 NaHCO₃, 4 KCl, 2 CaCl₂, 1 MgSO₄, 1.25 NaH₂PO₄, and 10 dextrose, equilibrated with 95% O₂-5% CO₂. The skin was removed, and the preparation was placed into an experimental chamber and secured with ear bars. The descending aorta was cannulated with a double-lumen catheter. One lumen of the catheter was connected to a peristaltic pump (Gilion Manipuls 3) and used to perfuse the descending aorta retrogradely with room-temperature perfusate containing 1.33% Ficoll type 70 (a nonionic polymer of sucrose, molecular weight of 70,000; Sigma-Aldrich F-2878) equilibrated with 40 Torr PCO₂ in O₂. The other lumen was attached to a pressure transducer to monitor perfusion pressure. Once cannulated, the speed of the peristaltic pump was increased so as to increase perfusion pressure to 60 mmHg over the first few minutes using a custom-built computer-controlled feedback system. A bilateral vagotomy was performed at the midcervical level. The common carotid arteries were tied off above the clavicles and cannulated proximal to the carotid bodies. A separate peristaltic pump with two channels was used to perfuse the common carotid arteries at ~15–20 ml·min⁻¹–carotid⁻¹ equating to ~90 mmHg. Up to this stage in the dissection, central and peripheral perfusions were from the same tonometer (~300 ml).

Independent perfusion of central (descending aorta) and peripheral (carotid arteries) circuits was then initiated by pulling fresh Ficoll-containing media from two different reservoirs of a custom-built tonometer. This custom-built system was designed to accommodate a common return, while preventing mixing of perfusate, once equilibrated in the two reservoirs. Between the reservoir and the prepara-
tion, the central perfusate passed through a bubble trap, heat exchanger, and a 25-µm filter. The carotid perfusate passed through a bubble trap and a heat exchanger. Perfusate leaked from the many cut vessels in the preparation and returned to the experimental tonometer where it was recycled and reequilibrated (total volume of perfusate was 750 ml). We tested this system before initiating the study protocol using a Clark-style polarographic PO2 electrode to ensure efficient equilibration and no cross contamination between reservoirs (unpublished measurements).

After the initiation of independent perfusion, the central perfusate was equilibrated with 40 Torr PCO2 in O2, and the peripheral perfusate was equilibrated with 40 Torr PCO2 and 100 Torr PO2 in N2. Note that, by this point, the perfusate was virtually blood free. Over the next 30 min, the temperature of the preparation and the central perfusion pressure was ramped to 32–33°C and 90 mmHg, respectively. Protocols were started at 70 ± 5 min after central cannulation. Gasses used to equilibrate perfusate were mixed by computer-controlled mass flow controllers (MKSI Instruments) and sampled to ensure accuracy (Sensormedics medical gas analyzer LB-2 and Sable Systems PA-1B O2 analyzer, calibrated daily). Gas concentrations reported here were accurate to within 0.1% (~1.3 Torr in Calgary). Note that no neuromuscular blocker was used due to the potential confounding effects on respiratory chemosensitivity (72).

**Electrophysiology**

The left phrenic nerve was dissected out with a small piece of diaphragm attached to its distal end and placed in a custom-made Plexiglas recording chamber. The nerve was bathed in perfusate and protected with petroleum jelly. Silver bipolar extracellular electrodes were used to record the phrenic neurogram, which was amplified (A-M Systems differential AC amplifier model 1700), filtered (low cut off of 300 Hz, high cut off of 5 KHz), rectified and integrated (amplitude demodulator, Saga Tech), and computer archived (Axon Instruments Digidata 1322A and Axoscope 9.0) at a sampling rate of 50 Hz, and analyzed offline.

**Data Analyses**

Only preparations that exhibited a ramping phrenic discharge (the peak of phrenic activity occurs in the last 50% of the duty cycle) and an inspiratory time (Tt) of ≤1 s under control conditions were included in the study. Preparations with a Tt of >1 s were considered apneustic. Analysis of phrenic neurogram characteristics was performed with software written in house by R. J. A. Wilson. The following respiratory variables were quantified from the integrated phrenic neurogram (refer to Fig. 1): period (Ttot), respiratory frequency (fR, 60 times the inverse of the period), Tt, expiratory duration (Te) (62), Tt-to-Te ratio, neural tidal volume (nVT), the peak phrenic amplitude; Ref. 22), neural minute ventilation (nVT, the product of fR and nVT), inspiratory ramp [IR; nVT divided by time to peak (Tp)], and eupneic index (Tp divided by Te).

**Protocols**

**Test for independence of central and peripheral perfusion circuits.** Six preparations were utilized to test for the independence of central and peripheral perfusion circuits. Under baseline conditions, the brain stem and carotid body perfusates were equilibrated with 28 Torr PCO2 (average)-balance O2 and 28 Torr PCO2 (average)-100 Torr PO2-balance N2, respectively. A mean PCO2 of 28 Torr was chosen to maximize the response to hypercapnic challenges without causing apnea. The following protocol was then applied: 5-min baseline, 5-min specific carotid body hypoxia (60 Torr PO2, PCO2 unchanged), 5-min washout, and 5-min specific carotid body hypercapnia (60 Torr PCO2, PO2 unchanged). These challenges confirmed the presence of 

![Fig. 1.](http://ajpregu.physiology.org/)
phrenic responses to both specific carotid body chemostimuli. The CSNs were then sectioned, and the protocol was repeated. The hypoxic carotid body perturbation tested whether the CSN transection was successful, and the hypercapnic carotid body perturbation was tested for contamination (i.e., whether peripheral perfusate stimulated brain stem chemoreceptors). Five minutes of specific brain stem hypercapnia (60 Torr PCO2, balance PO2) applied at the end of the protocol was used to ensure that the brain stem was still sensitive to PCO2 perturbations. For statistical comparison, nVE was calculated for the preceding minute (i.e., preceding baseline) and last minute of each challenge.

**Systemic PCO2 chemoresponse.** We determined the systemic PCO2 chemoresponse using data from nine preparations. For this protocol, the PO2 of central and peripheral chemoreceptors were held at >550 and 100 Torr, respectively. The PCO2 perturbations were applied randomly in ascending (n = 4) or descending (n = 5) order to both perfusion circuits in unison. The protocol consisted of a 10-min baseline period at 35 Torr PCO2 followed by six 10-min PCO2 perturbations of 15, 25, 35, 45, 55, and 65 Torr followed by a washout at 35 Torr PCO2. The apneic threshold was determined at the end of each protocol by lowering perfusate PCO2 until there was an absence of phrenic activity and then increasing PCO2 in 1-Torr increments until phrenic activity resumed. The apneic threshold was taken to be the level of PCO2 at or below which apnea could be maintained (11, 18). The entire protocol was analyzed and averaged in 2-min bins. In Fig. 3, raw or normalized mean phrenic variables from the last 2 min of each perturbation were plotted. For normalized data (nVT, nVE, IR), response values from each perturbation were normalized to the values at 35 Torr PCO2 in the middle of the protocol.

**Interruption chemostimuli.** Twenty-one preparations were used to quantify the phrenic time-dependent responses to specific carotid body IHX and IHC. Five preparations were used to determine the stability of the DPP over the protocol period. After the 70-min equilibration period, preparations were maintained for a further 70 min under baseline conditions: 30 Torr PCO2 in O2 for brain stem and 40 Torr PCO2 and 100 Torr PO2 in N2 for carotid bodies. In the remaining 16 preparations, the following 70-min protocol was superimposed on these baseline conditions: 5-min baseline, four 5-min specific carotid body perturbations, each separated by a 5-min washout, followed by a 30-min washout recovery period. The interruption carotid body perturbations were either hypoxic (40 Torr PO2 and 40 Torr PCO2 in N2) or hypercapnic (100 Torr PO2 and 60 Torr PCO2 in N2). Every phrenic burst over the protocol period was analyzed, and respiratory variables were averaged in 30-s bins. nVT, nVE, and IR data were normalized to the last minute of the initial baseline section (minute 4–5).

**Terminology.**

For statistical tests of frequency and nVT and nVE dynamics, bins of raw data were selected from each animal as follows (illustrated in Fig. 1C): initial baseline, minute 4–5 (2 bins) before the first perturbation (to which data were normalized for graphical representation); bout baseline, the last bin before each perturbation (for the first bout, the bout baseline is the same as the initial baseline); peak, the highest average bin for a given variable during each perturbation; bout end, the last bin of each perturbation; trough, the bin with the lowest value during each washout; and 5-min postbout recovery, the last bin of each 5-min recovery period (note that the 5-min postbout recovery bin for bouts 1–3 constitutes the bout baseline bin for bouts 2–4, respectively). The end point was taken as the average of the last two bins of the protocol (minute 69–70). These points were selected for each of the four perturbations.

Using this nomenclature, we define 1) short-term depression as the difference (Δ) between the peak frequency and bout end frequency, 2) PHxFD and PHcFD as when the frequency trough and/or the 5-min postbout recovery values differ from the initial baseline, 3) Δpeak of fR, nVT, or nVE as the difference between peak and the preceding bout baseline, and 4) Δoverall bout average of fR, nVT, or nVE as the difference between the average of all bins during each perturbation and the bout baseline.

**Statistical Tests.**

To test the independence of perfusion circuits, two one-factor repeated-measures (1F RM) ANOVAs were used, one for each phase of the experiment, i.e., before and after CSN transection. In each phase, nVE during the last minute of baseline, perturbations, and washout were compared. For each intermittent chemostimuli protocol (IHX and IHC), three 1F RM ANOVAs were used to test for differences in phrenic frequency between the initial baseline, end point, and each of the four 1) peaks, 2) troughs, and 3) 5-min postbout recovery. Similarly, frequency response Δ for peak, overall bout average, and short-term depression were tested using three two-factor repeated-measures (2F RM) ANOVAs. In these ANOVAs, one factor was the bout (4 levels) and the other was the constituent of the Δ (2 levels). For example, for Δpeak, the levels were the preceding bout baseline and peak. We chose this method over one that used three 1F RM ANOVA on derived Δ because, in addition to determining the significance of bout effect, the 2F RM ANOVA provides a test of the significance of the Δ itself as well as the interaction of the Δ and the bout. A similar approach was used to determine bout dependence of Δpeak and Δoverall bout average in nVT and nVE (data not shown, see DISCUSSION). In all cases, once significant differences had been determined, Student-Newman-Keuls (SNK) post hoc tests were used for pair-wise comparisons.

**RESULTS.**

**Characterization of the DPP.**

Test for independence of central and peripheral perfusion circuits. If the peripheral perfusate has no influence on the perfusion of the brain stem, then transecting the CSN should abolish all respiratory responses to changes in PCO2 of the peripheral perfusate. Two preparations developed apneasus after CSN transection and were not included in subsequent analyses. In the remaining four preparations, both peripheral hypoxia and peripheral hypercapnia induced increases in mean nVE (Fig. 2; preceding baseline compared with hypoxic response, 60 ± 14% increase, SNK post hoc test, q = 7.03, P = 0.002; preceding baseline compared with hypercapnic response, 51 ± 16% increase, SNK post hoc test, q = 5.44, P = 0.01). Transecting the CSN eliminated both responses (preceding baseline compared with hypoxic period after transection, SNK post hoc test, q = 2.47, P = 0.44; baseline compared with hypercapnic period after transection, SNK post hoc test, q = 0.88, P = 0.81). Although CSN-denervated preparations were unresponsive to peripheral challenges, they retained central respiratory chemosensitivity, increasing nVE when challenged with a central hypercapnic perfusate (preceding baseline compared with central hypercapnic response after transection, SNK post hoc test, q = 8.55, P < 0.001). If the two preparations that developed apneasus after CSN transection were included in the statistical analysis, similar results were obtained. These experiments confirm the independence of central and peripheral perfusion circuits.

**Systemic PCO2 chemoresponse.** To compare the PCO2 chemoresponse of the DPP to other vagotomized, decerebrate preparations, we challenged nine preparations with six 10-min PCO2 perturbations (Fig. 3). During each perturbation, the brain...
The PO2 of the brain stem and carotid body perfusates were kept constant throughout, 550 Torr and 100 Torr, respectively. The mean apneic threshold obtained was 22.44 ± 1.78 Torr. However, only six preparations were apneic at 15 Torr PCO2, and three preparations remained apneic even at 25 Torr PCO2. All nine preparations had phrenic activity at 35, 45, 55, and 65 Torr PCO2. Above apnea, an increase in PCO2 produced increases in fR, nVT, nVE, and IR. The rate of increase diminished markedly above 45 Torr, with the middle of the dynamic range being at ~30 Torr PCO2. The frequency response was accompanied by a decrease in Ti/Te and was accounted for predominantly by a decrease in Ti. The shape of phrenic bursts was PCO2 invariant (as indicated by the stable eupneic index).

It should be noted that, during this set of experiments, some preparations developed apneusis when held in the hypocapnic range. Data from these preparations were not included in the analysis. Because the perfused brain stem retains vascular responses to PCO2 (71), we consider it likely that severe hypocapnia in some cases induced vasoconstriction sufficient to cause ischemia-induced pontine damage (70).

Stability of the DPP. To characterize any changes that may occur in phrenic variables under control conditions, a prerequisite for using the DPP to study the time course of responses to intermittent chemostimuli, we performed a series of time controls. Figure 4 shows respiratory variables over the 70-min protocol period that began 70 min after the initiation of artificial descending aorta perfusion. The conditions were those used as baseline for the remainder of this study: brain stem and carotid body received the same level of PCO2 in unison. The PO2 of the brain stem and carotid body perfusates were kept constant throughout, >550 Torr and 100 Torr, respectively. Unless indicated by numbers above data points, n = 9. For phrenic shape variables (Ti, Ti, Ti/Te, and EI), we excluded animals from the means once they developed apnea to prevent values of zero from distorting the mean. ○ Apneic threshold [22.44 ± 1.78 (SE), n = 9]. • Mean for fR, nVT, nVE, and IR at 15 Torr PCO2, including zero values from apneic animals. Note that nVT, nVE, and IR data are normalized to the value at 35 Torr PCO2.
perfusate equilibrated with 30 Torr PCO2-balance O2 and carotid body perfusate equilibrated with 40 Torr PCO2-100 Torr PO2-balance N2.

Over the first 5 min of the protocol period (i.e., 75 min after reestablishment of central perfusion), timing variables were still recovering from dissection. After this period, these variables stabilized considerably compared with the 5-min baseline period. Accordingly, we compared the 5th min (indicated by arrows in Fig. 4) with the end point (i.e., the 70th min of the protocol period). Note that phrenic frequency increased by 12.4%, whereas nVT decreased by 21%, resulting in a net decrease in nV˙E of 11.3%. Throughout the protocol, the eupneic-like phrenic bursts retained their shape, having a stable eupneic index and a T1 of 0.7–0.8 s.

**Frequency Response to Bouts of Specific Carotid Body Hypoxia or Hypercapnia**

The responses to IHX or IHC perturbations were investigated by superimposing four 5-min bouts of specific carotid body hypoxia (40 Torr PO2-40 Torr PCO2-balance N2; n = 9) or hypercapnia (100 Torr PO2-60 Torr PCO2-balance N2; n = 7) on the baseline conditions defined above. Each bout was separated by 5 min. Figures 5 (IHX) and 6 (IHC) show how respiratory variables respond. For statistical analysis, we focused on three of the respiratory variables shown: fR, nVT, and nV˙E.

Figure 7 summarizes how frequency responds in terms of the parameters that we defined in Terminology, above. Each of the four hypoxic bouts elicits an increase in frequency (peak
compared with initial baseline and end point, 1F RM ANOVA: $F = 5.17, P < 0.001$; Fig. 7A). The peak obtained during hypoxia was independent of bout (SNK post hoc test, pair-wise comparison of bouts: $q < 1.09, P > 0.87$). After termination of bouts, the activity between bouts decreased below the initial baseline (i.e., PHxFD, trough compared with initial baseline and end point, 1F RM ANOVA on ranks: $P < 0.001$). PHxFD appeared to have some bout dependence (Fig. 7C, open bars). However, the effect of bout was weak (SNK post hoc test comparing the trough for bouts 1 and 3: $q = 3.264, P = 0.066$).

In one animal, the frequency increased by 40% from the initial baseline to the end point, a drift that is more than three times the norm for this data set. If this animal was excluded from the analysis, then the difference between bouts is more apparent (SNK post hoc test comparing trough of bouts 1–3 and 2–3: $q = 6.05$ and $q = 7.40$, respectively; $P < 0.05$). In addition, we compared the 5-min postbout recovery with initial baseline and end point. Bouts 1 and 2 were different from bouts 3 and 4 (SNK post hoc test: $q > 4.00, P < 0.05$). Together, these data suggest that the magnitude of PHxFD increases with repeated bouts of hypoxia.

As with hypoxia 1) bouts of specific carotid body hypercapnia caused an increase in frequency (peak compared with initial baseline and end point, 1F RM ANOVA: $F = 6.68, P < 0.001$; Fig. 7B), 2) peaks obtained during hypercapnia were independent of bout (SNK post hoc test, pairwise comparison of bouts: $q < 0.73, P > 0.64$), and 3) the activity declined after hypercapnic bouts (PHcFD, trough compared with initial baseline and end point, 1F RM ANOVA: $F = 3.14, P < 0.03$). However, unlike with hypoxia, there was no evidence of a bout effect on the trough (SNK post hoc test, pairwise comparison of troughs: $q < 1.53, P > 0.43$). Furthermore, the 5-min

Fig. 7. Summary of frequency responses to intermittent chemostimuli: peak, trough, and 5-min postbout recovery. A and C: specific carotid body hypoxia. B and D: specific carotid body hypercapnia (see Figs. 5 and 6 for details of challenges). A and B: during bout responses (solid bars). C and D: postbout responses (light gray bars show trough, dark gray bars show 5-min postbout recovery). Note that 1) in both A and B the peak frequency is not bout dependent, 2) in both C and D, troughs are significantly different from initial baseline (open bars labeled baseline) and end point (open bars labeled end), demonstrating PHxFD and PHcFD, and 3) there is a progressive increase in the magnitude of both trough and 5-min postbout recovery for hypoxia (C) but not for hypercapnia (D). All values are normalized to initial baseline. *Significantly different from initial baseline and end point. †Significant differences compared with bouts 1 and 2.
postbout recovery frequency was not different from the initial baseline or the end point (1F RM ANOVA on ranks: \( P = 0.51 \)). This suggests that the decline in frequency after termination of a bout of 60 Torr \( P_{\text{CO}_2} \) (PHxFD) had a faster recovery back to the baseline level than after a bout of 40 Torr \( P_{\text{O}_2} \) (PHxFD).

In addition to analyzing frequency responses relative to initial baseline, we also explored the effects of bout-by-bout changes in frequency, referred to here as initial baseline, we also explored the effects of bout-by-bout (PHxFD).

Conversely, the hypercapnic effects of frequency were bout dependent (2F RM ANOVA, interaction effect of \( P < 0.001 \), and the overall bout average from bout baseline also increased progressively with each bout (SNK post hoc tests, pairwise comparison of bouts: \( q > 3.101, P < 0.035 \), Fig. 8A). Conversely, the hypercapnic \( \Delta \) peak and overall bout average were independent of bout (2F RM ANOVAs, interaction effect of \( P = 0.941 \); Fig. 8B). Furthermore, although there was no significant decline in frequency from peak to bout end during hypoxic perturbations (i.e., short-term depression was not significant, 2F RM ANOVA, peak to bout end: \( F = 2.475, P = 0.154 \); Fig. 8A), there was a significant decline from the peak to the bout end during hypercapnic perturbations (2F RM ANOVA, peak to bout end: \( F = 36.026, P < 0.001 \); Fig. 8B).

We also performed comparisons using \( t \)-tests to confirm \( J \) that the frequency at the initial baseline (minute 4–5) was similar in the time control and experimental groups and 2) that complete recovery occurred from frequency depression after IHX and IHC by the end point (minute 69–70). The baseline frequency in the time course control group was not significantly different from the initial baseline frequency of the IHX or IHC groups (\( P = 0.756 \) and 0.566, respectively). Furthermore, the end point frequency of the time course control group was not significantly different from the end point of the IHX or IHC groups (\( P = 0.141 \) and 0.737, respectively).

In summary, hypoxia and hypercapnia at the levels that we chose to investigate have different time-dependent effects on frequency. Most noticeably, we found that a progressive augmentation in the frequency response occurs with repeated bouts of hypoxia but not with hypercapnia. The progressive augmentation was caused by a bout-dependent increase in the magnitude of PHxFD (depression), and thus a larger response difference, and not an increase in the peak frequency during the perturbation. Although a mild posthypcapnia frequency decline was elicited, it was not bout dependent. Conversely, hypercapnia elicited short-term depression of frequency during each bout, whereas hypoxia did not.

\( nV_T \) and \( nV_E \) Response to Bouts of Specific Carotid Body Hypoxia or Hypercapnia

Bouts of hypoxia increase both the \( nV_T \) \( \Delta \) peak and \( nV_T \) overall bout average significantly (2F RM ANOVA \( \Delta \) peak effect: \( F = 6.309, P = 0.036 \); overall bout average: \( F = 5.698, P = 0.044 \)). Conversely, bouts of hypercapnia affected the \( nV_T \) \( \Delta \) peak response (2F RM ANOVA \( \Delta \) peak effect: \( F = 12.831, P = 0.012 \) but not the \( nV_T \) overall bout average (2F RM ANOVA overall bout average effect: \( F = 2.765, P = 0.147 \)). However, no progressive augmentation was apparent in \( nV_T \) (i.e., neither \( nV_T \) \( \Delta \) peak nor \( nV_T \) overall bout average changed significantly with bout) (2F RM ANOVAs, interaction between \( \Delta \) peak and bout for hypoxia: \( F = 0.424, P = 0.738 \); for hypercapnia: \( F = 1.686, P = 0.206 \); 2F RM ANOVAs, interaction between overall bout average and bout, for hypoxia: \( F = 0.709, P = 0.556 \); for hypercapnia: \( F = 1.005, P = 0.413 \)).

Finally, to determine whether progressive augmentation in frequency with hypoxic bouts translated to \( nV_E \), we tested whether \( nV_E \) \( \Delta \) peak and \( nV_E \) overall bout average were dependent on bout. Progressive augmentation was evident in both measures of the hypoxic response (2F RM ANOVAs, interaction of \( \Delta \) peak and bout: \( F = 3.675, P = 0.026 \); for \( F = 3.743, P = 0.024 \)). Thus, with hypoxia, \( nV_E \) \( \Delta \) peak for bouts 3 and 4 are greater than for bout 1 (SNK post hoc tests, for \( \Delta \) peak: \( q > 3.9, P < 0.027 \)). Additionally, \( nV_E \) overall bout average for bouts 3 and 4 are greater than for bout 1, and bout 4 is greater than bout 2 (SNK post hoc tests, for \( \Delta \) overall bout average: \( q > 3.99, P < 0.022 \)).

As expected, hypercapnia elicited no bout dependence in either \( nV_E \) \( \Delta \) peak or overall bout average (2F RM ANOVAs: interaction of \( \Delta \) and bout effects, \( F = 0.216, P = 0.884 \) and \( F = 0.266, P = 0.849 \), respectively).

![Fig. 8. Summary of differences (Δ) in frequency responses to intermittent chemostimuli: Δpeak, Δoverall bout average, and STD, i.e., Δ from peak to bout end. Note that there is a progressive augmentation in the Δpeak and Δoverall bout average to repeated stimuli with hypoxia but not with hypercapnia. Conversely, statistically significant STD only occurred with hypercapnia. All values are normalized to initial baseline. †Significant difference compared with bout 1. ††Significant difference compared with bouts 1, 2, and 3. #Significant STD.](http://ajpregu.physiology.org/10.1152/ajpregu.00246.2005)
**Discussion**

Using an in situ DPP, we report two novel findings: 1) specific carotid body hypoxia and hypercapnia produced poststimulus frequency decline and 2) PHxFD magnitude increased with bout leading to progressive augmentation in frequency, whereas PHcFD was not bout dependent. Previous studies in anesthetized and awake rats indicated that PHxFD and PHcFD can occur in the absence of the carotid bodies (7, 15). In light of our results, we propose that, in the rat, multiple mechanisms can act in parallel to mediate these time-dependent responses.

Our demonstration of PHxFD and PHcFD resulting from specific carotid body chemostimulation is consistent with previous findings showing that 1) perfused, decerebrate, nonanesthetized preparations exposed to systemic hypoxia (carotid body and brain stem in unison) exhibit these phenomena (21) and 2) nonspecific electrical stimulation of the CSN in anesthetized rats also elicit poststimulus frequency decline (35). However, in our study, we found that sequential bouts of specific carotid body hypoxia, but not specific carotid body hypercapnia, progressively increased the magnitude of the poststimulus frequency decline. These findings differ from those with electrical stimulation in which the magnitude of poststimulus frequency decline was independent of bout (35).

**Critique of the DPP**

A number of different techniques have been developed to attempt to quantify the contribution of central and peripheral chemoreceptors to ventilatory responses (e.g., Refs. 8, 12, 55, 61). We developed the DPP as an alternative to, rather than a replacement of, previous preparations because it 1) allows the study of respiratory chemosensitivity independent of heart rate and systemic blood pressure, 2) allows the use of rats, which are being used increasingly as an experimental model for studies of the control of breathing, and 3) increases the feasibility of addressing a greater range of questions, owing to the fact that the DPP requires less sophisticated surgery and can be prepared rapidly. Being decerebrate, the DPP avoids the nonspecific, depressive effects of anesthetics and paralytic agents on respiratory chemoreceptors (28, 72). Although most of the other preparations used for these kinds of studies are perfused with blood, the DPP is distinct in that the brain stem and the carotid body are artificially perfused with defined media. Perfusion is nonpulsatile, facilitating cellular recordings. We have shown previously that artificially perfused brain stems are well oxygenated and have a tissue pH that closely matches the pH of the perfusate, and thus we can precisely regulate the stimulus acting on the chemoreceptors. In considering our results however, one should note that, in vivo, blood is likely to play an important role in determining systemic chemosensory responses, in that it is involved in the transport of gases, the buffering of pH, and the regulation of the microvasculature. When considering the DPP as an alternative to other preparations used for study of chemoreceptor interaction, additional cavets to consider are 1) the time window in which the DPP is stable is limited to a few hours; 2) the DPP is hypothermic (33°C), decerebrate, and vagotomized; and 3) sympathetic activation produced by carotid body stimulation could cause changes in brain stem perfusion, which in turn might change brain stem tissue Po2 and Paco2. These are briefly discussed in relation to the present study.

**Stability of the DPP**

We conducted time-trial control experiments to determine the stability of the DPP (Fig. 4). The phrenic amplitude (nVR) decremented (~21%) progressively over the entire 65 min (Fig. 4E). Whether this amplitude rundown reflects a physiological process (such as decay of long-term facilitation after transient hypoxia during dissection) or rundown in the viability of the preparation is unclear. However, the eupneic index (Tp/Ti) illustrates that the peak of the integrated phrenic neurogram occurs late in the duty cycle and remains ~0.7 throughout the protocol period. This suggests that the respiratory control circuit remains well oxygenated. Ti and Tc also remained stable, decreasing by just ~0.1 s over 70 min, resulting in a modest increase in burst frequency (~12.4%).

Most of the change in frequency occurred within the 5-min baseline period, after which it stabilized considerably. In the present study, we were primarily concerned with frequency. The time-dependent responses that we report here (short-term depression, poststimulus frequency decline, and progressive increase in PHxFD with bout) involve decreases in frequency. Thus, if anything, our results may underestimate the magnitude of these phenomena.

**Possible Effects of Decerebration**

Decerebration has been shown in several preparations to reduce the apneic threshold, shifting the CO2 chemoresponse curve in the hypocapnic direction (36, 46, 50). Hayashi and Sinclair (36), for example, showed that anemic decerebration in vagally intact rats leads to sustained increases in fR, VT, and V˙E and hypocapnia and a 90% reduction in the V˙E response to 5% inspired CO2. In 5 of 13 decerebrate rats that they studied, V˙E decreased below baseline values at a peak arterial PCO2 of 55 Torr due to a reduction in frequency. Together, these data suggest that the maximum chemosensitivity after decerebration occurs at a substantially lower level of PCO2 (36). We characterized the systemic PCO2 chemoresponse of the DPP and found it was also shifted in the hypocapnic direction. The apneic threshold of the nine preparations ranged between 10 and 28 Torr PCO2 (22.44 ± 1.78 Torr PCO2, ~10 Torr lower than in intact anesthetized rats; Ref. 11), and the asymptotes of the chemoresponse curves for fR, nVR, and nV˙E were ~45 Torr. Consequently, while testing the effects of specific carotid body chemostimulation, we chose to hold the brain stem at 30 Torr PCO2 to ensure the preparation was within its chemosensitive range.

**Possible Effects of Vagotomy**

In the DPP, the lungs are deflated and the vagi are cut. In the WHBP, from which the DPP is derived, the lungs are also deflated, but the vagi are intact. St.-John and Paton (63) investigated the possibility that tonic vagal inputs from the deflated lung of the WHBP influence respiratory burst activity. Vagotomy had no effect on the systemic CO2 chemoresponse of their preparation. However, phasic vagal input has powerful effects on respiratory timing (34, 74), may change the gain of respiratory chemosensitivity (42), and may therefore influence the integration of peripheral and central chemoreceptors in vivo.
Possible Effects of Sympathetic Activation on Brain Stem Perfusion

Sympathetic activity produced by carotid body stimulation might conceivably vasoconstrict the cerebral vasculature, causing tissue hypoxia and hypercapnia. Although central hypoxia and hypercapnia perfusion have direct effects on the cerebral vasculature, the central effects of sympathetic activation on the cerebral vasculature caused by carotid body stimulation are controversial (37, 49, 68). In all likelihood, these effects do not affect brain stem regions (1). However, carotid body stimulation does cause considerable vasoconstriction in the peripheral vasculature. In the present study, we used a Proportional-Integral-Derivative feedback controller to clamp the perfusion pressure of the central circuit supplying the brain stem, forelimbs, and trunk of the preparation. Using this system, we compensated for changes in peripheral vascular resistance in the forelimbs and trunk by changes in flow rate delivered by the peristaltic pump. Thus brain stem perfusion likely remains constant. As noted above, during hypoxic challenges delivered through the carotid artery, the eunepic index decreased only slightly (from 0.73 ± 0.026 to 0.69 ± 0.034). Furthermore, Ti also decreased, an effect inconsistent with brain stem hypoxia. These considerations, along with the data from the independent perfusion experiment, suggest that hypoxia perfused through the peripheral circuit was specific to the carotid body and did not cause any secondary brain stem hypoxia.

Amplitude and Timing Responses to Systemic PCO2 in the DPP

Zhou et al. (74) reported that, in a decerebrate, vagotomized rat preparation, most of the ventilatory response to increased PCO2 is mediated by increases in phrenic amplitude. Phrenic burst amplitude appears to play a comparable role in the DPP (and the WHBP; Ref. 63), dominating the nVE response to changes in systemic PCO2 (Fig. 3). In nondecerebrate animals, ventilatory responses to PCO2 include changes in tidal volume and frequency (e.g., Ref. 15). Nielson et al. (50) showed that following decerebration in dogs, the frequency response to hypercapnia is more attenuated and variable than the amplitude response. Thus, in the DPP, decerebration likely explains the dominance of the amplitude response and the variability in the frequency response, which is particularly apparent in the hypocapnic range. St.-John and Paton (63) reported similar frequency variability in the WHBP. Of note is the fact that, in the DPP, the IR (nVE/Tp) shows a more linear response to PCO2 than nVE and continues increasing beyond the nVE asymptote of 45 Torr. It appears, therefore, that the IR has a dynamic CO2 chemosensitive range distinct from the other variables that we examined.

Specific Carotid Body Chemostimuli Elicit Poststimulus Frequency Decline in the DPP

A central finding of this study is that both specific carotid body hypoxia and hypercapnia are sufficient to elicit poststimulus frequency decline (PHxFD) in the DPP. These results are consistent with previous findings demonstrating a similar phenomenon after termination of electrical CSN stimulation in the anesthetized rat (35). Together, these findings suggest that central hypoxia may not be necessary to initiate PHxFD, although central hypoxia may itself be sufficient (15).

Several studies have demonstrated the importance of the noradrenergic A5 pontine region in PHxFD. Lesions to this region abolish PHxFD in both the anesthetized, artificially ventilated and intact awake rats (13, 59), whereas electrical stimulation causes prolongation of TPE (a hallmark of PHxFD in the DPP and other preparations). Furthermore, systemic application of α-adrenoreceptor antagonists blocks PHxFD, although whether this is through a central or peripheral mechanism is presently unclear (2, 14). Recent work suggests that the A5 region may contain neurons that have an endogenous sensitivity to hypoxia and hypercapnia (39). The A5 is also excited by CSN activation (27, 33). Thus this region may be an integrator for PHxFD, responding to specific carotid body hypoxia and/or central hypoxia.

Specific Carotid Body Hypoxia Produces Progressive Augmentation

The second important finding of the present study is that PHxFD increased with each bout of hypoxia (40 Torr PO2). In anesthetized, artificially ventilated rats exposed to systemic isocapnic hypoxia, Bach et al. (2) also observed that PHxFD exhibited some bout dependence; however, in their preparation, PHxFD diminished with repeated bouts. In our experiments, the increasing magnitude of PHxFD gave rise to progressively augmenting frequency (and nVE) responses to subsequent bouts. This is a different manifestation of progressive augmentation to that described previously (30, 66). For example, Turner and Mitchell (66) observed progressive augmentation of fG and nVE in awake goats exposed to episodic normocapnic hypoxia. However, unlike the progressive augmentation that we described in which the Δpeak frequency increased, in the goat, the Δpeak frequency was unchanged. Instead, in Turner and Mitchell’s study, the absolute peak frequency increased (i.e., peak frequency compared with baseline before first bout). This progressive increase in frequency, they concluded, was a consequence of an augmenting bout baseline (underlying long-term facilitation). We have yet to observe long-term facilitation in systematic experiments using the DPP. Pregossi and Mitchell (30) found that treatment with the serotonin receptor antagonist methysergide blocked long-term facilitation but progressive augmentation of phrenic amplitude remained. Thus long-term facilitation and progressive augmentation appear to result from differing physiological mechanisms (30).

The different manifestations of progressive augmentation observed may be due to the experimental perturbation utilized: although we gave specific carotid body perturbations, Turner and Mitchell (66) applied systemic hypoxia. Additionally, as illustrated by progressive augmentation in VT in the duck, the nature of progressive augmentation may differ between species (48). It should also be noted that nonspecific electrical stimulation of the CSN does not produce progressive augmentation (35), suggesting that progressive augmentation resulting from peripheral inputs is either masked by anesthetics or unmasked by decerebration.

Specific Carotid Body Hypoxia and Hypercapnia Have Different Effects

A third finding of the present study is that specific carotid body hypoxia and hypercapnia produced distinct results. In
contrast to the effects of specific carotid body hypoxia, the frequency decline after specific carotid body hypercapnia (i.e., PhcFD) was bout invariant, and no progressive augmentation occurred. These differences may suggest that hypoxia and hypercapnia are coded by distinct populations of axons within the CNS and/or different temporal patterns of activity. Although there is no evidence that the carotid body uses a spatial code to transmit information regarding different sensory modalities to the central nervous system (43, 69), we have recently found that bouts of hypoxia and hypercapnia produce very different patterns of CNS activity. For example, after bouts of moderate to severe hypoxia, CNS activity fell below the prebout baseline activity (i.e., sensory posthypoxic decline; Ref. 17). However, this was absent after bouts of severe hypercapnia.

Alternatively, the differences in responses to bouts of hypoxia and hypercapnia in the present study may suggest that these phenomena are dependent on the degree to which the carotid body is activated. Although the level of chemostimuli that we chose to use produced similar phrenic burst frequencies (~15% over initial baselines, compare Fig. 7A and Fig. 7B), the nVt and nVe responses to specific carotid body hypoxia (40 Torr Po2) were noticeably greater than that for hypercapnia (60 Torr PCO2, compare raw data in Figs. 5 and 6).

Comparison to Previous Reports of PHxFD

The observation that a poststimulus frequency decline is elicited by many different mechanisms may illustrate its adaptive importance in the control and stability of breathing. This hypothesis assumes that the time-dependent effects that we observed are the same phenomena elicited both in other reduced preparations and in intact unanesthetized animals. In the DPP, maximum PHxFD after the first bout occurred ~1 min after termination of hypoxia. The recovery phase, over which phrenic activity returned to the prehypoxic baseline level, was 75% complete within 5 min. This was similar to the time course observed by Hayashi et al. (35) after electrical stimulation of the CNS in anesthetized, artificially ventilated rats. In addition, they found that maximum PHxFD after systemic hypoxia was also achieved ~1 min after termination of hypoxia, but they did not report the time course of recovery. Coles et al. (15) reported poststimulus frequency decline in intact spontaneously breathing rats, after both hypoxic and hypercapnic challenges, but did not report when the maximum occurred. However, recovery in both cases was complete by 5 min. These similarities, which occurred after stimuli that differed in nature, magnitude, and duration, lead us to speculate that these are manifestations of the same phenomenon. Interestingly, posthypoxic ventilatory decline occurs in awake and sleeping humans (6, 3, 32). For example, Badr et al. (3) showed that, during sleep, posthypoxic ventilatory decline was mediated by decreases in both Vt and frequency.

Conclusions

In conclusion, this is the first demonstration that intermittent specific carotid body chemostimulation (hypoxia, 40 Torr Po2, or hypercapnia, 60 Torr PCO2) is sufficient to elicit phrenic time-dependent effects in the rat. These effects include short-term depression, PHxFD, and PhcFD. Furthermore, the magnitude of PHxFD increases with bout, leading to progressive augmentation of each successive phrenic response in fP and nVe. These data suggest that, in intact rats, these phenomena are elicited, at least in part, by central integration of specific afferent information. Given that 1) poststimulus frequency decline can also be elicited in carotid sinus denervated non-anesthetized rats and 2) a similar pattern of activity is present in the CNS after chemostimulation of the carotid body, there would appear to be redundant mechanisms facilitating time-dependent responses, suggesting they may have significant adaptive importance in the respiratory chemoreponse.

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