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The ventilatory response to environmental hypercarbia in the South American rattlesnake, *Crotalus durissus*

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Abstract To study the effects of environmental hypercarbia on ventilation in snakes, particularly the anomalous hyperpnea that is seen when CO_2 is removed from inspired gas mixtures (post-hypercapnic hyperpnea), gas mixtures of varying concentrations of CO_2 were administered to South American rattlesnakes, *Crotalus durissus,* breathing through an intact respiratory system or via a tracheal cannula by-passing the upper airways. Exposure to environmental hypercarbia at increasing levels, up to 7% CO_2, produced a progressive decrease in breathing frequency and increase in tidal volume. The net result was that total ventilation increased modestly, up to 5% CO_2 and then declined slightly on 7% CO_2. On return to breathing air there was an immediate but transient increase in breathing frequency and a further increase in tidal volume that produced a marked overshoot in ventilation. The magnitude of this post-hypercapnic hyperpnea was proportional to the level of previously inspired CO_2. Administration of CO_2 to the lungs alone produced effects that were identical to administration to both lungs and upper airways and this effect was removed by vagotomy. Administration of CO_2 to the upper airways alone was without effect. Systemic injection of boluses of CO_2-rich blood produced an immediate increase in both breathing frequency and tidal volume. These data indicate that the post-hypercapnic hyperpnea resulted from the removal of inhibitory inputs from pulmonary receptors and suggest that while the ventilatory response to environmental hypercarbia in this species is a result of conflicting inputs from different receptor groups, this does not include input from upper airway receptors.

Keywords Breathing pattern · Hypercapnia · Intrapulmonary chemoreceptors · Snake, *Crotalus durissus* · Upper airway receptors

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Introduction

The ventilatory responses to environmental hypercarbia of many lizards and snakes appear peculiar. While low levels of environmental CO_2 almost always cause ventilation to increase, in many lizards (Boelaert 1941; Nielsen 1961; Templeton and Dawson 1963; Pough 1969; Ballam 1984; Klein et al. 2002) and snakes (Glass and Johansen 1976; Gratz 1979; Coates and Ballam 1989) breathing is depressed by higher levels of environmental CO_2 ($>3\%$). In general, CO_2 always causes tidal volume to increase but the higher concentrations usually result in a substantial fall in breathing frequency and total ventilation. More surprisingly, return from hypercarbia to air is accompanied by a marked transient increase of ventilation relative to values during hypercarbic exposure. This rebound overshoot in ventilation has been termed a post-hypercapnic hyperpnea.

All species of lizards and snakes are believed to possess peripheral arterial chemoreceptors and it is assumed that all possess central CO_2/pH-sensitive receptors as well. Stimulation of these receptors by increases in arterial levels of CO_2 (hypercapnia) results in an elevation in

ventilation (see Milsom 1995 for review). Both lizards and snakes possess intrapulmonary chemoreceptors that are inhibited by increasing levels of CO_2 and it has been shown that high levels of environmental CO_2 (hypercarbia) act on these chemoreceptors to elevate tidal volume and reduce breathing frequency (Fedde et al. 1977; Furilla and Bartlett 1988). Both lizards and snakes also possess upper airway chemoreceptors in the nasal epithelium whose discharge is stimulated by CO_2 and continuous exposure to hypercarbia acts on these receptors to dramatically inhibit breathing frequency with little effect on tidal volume (Coates and Ballam 1987, 1989). Snakes also appear to possess a further group of vomeronasal receptors that produce an increase in breathing frequency and ventilation in response to environmental hypercarbia (Coates and Ballam 1989).

The physiological roles of these various CO_2-sensitive airway receptors are not known. It has been proposed that the intrapulmonary chemoreceptors are involved in breath-by-breath control of breathing, both enhancing inspiration in a positive feedback fashion once a breath has been initiated, as well as contributing to inspiratory termination much as pulmonary stretch receptors do in mammals (Banzett and Burger 1977; Milsom et al. 1981; Cross et al. 1980; Furilla and Bartlett 1989). The upper airway receptors are not involved in breath-by-breath control of ventilation. While no role for the modulatory effects of the vomeronasal receptors has yet been proposed, it has been suggested that the receptors found in the nasal epithelium may function to detect changes in environmental CO_2 originating from prey or predators (Ballam 1985; Coates and Ballam 1987, 1989) although why this should lead to a net inhibition of breathing and change in breathing pattern is not at all clear.

Differences in the ventilatory responses to CO_2 of various species of lizards and snakes are now thought to be due to differences in afferent sensitivities and/or central nervous system processing of afferent information from different receptor groups. Depending on the balance, a wide spectrum of responses ranging from an increase to no change or a decrease in ventilation may ensue. It is generally in those species that show no increase, or a reduction in breathing frequency in response to inhaled CO_2, that the post-hypercapnic hyperpnea is seen when CO_2 is removed from the gas mixture (Nielsen 1961, Templeton and Dawson 1963; Glass and Johansen 1976; Nolan and Frankel 1982). This has been interpreted to reflect the transient expression of the stimulatory effect of systemic hypercapnia, which was masked by the inhibitory effect of the tonically elevated airway CO_2, as the airway inhibition is removed more quickly than the systemic CO_2 load (Nielsen 1961; Templeton and Dawson 1963). The post-hypercapnic hyperpnea is reduced or eliminated at higher temperatures when CO_2 washout from blood, lungs and airways is faster due to increased pulmonary perfusion and ventilation (Klein et al. 2002).

The goals of the present study were to examine the post-hypercapnic hyperpnea more closely with two specific questions in mind. To begin with we wanted to examine the magnitude of the post-hypercapnic hyperpnea as a function of the level of environmental hypercarbia and the magnitude of the hypercapnic ventilatory response. The literature reports conflicting data concerning a possible correlation between the level of environmental hypercarbia and the magnitude of the response to CO_2 removal (Templeton and Dawson 1963, Nielsen 1961; Nolan and Frankel 1982; Klein et al. 2002). We hypothesized that if the post-hypercapnic hyperpnea represents the transient expression of a stimulatory effect of systemic hypercapnia as an airway inhibition is removed, then this rebound effect should be greater in instances where both the excitatory effect of the systemic hypercapnia (i.e. the level of CO_2) and the inhibitory effect on the airway CO_2-sensitive receptors (as indicated by the reduction in steady-state breathing frequency during sustained hypercarbia) were greater. Secondly, we wanted to examine the relative roles of upper airway versus pulmonary receptors in contributing to this response.

Materials and methods

Experiments were performed on ten rattlesnakes, *Crotalus durissus*, weighing 599 ± 52 g (mean $\pm$ SE). All snakes were captured in the state of São Paulo, Brazil and reared in captivity. The snakes had been fed a diet of mice but fasted for 2 weeks prior to experimentation. They were maintained at room temperature ($\sim$25°C) both before and during experiments.

Surgery

For anesthesia, each snake was placed into a plastic bag flushed with pure CO_2 until all righting and withdrawal reflexes were abolished (Wang et al. 1993). Animals continued to breathe CO_2 through a mask while the right subclavian artery was exposed by a short (2–3 cm) ventro-lateral incision and a non-occlusive catheter (PE 50) was inserted through the artery into the dorsal aorta. The incision was then closed. For series I, in eight snakes a face mask constructed from the bottom half of a 60-ml syringe barrel was then attached to the snake by a latex rubber cuff, and sealed at the neck using duct tape (series I; Fig. 1). A period of at least 12 h was allowed for recovery after surgery before any measurements were made. Following completion of this series of experiments, six of these same snakes were re-anesthetized, the mask removed, and the snakes intubated. The tracheal cannula was sewn in place with a purse string suture sealing the glottis around the cannula and the cannula was run out through the skin behind the angle of the jaw. A secondary, small mask was also glued over the nostrils of these animals at this time (series II; Fig. 1). Another period of at least 12 h (overnight) was allowed for recovery after this surgery and measurements were made the following day. Following completion of this series of experiments, four of these same animals were re-anesthetized and their vagus nerves were sectioned high in the neck through small lateral incisions (series III). The incisions were then closed and another period of at least 12 h (again, overnight) was allowed for recovery before any measurements were made the following day.

Blood pressure and blood gas measurements

The arterial catheter was connected to a Deltran pressure transducer kept level with the heart of the animal. A water column was used to calibrate the transducer before and after each experiment.

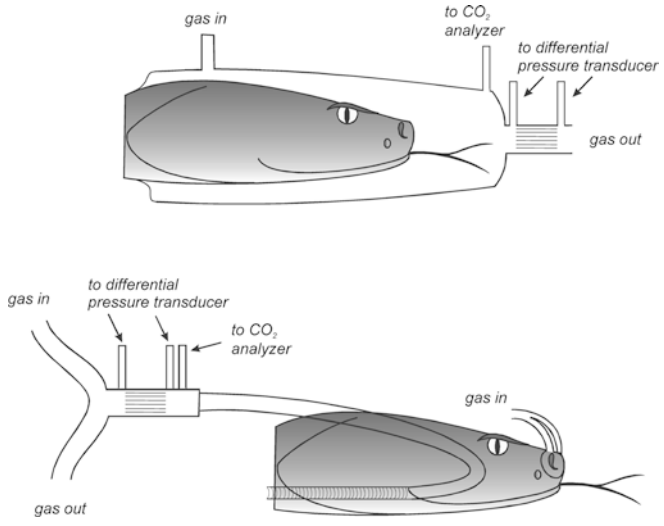

Fig. 1 Schematic diagram showing the experimental setup for measuring ventilation in series I (*upper*) and series II/III (*lower*)

The pressure signal was amplified and stored on computer (Sable Systems v. 2.0 for Datacan V sampling at a rate of one sample every 0.22 s).

For blood sampling, pressure measurements were interrupted and samples (0.6 ml) were withdrawn into heparinized 1-ml syringes via a three way stopcock. The samples were immediately analyzed for PO_2, and pH using a Radiometer BMS3 system. They were also analyzed for total O_2 and CO_2 content using a Tucker chamber and Cameron chamber, respectively (Tucker 1967; Cameron1971). The O_2 electrodes were calibrated using pure nitrogen and humidified air and the pH electrode was calibrated using Radiometer precision buffers (S1500 and S1510). PCO_2 was calculated from the pH and total CO_2 content using the Henderson-Hasselbach equation and using pK' and CO_2 solubility values derived from Heisler (1989). All samples were also analyzed for hematocrit.

Ventilation measurements

Pulmonary ventilation was measured using the pneumotachographic method in all instances. In Series I, the mask was ventilated at 3000 ml min^{-1} with the gas entering the mask near the back of the head of the snake and exiting the mask via a Fleisch tube attached at the front (Fig. 1). In series II and III the Fleisch tube was attached between the tracheal cannula and a gas line through which gas flowed at 3000 ml min^{-1} (Fig. 1). A grid provided laminar flow within the Fleisch tube to achieve a direct relationship between the instantaneous values for flow and the pressure difference across the grid. The pressure difference was monitored by means of a differential pressure transducer (Sable PT100) connected to a computer data acquisition system (Sable Systems v. 2.0 for Datacan V sampling at a rate of one sample every 0.22 s). The pneumotachographs were calibrated before and after each experiment by injecting known volumes at different flow rates through each system from a syringe inserted into the system in lieu of the snake. In series II and III, gas was also delivered to the nose of the animal at a rate of 500 ml min^{-1}. This gas would enter the nares whenever the nostrils opened and exit the mouth via the internal nares (Fig. 1).

Inspired levels of CO_2 were monitored using a gas analyzer (Sable Systems CA-1B CO_2 Analyzer). In series I, gas was sampled from the inside of the facemask, just before the outflow, at 100 ml min^{-1} (Fig. 1). This also served to eliminate dead space within the mask. In series II gas was sampled from the tracheal cannula (Fig. 1), also at 100 ml min^{-1}. The gas analyzer was calibrated with air and CO_2 mixtures produced from bottled gases by a gas-mixing pump (GF-3MP, Cameron Instruments).

Experimental protocol

Series I

In this series, snakes equipped with a face mask were allowed to rest, breathing air overnight in an isolated, temperature controlled (25°C) chamber. On the day of the experiment, all equipment was calibrated and then after recording baseline values for at least 2 h, the air was replaced with 3%, 5%, or 7% CO_2 for 1-h periods in random order. The gases were produced with a gas-mixing pump (GF-3MP, Cameron Instruments). Animals were returned to breathing air for at least 1 h between each CO_2 run. In this series, all inspired gases would transit the entire respiratory system.

Series II and III

In these series, the snakes were equipped with a tracheal cannula that was attached, via a T-connector, to a gas line running through the chamber and exhausting outside the chamber. In this way, the composition of the gas the snake inspired into its lungs could be controlled independently of the gas surrounding the body and upper airways. A second gas line was connected to the small secondary mask attached over the nostrils and in this way the composition of the gas administered to the upper airways could be controlled independently of the gas going to the lungs. These animals were also allowed to rest, breathing air overnight in an isolated chamber. On the day of this experimental series, after recording baseline values for at least 2 h, the air was replaced with 5% CO_2 (balance air) provided from a gas mixing pump (Cameron GF-3MP) and could be delivered to either the lungs only, the upper airways only or to both the upper airways and lungs, in random order, for 1 h each. Animals were always administered air to both lungs and upper airways for at least one hour between CO_2 runs. In series III (vagotomized animals), the 5% CO2 was administered to the lungs only.

Series IV

In this series two animals, fitted with a mask, had 10 ml of blood withdrawn and tonometered with 100% CO_2. The arterial cannula, in both instances, had been advanced to sit immediately outside the valves of the heart within the right systemic arch (checked visually at autopsy). These animals were subsequently administered rapid injections (roughly 30–60 s) of either 2 ml of normal blood (withdrawn and subsequently reinjected with no CO_2 added) or 2, 3, or 4 ml of tonometered blood, while all cardiorespiratory variables were recorded continuously.

Calculations and statistics

Blood gases and hematocrit (Hct) were measured at the end of each initial air run as well as at the end of each CO_2 run in all series. All cardio-respiratory variables were measured continuously under steady state control (normocapnia) and hypercarbic conditions and analyzed during the last 15 min of 1-h exposures and during the first 30 s and each minute (up to 20 min) of the recovery period following the return to inspiring air after hypercarbic exposures. For ventilation, measures were made of the tidal volume, length of each expiration (T_{exp}), inspiration (T_{insp}), the pause between subsequent breaths (T_{NVP}), and the corresponding overall breathing frequency. Total ventilation was calculated by multiplying the mean tidal volume by the breathing frequency. Heart rate was counted from the blood pressure trace.

Data were compared using a one-way repeated measures analysis of variance combined with Tukey's test for differences between individual means. All values expressed as percentage change were arcsin transformed before the analysis was run. Significance was accepted at $P < 0.05$.

Results

Table 1 compares the levels of all resting cardio-respiratory variables for animals breathing air in each of the treatment groups (series). Animals in series I wore a mask while those in series II were intubated and hence their glottis was bypassed. Since reptiles normally pause between breaths, at end-inspiration with a breath-hold maintained against a closed glottis, intubation leads to a reduction in resting lung volume and a change in breathing pattern. Animals now begin each breath with a small, active expiration (to below resting lung volume) followed by an active inspiration; but, instead of the inspiration being followed by a breath-hold, it is followed by a passive expiration. The net result of this manipulation was a slowing of the breathing rate (primarily due to an increase in the pause between breaths), and an increase in tidal volume with no net effect on total ventilation (Table 1). This was accompanied by a small, but significant, increase in heart rate but no change in arterial blood pressure (Table 1). Animals in series III were also intubated and were vagotomized. Amongst other things, this will have removed all feedback from pulmonary receptors. The net result was a dramatic slowing of breathing frequency but no further change in tidal volume (Table 1). Total ventilation, overall, was reduced (again primarily due to an increase in the pause between breaths). This also removed parasympathetic vagal tone to the heart and resulted in an increase in heart rate. Despite this, arterial blood pressure was still maintained constant (Table 1).

Table 1 Cardio-respiratory variables for animals breathing air in the different treatment protocols

	Series I	Series II	Series III
Arterial blood pressure (mmHg)	34.41 ± 3.30	33.97 ± 3.17	33.55 ± 2.33
Heart rate (beats min^{-1})	23.84 ± 1.33	37.99 ± 3.67*	52.43 ± 3.86*, **
Breathing frequency (breaths min^{-1})	3.69 ± 0.25	2.26 ± 0.19*	0.66 ± 0.10*, **
Tidal volume (ml kg^{-1})	12.82 ± 1.52	22.52 ± 2.71*	18.00 ± 4.50
Total ventilation (ml kg^{-1} min^{-1})	47.25 ± 1.09	50.98 ± 1.29	11.81 ± 0.87*, **
Expiratory time (s)	2.74 ± 0.51	3.57 ± 0.51	4.49 ± 0.14
Inspiratory time (s)	3.31 ± 0.28	3.98 ± 0.35	3.45 ± 0.75
Non-ventilatory period (s)	12.19 ± 1.12	27.98 ± 5.28*	99.27 ± 13.93*, **
Mass (g)	591 ± 46	596 ± 59	608 ± 71

Series I: wearing a mask; series II: with an endotracheal tube and nose mask; series III: with an endotracheal tube and nose mask following vagotomy

*Denotes values that are significantly different from values in series I;

**indicates values in series III that are significantly different from values in series II

Steady-state responses to CO_2

Series I

This series was designed to examine the effects of different levels of environmental hypercarbia on breathing in animals wearing the mask. Figure 2 shows breathing traces from one snake in series I illustrating the patterns of breathing present under steady state conditions after 1-h exposures to different levels of CO_2. Table 2 shows the changes in blood gases and pH that occurred during exposure to each level of hypercapnia, while Fig. 3 and Table 3 present the corresponding effects of each exposure on cardiovascular and ventilatory variables. Increasing levels of hypercarbia led to a progressive increase in tidal volume and fall in breathing frequency (Fig. 3). The net effect was a small increase in total ventilation that was greatest (and only significant) when animals were breathing 5% CO_2 (Fig. 3). While there were no significant changes in inspiratory time, the expiratory interval and the pause between breaths (non-ventilatory period) progressively increased (Table 3). This was also accompanied by an increase in heart rate and a progressive rise in arterial blood pressure that became significant when animals were breathing 7% CO_2 (Fig. 3).

Series II and III

In series II and III, snakes breathing spontaneously were induced to inspire 5% CO_2 either through the upper airways and lungs, into the lungs alone or through the upper airways alone in an attempt to resolve the location of the receptors responsible for the changes in breathing

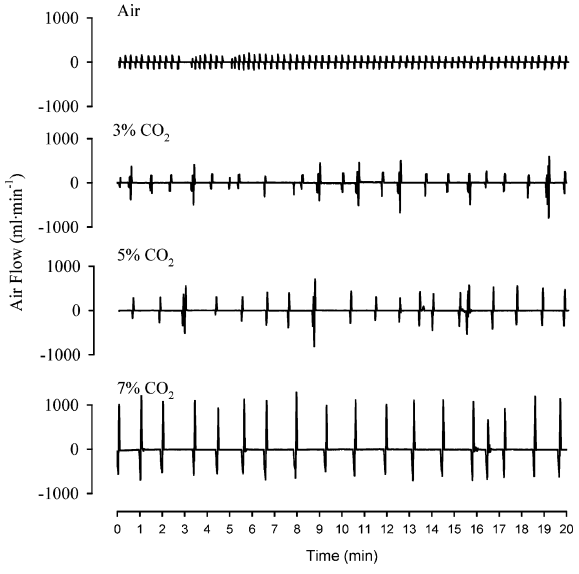

Fig. 2 Traces of respiratory air flow (inspiration is *up*) from one snake (series I) illustrating steady-state responses in ventilation to various levels of hypercarbia

Table 2 Values for blood gases and hematocrit (Hct) for animals wearing a mask and breathing various concentrations of CO_2

	Air	3% CO_2	5% CO_2	7% CO_2
PO_2 (mmHg)	75.4 ± 5.0	84.9 ± 6.1	95.5 ± 2.4*	93.6 ± 4.6*
O_2 content (vol %)	10.3 ± 1.1	9.7 ± 0.9	8.9 ± 0.5	7.6 ± 0.7
pH	7.48 ± 0.03	7.32 ± 0.05*	7.18 ± 0.03*	7.11 ± 0.03*
PCO_2 (mmHg)	17.0 ± 1.1	29.4 ± 2.9*	40.9 ± 2.9*	50.5 ± 4.8*
CO_2content (mmol l^{-1})	15.0 ± 0.9	18.4 ± 1.9	18.6 ± 0.6	20.1 ± 1.4*
Hct (%)	24.8 ± 1.3	25.2 ± 1.7	28.6 ± 1.4	27.3 ± 1.6

*Denotes values that are significantly different from those recorded while animals were breathing air

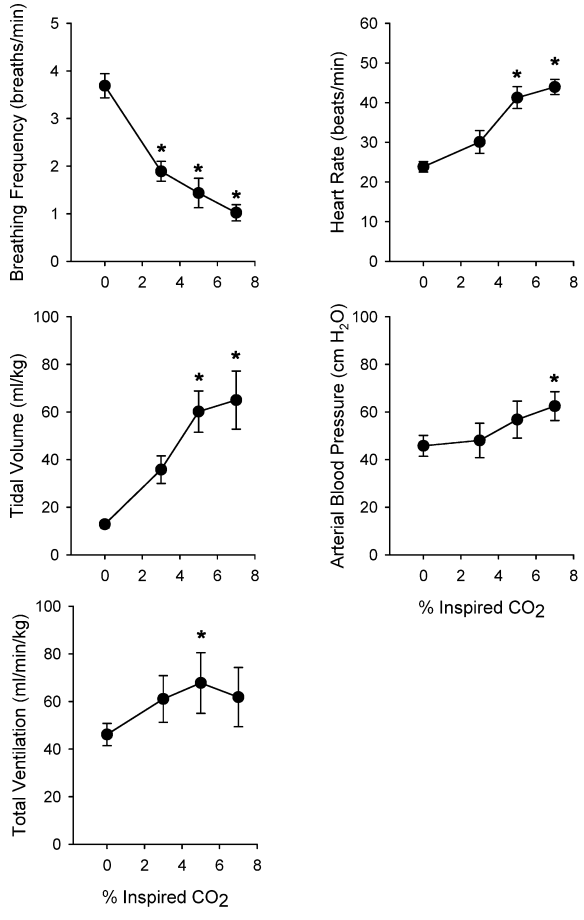

Fig. 3 Effect of 1 h exposure to different levels of hypercarbia on breathing frequency, tidal volume, total ventilation, heart rate and arterial blood pressure in snakes breathing through a mask (series I). Values are mean ± SEM ($n = 8$); *asterisk* denotes significant difference from values in animals breathing air

pattern seen when CO_2 was administered through the mask. The effects of each treatment on blood gases, for all Series, are shown in Table 4. Note there were no changes in blood gases when the 5% CO_2 was delivered to the upper airways alone since this gas did not come in

Table 3 Respiratory intervals for animals wearing a mask (series I) and breathing various concentrations of CO_2

	Expiratory time (s)	Inspiratory time (s)	Non-ventilatory period (s)
Air	2.74 ± 0.51	3.31 ± 0.28	12.2 ± 1.1
3% CO_2	3.83 ± 0.44	3.69 ± 0.18	31.4 ± 3.8
5% CO_2	3.98 ± 0.42	3.74 ± 0.42	47.4 ± 8.2*
7% CO_2	4.13 ± 0.55*	3.41 ± 0.38	70.2 ± 10.5*

*Denotes values that are significantly different from those recorded while animals were breathing air

contact with the pulmonary exchange surface. Also note that the reduction in overall breathing seen in vagotomized animals (Table 1) led to a fall in PO_2 and CO_2 retention under normocarbic conditions (Table 4). None-the-less, inspiration of 5% CO_2 still led to a significant rise in PCO_2 in the vagotomized animals (Table 4).

When 5% CO_2 was administered to both the lungs and upper airways in these experiments, the reductions in breathing frequency and increases in tidal volume were similar to those seen when the CO_2 was administered through the mask in series I (Fig. 4). The decrease in frequency was larger, however, and the rise in tidal volume smaller such that there was now no significant increase in total ventilation. The results were identical when CO_2 was administered to the lungs alone. Administration of CO_2 to the upper airways alone had no effect on ventilation (Fig. 4). Following vagotomy (series III), the administration of 5% CO_2 to the lungs alone led to a significant rise in total ventilation due primarily to a (non-significant) increase in tidal volume (Fig. 4). Breathing frequency did not fall during CO_2 exposure post-vagotomy.

In the case of animals in which the 5% CO_2 gas mix was administered to both the lungs and upper airways (''mask'' and ''both'' in Fig. 4) or the lungs alone, there was a small but non-significant increase in heart rate and arterial blood pressure. This trend was absent in animals in which the 5% CO_2 was delivered to the upper airways alone (and hence did not come in contact with the blood; Table 4) and in vagotomized animals where the heart rate was already elevated due to an absence of cardiac parasympathetic tone (see Table 1).

Series IV

In series IV, two animals received bolus Injections of blood equilibrated with 100% CO_2 into the aortic arch just outside the heart, in an attempt to mimic the effects of a ''metabolically produced CO_2 load'' (hypercapnia versus hypercarbia). The results were similar in both cases and data for one individual are shown in Fig. 5. Bolus injections of CO_2-rich blood just outside the heart invariably led to immediate, transient increases in both breathing frequency and tidal volume (Fig. 5).

Table 4 Levels of blood gases and pH for animals inspiring air and 5% CO_2 via different routes

	PO_2(mmHg)		O_2 content (vol %)		pH		PCO_2 (mmHg)		CO_2 content (mmol l^{-1})	
	Air	5% CO_2	Air	5% CO_2	Air	5% CO_2	Air	5% CO_2	Air	5% CO_2
Mask	75.4 ± 5.0	95.5* ± 2.4	10.3 ± 1.1	8.9 ± 0.5	7.48 ± 0.03	7.18* ± 0.03	19.7 ± 0.8	40.9* ± 3.2	15.0 ± 0.9	18.6* ± 0.6
Both	56.5 ± 7.0	75.0 ± 10.1	8.2 ± 1.2	6.9 ± 1.2	7.44 ± 0.04	7.12* ± 0.04	18.3 ± 1.3	45.5* ± 3.0	15.0 ± 1.5	18.7* ± 1.0
Lungs	56.5 ± 7.0	62.0 ± 12.2	8.2 ± 1.2	6.3 ± 1.4	7.44 ± 0.04	7.19* ± 0.08	18.3 ± 1.3	41.9* ± 6.6	15.0 ± 1.1	18.9* ± 0.6
Upper Airways	56.5 ± 7.0	53.9 ± 5.2	8.2 ± 1.2	8.1 ± 1.2	7.44 ± 0.04	7.39 ± 0.05	18.3 ± 1.3	20.3 ± 1.4	15.0 ± 1.1	14.9 ± 0.8
Vagotomy	49.5 ± 12.8	74.1* ± 4.92	4.7 ± 1.2	5.3 ± 0.5	6.99 ± 0.05	6.94 ± 0.08	43.4 ± 3.4	59.7* ± 4.7	14.3 ± 2.2	17.0* ± 1.3

Mask: to both lungs and upper airways via a mask; *Both*: to both lungs and upper airways simultaneously via an endotracheal tube and nose mask; *Lungs*: to the lungs alone via an endotracheal tube; *Upper airways* to the upper airways alone via a nose mask; *Vagotomy* to the lungs alone via an endotracheal tube following vagotomy

*Denotes values that are significantly different from those recorded while animals were breathing air in that treatment group

"Post-hypercapnic hyperpnea"

Series I

Figure 6 illustrates the post-hypercapnic hyperpnea in one animal following one hour periods of breathing 3%, 5%, and 7% CO_2 via a mask (series I). The results for all animals are shown quantitatively in Fig. 8 (left-hand panels). The higher the level of CO_2, the lower the level of respiratory frequency. Once the CO_2 was removed, breathing frequency immediately increased to roughly the same maximum level, regardless of the level of CO_2 the animal had been breathing, before slowly declining back to typical air breathing values (Fig. 8, left centre panel). The higher the level of CO_2, the greater the tidal volume at the end of the hypercarbic episode. Within the first breath after the CO_2 was removed from the gas stream, however, tidal volume increased further and did so in a dose-dependent fashion (Fig. 8, left top panel). The net result was a dramatic overshoot in total ventilation that was also dose dependent. (Fig. 8, bottom left panel).

Series II and III

Figure 7 illustrates the post-hypercapnic hyperpnea for one intubated animal and quantitative data for all animals are shown in Fig. 8 (right-hand panels). The post-hypercapnic hyperpnea for the animals receiving 5% CO_2 to both lungs and upper airways or to the lungs alone was very similar to that of the animals wearing a mask in series I (Fig. 8; compare right- and left-hand panels). There was no effect of removing CO_2 from the air stream delivered to the upper airways alone (Fig. 8) and in the vagotomized animals both tidal volume and total ventilation rapidly returned to normocarbic values (Fig. 8). There was no post-hypercapnic hyperpnea in either of these latter two cases.

Discussion

Critique of methods

Animals in series I wore a mask while those in series II and III were intubated and their glottis was bypassed during breathing. This led to a significant change in resting breathing pattern (reduced frequency but increased tidal volume) but not in total ventilation (Table 1). Reptiles normally pause between breaths at

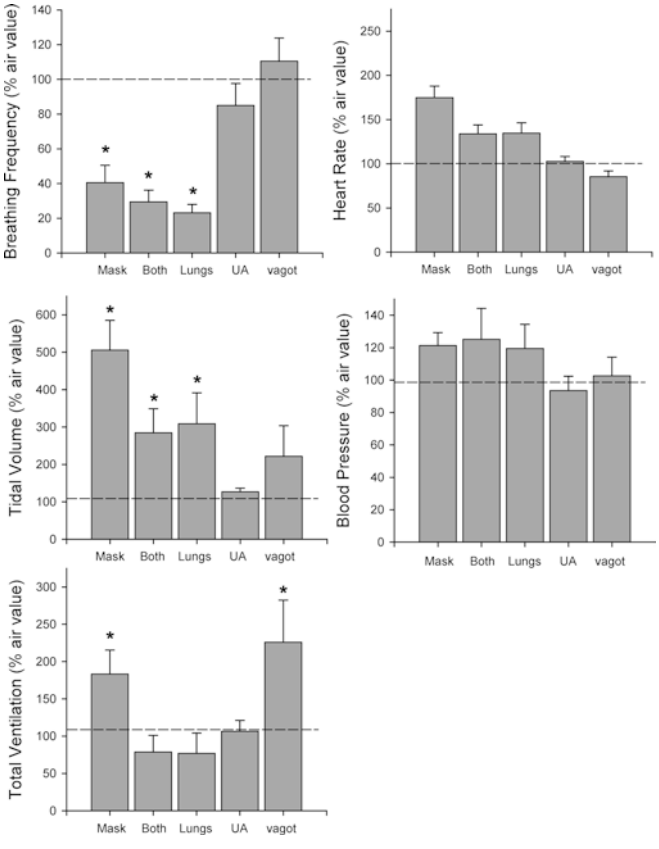

Fig. 4 Effect of 1 h exposure to 5% CO_2 on breathing frequency, tidal volume, total ventilation, heart rate and arterial blood pressure in snakes inspiring CO_2 via different routes (to both lungs and upper airways via a mask (*Mask*; $n = 8$); to both lungs and upper airways simultaneously via an endotracheal tube and nose mask (*Both*, $n = 6$); to the lungs alone via an endotracheal tube (*Lungs*, $n = 6$), to the upper airways alone via a nose mask (*UA*, $n = 6$), and to the lungs alone via an endotracheal tube following vagotomy (*Vagot*, $n = 4$)). Values are expressed as a percentage of the values for animals breathing air before CO_2 exposure; *asterisk* denotes values that are significantly different from those recorded while animals were breathing air in that treatment group

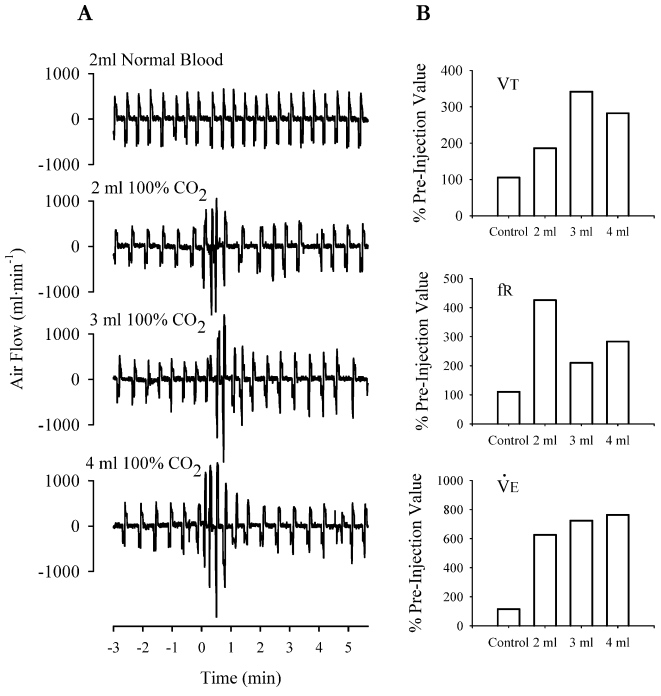

Fig. 5 A *Left panels*: traces of respiratory air flow from one snake (series IV) illustrating the changes in ventilation that occurred in response to bolus injections of different volumes of normal blood or blood that had been tonometered with 100% CO_2. Injections took place at time 0. **B** *Right panels*: the changes in tidal volume, breathing frequency and total ventilation that occurred at the peak of the response in this individual as a result of the bolus injections shown on the left expressed as a percentage of the pre-injection value

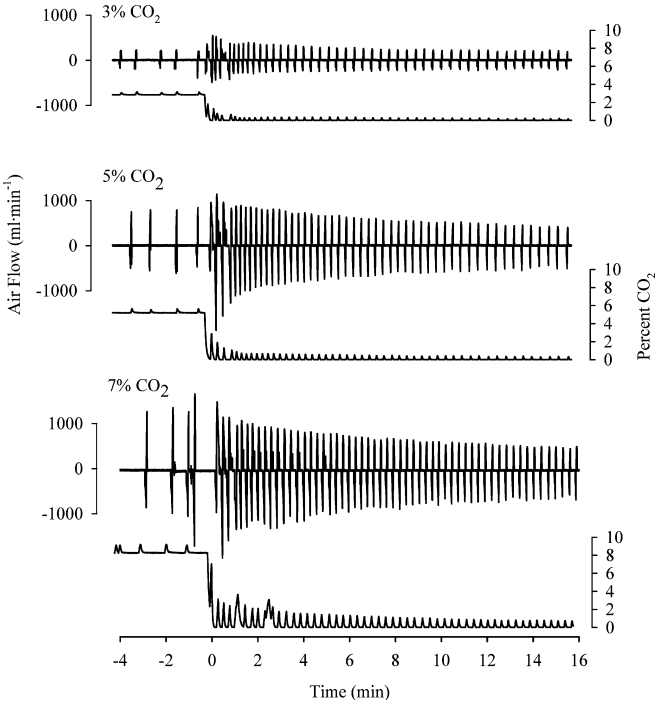

Fig. 6 Traces of respiratory air flow, and mask CO_2 levels from one snake (series I) illustrating the post-hypercapnic hyperpnea in ventilation that occurred during the return to air after breathing various levels of CO_2. The switch back to breathing air occurred at time 0

end-inspiration with a breath-hold maintained against a closed glottis. Following intubation, this was not possible since the lungs passively deflated following active inspiration with no glottal trapping. Slow, deep breathing such as this is seen in many vertebrates as a consequence of a reduction in lung volume and tonic pulmonary receptor feedback (Milsom 1990) and may represent an attempt to reinflate the lungs (Sanders and Milsom 2001). Nonetheless, intubated animals responded to inspiration of CO_2 in much the same manner as animals with an intact glottis (''both'' versus ''mask'', Fig. 4). While there was no longer a significant increase in total ventilation, primarily because the increase in tidal volume was proportionately smaller in the intubated animals, this most likely reflected the large tidal volume seen in normocarbia following intubation (Table 1). The maximum tidal volumes seen in animals breathing 5% CO_2 were similar in both cases (65.8 ± 17.9 and 60.13 ± 8.7 ml kg^{-1}, respectively). Both groups showed significant reductions in breathing frequency and significant increases in tidal volume during hypercapnia and a significant post-hypercapnic hyperpnea, and thus the data obtained using this technique were adequate for addressing our questions.

Besides being intubated, animals in series III were also vagotomized. This will have removed all feedback from pulmonary receptors and the net result was a dramatic further slowing of breathing frequency, but no further change in tidal volume (Table 1). Total ventilation, overall, was reduced (primarily due to an increase in the pause between breaths). This change in breathing pattern led to a fall in PO_2 and rise in PCO_2 although neither was significant (Table 4). We considered how we would interpret our results if there was an absence of a hypercarbic ventilatory response in these animals, since it would be possible that it could have arisen due to an inability to slow frequency or increase volume further in animals that were already marginally hypercarbic. We were reassured, however, when these animals did produce a significant response that now consisted of a rise in total ventilation on exposure to 5% CO_2 and these data have proven useful for interpreting our results.

Our data show that exposure to environmental hypercarbia at levels up to 7% CO_2 results in only modest increases in net ventilation in the South American rattlesnake, *C. durissus*. It does, however, produce a dramatic change in breathing pattern with increasing levels of CO_2 leading to a progressive decrease in breathing frequency and increase in tidal volume. On return to breathing air, there are immediate and transient increases in tidal volume and breathing frequency that lead to a pronounced increase in ventilation. This post-hypercapnic hyperpnea is greater when animals have been breathing higher levels of CO_2 and both the

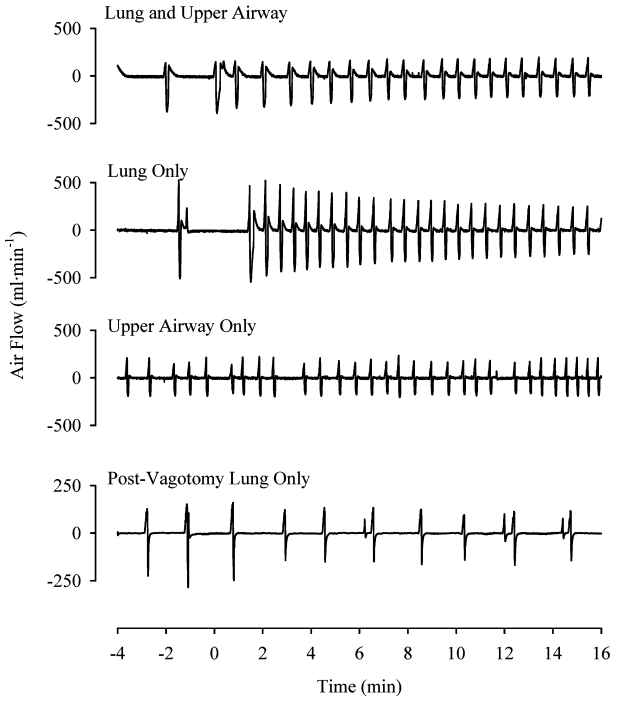

Fig. 7 Traces of respiratory air flow from one snake (series II and III) illustrating the post-hypercapnic hyperpnea in ventilation that occurred during the return to air after breathing 5% CO_2 by different routes (to both lungs and upper airways simultaneously via an endotracheal tube and nose mask (*Lungs and Upper Airway*, $n=6$); to the lungs alone via an endotracheal tube (*Lungs Only*, $n=6$), to the upper airways alone via a nose mask (*Upper Airways Only*, $n=6$), and to the lungs alone via an endotracheal tube following vagotomy (*Post-Vagotomy Lungs Only*, $n=4$)). The switch back to breathing air occurred at time 0

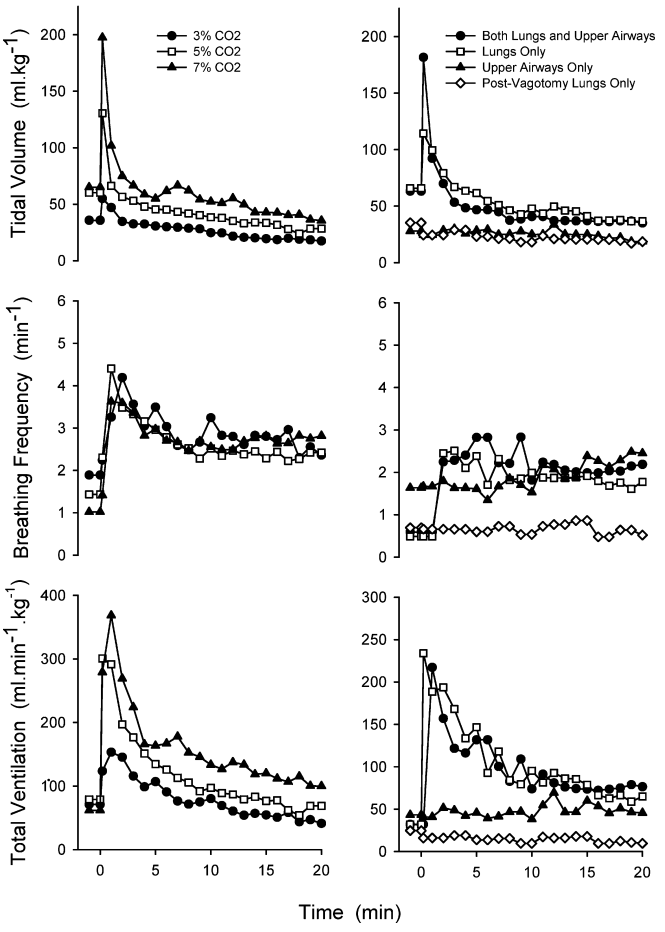

Fig. 8 *Left panels*: the changes in tidal volume, breathing frequency and total ventilation that occurred in all snakes in series I ($n=8$) during the return to air after breathing different levels of CO_2 for 1 h ($n=8$). *Right panels*: the changes that occurred in tidal volume, breathing frequency and total ventilation in all snakes in series II and III during the return to air after breathing 5% CO_2 by different routes for 1 h (to both lungs and upper airways simultaneously via an endotracheal tube and nose mask (*Both Lungs and Upper Airway*, $n=6$); to the lungs alone via an endotracheal tube (*Lungs Only*, $n=6$), to the upper airways alone via a nose mask (*Upper Airways Only*, $n=6$), and to the lungs alone via an endotracheal tube following vagotomy (*Post-Vagotomy Lungs Only*, $n=4$). The switch back to breathing air occurred at time 0

changes in breathing pattern and the post-hypercapnic hyperpnea arise from an interaction between the effects of CO_2 acting on receptors at multiple sites.

Steady-state response

It is becoming clear that during the steady-state response to environmental hypercarbia in snakes and lizards there are conflicting excitatory and inhibitory inputs arising from different receptor groups. The net effect of these inputs on total ventilation is a result of the relative strengths of each input. Thus, inhalation of CO_2 in different species of snakes and lizards produces a variety of responses. In some lizards (*Crotaphytus collaris*, Templeton and Dawson 1963; *Lacerta* sp., Boelaert 1941; Nielsen 1961; *Tupinambis*, Ballam 1984; *Uma notata* and *Dipsosaurus dorsalis*, Pough 1969) and snakes (*Acrocordus*, Glass and Johansen 1976; *Natrix*, Gratz 1979; *Thamnophis*, Coates and Ballam 1989) breathing is depressed at higher levels of environmental CO_2 due to reductions in breathing frequency. In other snakes and lizards minute ventilation continues to increase when gas mixtures containing increasing levels of CO_2 (2–10%) are inspired (*Coluber constrictor*, Nolan and Frankel 1982; *Drymarchon*, Randall et al. 1944; *Varanus exanthematicus*, Glass and Wood 1983; *Uromastyx aegypticus*, Klein et al. 2002). The results for *Crotalus durissus* fall somewhere in between with total ventilation increasing with hypercarbia up to 5% CO_2 and only then beginning to fall, again due to reductions in breathing frequency.

"Post-hypercapnic hyperpnea"

In the lizards, *Crotaphytus collaris, Lacerta viridis* and *Uromastyx aegypticus* (Templeton and Dawson 1963; Nielsen 1961; Klein et al. 2002) the snakes, *Acrochordus javanicus* and *Coluber constrictor* (Glass and Johansen

1976; Nolan and Frankel 1982) the anuran amphibian, *Rana catesbeiana* (Kinkead and Milsom1996) and the South American lungfish, *Lepidosiren paradoxa* (Sanchez and Glass 2001), an immediate hyperpnea is seen when inspired hypercarbic gas is replaced with a normocarbic gas mixture (a post-hypercapnic hyperpnea). *C. durissus* can now be added to this list. Previous investigators have interpreted this response to suggest that during conditions of environmental hypercarbia, the stimulating effect of systemic hypercapnia is masked by an inhibitory effect of tonically elevated airway CO_2 (Boelart 1941; Nielsen 1961;Templeton and Dawson 1963; Nolan and Frankel 1982; Coates and Ballam 1989; Kinkead and Milsom 1996; Klein et al. 2002). When animals begin to breathe normocarbic air again, arterial CO_2 levels will still be elevated for some time but the level of CO_2 in the airways will be elevated only during expiration. Arterial levels of CO_2 and end-expiratory levels of CO_2 will fall slowly as whole body CO_2 stores are lowered and CO_2 is eliminated, whereas inspired CO_2 levels will fall immediately. We hypothesized initially that if this were so, then the post-hypercapnic hyperpnea should be greater in instances where both the excitatory effect of the systemic hypercapnia (i.e., the level of CO_2) and the inhibitory effect on airway CO_2-sensitive receptors (as indicated by the reduction in steady-state breathing frequency during sustained hypercarbia) were greater. This was indeed the case in the present study. The post-hypercapnic hyperpnea was greater when the level of CO_2 inspired before the return to breathing air had been greater. The literature contains reports suggesting both that there is (Templeton and Dawson 1963) and is not (Nielsen 1961; Nolan and Frankel 1982;) a correlation between the level of environmental hypercarbia and the magnitude of the response to CO_2 removal. Interestingly, Klein et al. (2002) found that there was a correlation when the data were expressed in absolute terms but that there was not when the data were expressed in relative (proportionate change) terms. Our data support the former report, regardless of how the data are expressed. Why consistent results have not been found in all studies is hard to say but this may reflect the kinetics of the receptor responses. It is possible that receptor kinetics are such that there is a maximum response that can be produced and in some species this is produced at lower levels of inspired CO_2 masking this correlation.

The magnitude of the inhibitory effect of CO_2 acting on the airway receptors in *C. durissus* can, perhaps, best be seen in Fig. 9. While total ventilation only increased modestly during hypercarbia (and this was significant only at 5% CO_2), there was a steep, linear relationship between ventilation and the inspired CO_2 levels during the onset (1st minute) of the post-hypercapnic hyperpnea. These data suggest that both breathing frequency and tidal volume were reduced by the inhibitory effects of CO_2, and, that the net effect was extremely large. If our hypothesis is correct, the responses seen during the 1st minute of the post-hypercapnic hyperpnea most likely are an accurate indication of the "unmasked" level of excitation being provided by systemic (arterial and central) chemoreceptors.

Our data also support previous reports indicating that the site of this inhibitory input differs from species to species. Previous studies designed to investigate the effects of elevated CO_2 on upper airway receptors have shown that CO_2 acting on these receptors, specifically, inhibits ventilation in the tegu lizard (Ballam 1985; Coates and Ballam 1987), and the garter snake (Coates and Ballam 1989). A series of denervation experiments have isolated the upper airway receptors responsible for reflex inhibition of ventilation during CO_2 inhalation to the nasal sensory epithelium in both species (Ballam 1984, 1985; Coates and Ballam 1987). Furthermore, it has been shown in the tegu lizard (Ballam and Coates1989; Coates et al. 1991) that CO_2 delivered to these receptors during only the inspiratory or expiratory phase of the ventilation cycle did not inhibit ventilation. Ventilation was only depressed if the elevated levels of CO_2 were sustained. When CO_2 was removed from the inspired gas, the depression of ventilation due to the tonic stimulation of the upper airway receptors was replaced by a phasic stimulation which no longer inhibited ventilation.

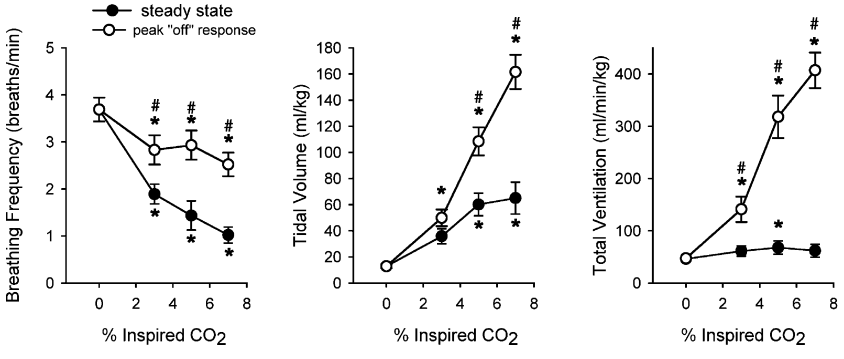

Fig. 9 Comparison of the levels of breathing frequency, tidal volume and total ventilation in all snakes in series I during steady-state exposure to various levels of CO_2 as well as during the 1st minute after the return to breathing air (peak "off" response) from each level of CO_2. Values are mean ± SEM ($n = 8$); *asterisk* denotes significant difference from values in animals breathing air in each treatment group, # denotes a significant difference between animals in the different treatment groups breathing the same level of CO_2

This was not the case in the South American rattlesnake. Figures 4, 7 and 8 show that neither the steady-state response, nor the post-hypercapnic hyperpnea arise from receptors in the upper airways. This is consistent with several other studies that have shown that vagotomy, which only removes feedback from lung receptors and not upper airway receptors, eliminates the post-hypercapnic hyperpnea (Boelert 1941; Nielsen 1961; Templeton and Dawson 1963; Gatz et al. 1975; Glass and Johansen 1976; Nolan and Frankel 1982). It is interesting to note that in garter snakes (Coates and Ballam 1989), total ventilation was depressed by hypercarbia and that upper airway receptors were involved while in the rattlesnake, breathing was modestly elevated and that upper airway receptors were not involved.

Two other lines of evidence support our conclusion that both the inhibitory effects of CO_2 on breathing in the steady state, and the post-hypercapnic hyperpnea, arise from receptors within the lungs. Following vagotomy, where CO_2 will enter the blood stream without altering pulmonary receptor discharge, total ventilation increased and the post-hypercapnic hyperpnea was absent. Furthermore, the response to injection of CO_2 rich blood into the systemic circulation just outside the heart (Fig. 5) was one of increases in both breathing frequency and tidal volume. These data suggest that the effect of CO_2 acting on systemic (arterial and central) chemoreceptors is one of overall excitation of ventilation and that the inhibition of ventilation must come from receptors within the lung itself.

Recent recordings of total vagus nerve discharge arising from the lungs suggest that the majority of the sensory input arising from the lungs comes from intrapulmonary chemoreceptors in this species and very little arises from pulmonary stretch receptors (Sundin et al. 2001). Intrapulmonary chemoreceptors have been described in reptiles, in snakes, lizards and alligators (see Milsom 1995 for review). They are located within the lung, innervated by the vagus nerve and have discharge that is inversely proportional to CO_2 levels. Moreover, they exhibit rate sensitivity with an overshoot in activity accompanying the sudden removal of CO_2 and an undershoot in activity with the sudden addition of CO_2. While the actual role of these receptors remains in question, it has previously been suggested that part of the inhibition of ventilation during environmental hypercarbia arises from inhibition of this receptor group (Ballam 1985; Ballam and Coates 1989; Coates and Ballam 1989).

Perspectives

The interactions between the various receptor groups in these reptiles appear designed to produce a robust response to metabolically produced CO_2 (hypercapnia), but either a very modest, or an inverse response to environmental CO_2 (hypercarbia). Does this simply reflect the fact that hypercarbia is not a common physiological situation for these animals and thus the response to hypercarbia is an anomalous response of a system designed for other functions, or is there some biological significance to the hypercarbic response?

The upper airway receptors have been shown not to be involved in breath-by-breath control of ventilation in reptiles and it is not yet clear why receptors with such profound effects on ventilation and breathing pattern exist. The detailed role of intrapulmonary chemoreceptors also remains in question. It has been proposed that the intrapulmonary chemoreceptors are involved in breath-by-breath control of breathing. They have been proposed to contribute both to inspiratory termination much as pulmonary stretch receptors do in mammals (Banzett and Burger 1977; Milsom et al. 1981) and to enhance inspiration in a positive feedback fashion once a breath has been initiated, much as pulmonary stretch receptors do in mammals (Cross et al. 1980; Furilla and Bartlett 1989). As such, they normally monitor the washout or dilution of CO_2 in the respiratory passages during inspiration, which is a function of the rate and depth of each inspiration. It has been suggested, in the context of the hypercarbic ventilatory response, that both groups of receptors may play a role in altering breathing pattern to enhance the efficiency of CO_2 excretion under conditions of environmental hypercarbia (larger tidal volume, lower frequency, and hence reduced dead space ventilation). It has also been suggested that they may be designed to detect changes in environmental CO_2 originating from prey or predators (Ballam 1985; Coates and Ballam 1987, 1989) although why this should lead to an inhibition of breathing is not clear. While this may reduce body wall movements making the waiting predator more difficult to detect, the size of each breath and associated body wall expansion that occurs when they do breathe is enhanced. Whatever the case, the biological significance of these overall responses and the species differences seen in the relative roles of different receptor groups remain intriguing questions for further research.

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