Acute systemic hypoxia activates hypothalamic paraventricular nucleus-projecting catecholaminergic neurons in the caudal ventrolateral medulla

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Submitted 6 June 2013; accepted in final form 8 September 2013

King TL, Kline DD, Ruyle BC, Heesch CM, Hasser EM. Acute systemic hypoxia activates hypothalamic paraventricular nucleus-projecting catecholaminergic neurons in the caudal ventrolateral medulla (CVLM). The hypothalamic paraventricular nucleus (PVN) modulates arterial chemoreflex responses and receives catecholaminergic projections from the CVLM, but it is not known whether the CVLM-PVN projection is activated by chemoreflex stimulation. We hypothesized that acute hypoxia (AH) activates PVN-projecting catecholaminergic neurons in the CVLM. Fluoro-Gold (2%, 60–90 nl) was microinjected into the PVN of rats to retrogradely label CVLM neurons. After recovery, conscious rats underwent 3 h of normoxia (21% O2, n = 4) or AH (12, 10, or 8% O2; n = 5 each group). We used Fos immunoreactivity as an index of CVLM neuronal activation and tyrosine hydroxylase (TH) immunoreactivity to identify catecholaminergic neurons. Positively labeled neurons were counted in six caudal-rostral sections containing CVLM. Hypoxia progressively increased the number of Fos-immunoreactive CVLM cells (21%, 19 ± 6; 12%, 49 ± 2; 10%, 117 ± 8; 8%, 179 ± 7; P < 0.001). Catecholamnergic cells coabeled with Fos immunoreactivity in the CVLM were observed following 12% O2, and containing CVLM. Hypoxia progressively increased the number of Fos-immunoreactive CVLM cells (21%, 19 ± 6; 12%, 49 ± 2; 10%, 117 ± 8; 8%, 179 ± 7; P < 0.001). Catecholamnergic cells coabeled with Fos.

chemoreflex; blood pressure; Fos; catecholaminergic neurons; retrograde label

PERIPHERAL CHEMOREFLEX ACTIVATION by systemic hypoxia increases ventilation, sympathetic nerve activity, and secretion of vasopressin and ACTH. These respiratory, autonomic, and endocrine responses are critical for the body’s homeostatic mechanisms and for maintaining adequate tissue oxygen levels (29, 44). Decreases in the partial pressure of oxygen in arterial blood are sensed by chemoreceptors located in the carotid bodies, and afferent information is relayed through the carotid sinus nerve to the brain stem (46). The nucleus tractus solitarii (nTS) is the first site of chemoreceptor afferent integration and modulation (2, 58). A monosynaptic projection from the nTS to the rostral ventrolateral medulla (RVLM) is believed to be the primary pathway involved in arterial chemoreflex function (24). However, other brain stem and forebrain nuclei also make important contributions to chemoreflex function (34), but the central nervous system pathways and their relative importance in response to systemic, acute hypoxia remain unclear.

The paraventricular nucleus of the hypothalamus (PVN) is an important integrative center containing neurons that regulate both autonomic and neuroendocrine function (69). Magnocellular neurons in the PVN regulate release of vasopressin and oxytocin from the posterior pituitary, whereas parvocellular PVN neurons are involved in autonomic nervous system and respiratory function, and secretion of corticotropin-releasing hormone (4, 27, 53). Neurons in the PVN receive multiple inputs and send direct projections to brain regions that play important roles in regulation of sympathetic outflow (1, 59, 62) and respiratory drive (75). Oxytocin- and vasopressin-containing PVN parvocellular neurons project to the RVLM, pre-Bötzing complex, phe-ric motor nucleus, and the intermediolateral cell column, and both neuropeptides modulate ventilation (34). PN neurons are activated by hypoxia and hypercapnia (5, 15), and may play a role in chemoreflex function. Lesion or inhibition of the PVN decreases ventilation and attenuates cardiovascular and sympathetic responses to chemoreflex activation (49, 54), indicating the PVN is required for full expression of the peripheral chemoreceptor reflex. Although these data indicate the PVN is important in central processing of chemoreceptor information, the neural pathways and neurotransmitters that convey peripheral chemoreceptor input to the PVN have not been fully delineated.

Catecholaminergic inputs to the PVN may influence autonomic responses during chemoreflex activation because blockade of adrenergic receptors in the PVN blunts the cardiovascular response to carotid chemoreceptor stimulation (38). Previous research in our laboratory (35) indicates that hypoxic stimuli activate nTS cells with projections to the PVN, especially catecholaminergic PVN-projecting neurons. The PVN also receives projections, including catecholaminergic input, from the caudal ventrolateral medulla (CVLM) (10, 60). These projections have been implicated in mediating neuroendocrine responses to hemorrhage (28) and stress (14), and could also play a role in responses to hypoxia. CVLM neurons are activated by chemoreflex stimulation (42, 43, 66), and it has been suggested that the CVLM contributes to neuroendocrine responses to hypoxia (66). In addition, a population of hypoxia-sensitive CVLM neurons responds to changes in arterial pressure (43), and the decrease in pressure observed during hypoxia may influence activation of these neurons. However, it is not known whether PVN-projecting CVLM cells, in particular catecholaminergic CVLM neurons with direct projections to the PVN, become activated in response to systemic hypoxia. Furthermore, whether

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In this study we used retrograde labeling to directly evaluate the caudal-to-rostral activation of PVN-projecting CVLM neurons due to increasing severity of acute hypoxia. Second, we determined whether these CVLM projection neurons are catecholaminergic. Finally, because acute hypoxia decreases arterial pressure and changes in pressure affect CVLM neuronal activation, we also assessed whether hypoxia-induced decreases in pressure influenced activation of these neurons during hypoxia. We hypothesized that the direct projection from CVLM catecholamine cells to the PVN is activated during hypoxia. Furthermore, because the peripheral chemoreceptors do not project directly to the CVLM, and thus afferent information must traverse several synapses before activating CVLM neurons, we hypothesized that these neurons would require higher intensities of hypoxia for activation. Our findings indicate that increasing the intensity of acute systemic hypoxia activates progressively more CVLM cells, including PVN-projecting catecholaminergic neurons. These results support the role of PVN-projecting CVLM neurons in physiological responses to hypoxia.

MATERIALS AND METHODS

Studies were performed on 23 male Sprague-Dawley rats (280–340 g). The rats were housed in a 12-h light/dark cycle at a constant temperature of 22°C and 40% humidity. Animals were given food and water ad libitum. All experimental procedures were conducted in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the University of Missouri Animal Care and Use Committee. Tissue from animals used in the current experiments was also used in previously published studies to evaluate effects of hypoxia on nTS neurons (35).

Surgical and Microinjection Procedures

The distribution and activation of neural inputs to the PVN from the CVLM were investigated by injecting the retrograde tracer Fluoro-Gold (FG; 2% in deionized water; Fluorochrome, Denver, CO) into the PVN using techniques similar to procedures previously published for retrograde tracer and microinjection experiments (35, 36, 45). All surgeries were performed under aseptic conditions. Anesthetized animals (Isoflurane; Baxter Healthcare, Deerfield, IL; 5% for induction and 2–2.5% for maintenance) were placed in a stereotaxic frame, the dorsal surface of the skull was exposed, and a small hole was made to access the brain. Coordinates for PVN microinjections were 1.8–2.0 mm caudal to bregma, ± 0.5 mm lateral from the midline, and 7.8 mm ventral to the dura. FG (60–90 nl) was microinjected bilaterally into the PVN. Incisions were closed, rats were treated with postoperative antibiotics, and returned to their home cage for at least 2 wk of recovery. To measure blood pressure in response to acute hypoxia, a separate group of rats (n = 4), telemetry devices (TA11PA-C40; Data Sciences International) were implanted via the abdominal aorta and animals were allowed to recover for at least 10 days. Arterial pressure, heart rate, and the strength of the telemetry signal were evaluated daily after surgery. Five days before immunohistochemistry experiments in which mean arterial pressure (MAP) was maintained constant during acute hypoxia, animals were also instrumented with a femoral venous catheter for drug administration.

Hypoxia Experimental Procedures

Before the experiment, conscious rats in their home cages were acclimated to the hypoxia chamber (Biospherix, Redfield, NY) for 1–3 h per day for 2 days to allow them to become accustomed to the environmental stimuli associated with the chamber. On the day of the experiment, conscious rats with previous PVN tracer microinjections were allowed to acclimate for 30 min in the chamber (21% O2). The gas mixture then was adjusted to bring the air in the chamber to 21% O2 (normoxic control; n = 4); 12% O2, 10% O2, or 8% O2 (n = 5 each group) and maintained at that percentage for 3 h via a negative feedback control system, similar to previous studies (5, 35, 36, 70).

Maintenance of Arterial Blood Pressure During Hypoxia

In the animals implanted with telemetry devices (n = 4), two sets of blood pressure measurements were obtained, each during 3 h of hypoxia. These animals were also acclimatized to the hypoxic chamber as described above but underwent hypoxia exposure at only 10% O2 for 3 h. In the initial hypoxic exposure, blood pressure was continuously measured and recorded using telemetry. After 2 wk, animals were instrumented with a femoral venous catheter and then allowed 1 wk for recovery. Then, animals were again exposed to 3 h of hypoxia at 10% O2, but this time blood pressure was maintained constant throughout the hypoxic period by infusing phenylephrine (0.6 mg/ml, 0.1–0.5 ml/h iv). Animals were perfused immediately following the experiment. Fos expression in these animals was compared with that in the group of FG-microinjected animals that were exposed to 10% hypoxia only.

Immunohistochemistry

Immediately following hypoxia experiments, animals were transcardially perfused with DMEM (125 ml pH 7.4; Sigma) bubbled with oxygen, followed by 0.01 M phosphate-buffered 4% paraformaldehyde (500 ml pH 7.4; Sigma). After postfixation, coronal sections were cut at 30 µm (brain stem) or 40 µm (forebrain) with a vibratome (VT 1000S; Leica, Germany). In all animals with retrograde tracer injections, forebrain sections were examined to verify the location of PVN injection sites using a rat brain stereotaxic atlas (50). Only animals in which the microinjections were localized to the PVN were used for immunohistochemistry protocols. A double-labeling technique similar to that previously described (35, 36) was used to identify immunoreactivity for Fos (a marker for neuronal activation) and tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, a marker for catecholaminergic neurons. Briefly, sections were incubated overnight in 1% normal donkey serum (NDS) and 0.3% Triton-0.1 M PBS containing primary antibodies against Fos (rabbit anti-Fos, 1:3,000; Calbiochem) and TH (mouse anti-TH, 1:1,000; Millipore). The following day, sections were rinsed then incubated for 2 h in Cy3-conjugated donkey anti-rabbit IgG and Cy2-conjugated donkey anti-mouse IgG (1:200; Jackson Immuno Research) with 1% NDS in 0.3% Triton-0.01 M PBS and covered-slipped. In addition, tissue from a subset of three of the rats subjected to 10% O2, a primary antibody against phenylethanolamine N-methyltransferase (sheep anti-PNMT, 1:2,000; Millipore) was included in the primary antibody solution, and Cy3-conjugated donkey anti-sheep IgG secondary antibody (1:200; Jackson ImmunoResearch) was used. Immunohistochemistry controls included omission of primary or secondary antibodies. Because so little PNMT was observed in the CVLM sections included in the current study, we verified the validity of our immunohistochemical technique by examining the next two more rostral sections, which would be expected to include both A1 and C1 neurons. In these sections, 23 ± 9% of all TH neurons were also PNMT-immunoreactive (IR). In addition, evaluation of the rostral RVLM indicated that a large proportion (94 ± 2%) of the TH-IR neurons also exhibited PNMT immunoreactivity. Antibody specificity for Fos (65), TH (3), and PNMT (21) have been verified previously.
Neurons in the CVLM that projected to the PVN were identified by the intrinsic fluorescence of retrograde tracer.

Microscopy and Image Analysis

We evaluated the CVLM on the basis of location relative to clearly recognizable anatomic structures, including lateral to the pyramidal tracts and inferior olives, medial to the spinal trigeminal tracts, dorsal to the lateral reticular nuclei, and ventral to the nucleus ambiguus (S0). Brain stem sections were examined upon an Olympus epifluorescent microscope (BX51). Filter sets for Cy2, Cy3, Cy5, or FG were used to visualize positive labeling. Image brightness and contrast were adjusted only for image clarity. Images in the same focal plane were captured under each filter set using a cooled monochrome digital camera (ORCA-AG; Hamamatsu, Bridgewater, NJ). Images were subsequently combined and analyzed with Image J (version 1.41; National Institutes of Health, Bethesda, MD) using a custom-made plug-in (GAIA Group, Novato, CA; http://gaiag.net/index.html). Two individuals blinded to the experimental protocol performed the counting, and counts for each section were averaged. The following criteria were used to identify positively labeled cells: Fos-IR nuclei were identified as highly condensed stained regions, with an ovoid shape and frequently with a clearly discernible unstained nucleolus. TH- and PNMT-positive cells were recognizable by staining of the cytoplasm, and visible processes, and a blank nuclear region. FG-positive cells displayed bright or punctate cytosolic labeling. When the above criteria were met under more than one filter set, the cells were considered double- or triple-labeled. Counts of positively labeled cells were made in six caudal-to-rostral levels of the CVLM: −15.00 to −14.10 mm relative to bregma (corresponding to −540 to +360 μm relative to calamus scriptorius).

RESULTS

PVN-Projecting CVLM Cells Have a Distinct Distribution and Many Are Catecholaminergic

Microinjection sites for FG within the PVN were verified histologically in all animals used in this study. Figure 1A depicts a representative PVN injection site; a summary of all sites was presented previously (35). An example of retrograde labeling in the CVLM is shown in Fig. 1B. Examination of the

Data Analysis

Immunohistochemical data were analyzed as the total (sum) of counts from all sections and also at each caudal-to-rostral level of the CVLM. The percentage of colabeled CVLM neurons was calculated by dividing the number of colabeled cells by the total number of cells of each individual phenotype. For example, the percentage of projecting cells activated was calculated as follows: (Fos and FG colabeled cells)/(total number of FG labeled cells) × 100. Statistical analyses were performed using SigmaPlot (12.3; Systat Software, San Jose, CA). All data are presented as means ± SE. Statistical significance was set at P < 0.05. One-way repeated measures ANOVA was used to compare the total number of neurons with a given phenotype, colabeling, and percentages of colabeling (e.g., TH-IR neurons expressing Fos) among experimental groups. Caudal-rostral distributions of labeling among hypoxic groups was analyzed by two-way repeated measures ANOVA; average distributions were analyzed by one-way repeated measures ANOVA. When appropriate, ANOVAs were followed by post hoc analysis using Fisher’s LSD test.
distribution of PVN-projecting cells in the CVLM indicated a distinct pattern, with a gradual increase in the number of labeled cells from caudal to rostral (Fig. 1C). Two-way repeated measures ANOVA revealed that the three most rostral sections examined in the CVLM contained a significantly greater number of PVN-projecting cells compared with more caudal sections. In contrast, catecholaminergic (TH-IR) cells were evenly distributed throughout the CVLM, similar to previous reports (10, 13, 59). Overall, about two-thirds (67%) of all PVN-projecting CVLM neurons were catecholaminergic. The distribution of catecholaminergic PVN-projecting neurons was similar to that of FG-labeled cells; the greatest proportion of TH- and FG-colabeled cells also occurred at intermediate and rostral levels of the CVLM (Fig. 1D). As expected, hypoxia had no effect on total number (Table 1) or distribution (Fig. 1) of FG, TH, or their colabeling in the CVLM.

**Graded Systemic Hypoxia Progressively Activates CVLM Cells**

We used Fos immunoreactivity to determine activation of CVLM cells by increasing intensities of hypoxia. Figure 2, A–D, includes examples of activated cells (Fos-IR, pseudocolored red) in brain stem sections containing the CVLM from animals that were exposed to 3 h of normoxia (21% O2) or different intensities of hypoxia (12%, 10%, or 8% inspired O2). In control animals, there were very few Fos-IR cells in the CVLM, suggesting a low level of acute activation under normoxic conditions. Increasing the intensity of hypoxia augmented the number of Fos-IR cells. Mean data showing the increase in total number of activated cells in the CVLM are depicted in Figure 2E (left). Significant activation of CVLM cells was first apparent after breathing 12% O2. Greater intensities of hypoxia increased the number of Fos-IR neurons in the CVLM, with the greatest activation occurring following exposure to 8% O2. Evaluation of the caudal-to-rostral extent of the CVLM indicated that activation of cells was evenly distributed in all hypothalamic regions, with an intensity-dependent effect of hypoxia evident throughout (Fig. 2E, right).

**Hypoxia Activates Catecholaminergic and PVN-Projecting Cells in the CVLM**

Although exposure to hypoxic gas mixtures induces Fos expression in CVLM catecholamine cells (66), the projections of these hypoxia-sensitive CVLM cells are not known. To investigate the phenotype of activated CVLM cells, we evaluated whether they were catecholaminergic (TH-IR) and/or PVN-projecting (FG-labeled).

**Catecholaminergic CVLM cells are activated following graded hypoxia.** Figure 3 includes examples of activated (Fos-IR) and catecholaminergic cells (TH-IR, pseudocolored green) in brain stem sections containing the CVLM from animals exposed to 3 h of normoxia (21% O2) or different intensities of hypoxia (12%, 10%, or 8% inspired O2). Very few TH-IR cells were activated following normoxia (Fig. 3A). Activation of catecholaminergic cells occurred in response to 12% O2 (Fig. 3B), and further increases in hypoxia caused markedly more activation (Fig. 3, C and D).

The mean number of catecholaminergic CVLM cells activated (Fos- and TH-colabeled) by exposure to hypoxia is shown in Figure 3E (left). Compared with normoxia, the number of colabeled cells increased significantly after breathing 12% O2 and increased further with a greater intensity of hypoxia. Because the CVLM contains many cell types, it is important to determine which specific cell populations are activated by hypoxia. Although Fos expression is used as a marker of cell activation, the localization of Fos-IR cells does not provide a measure of cell type. We used Fos immunoreactivity to determine activation of CVLM cells by increasing intensities of hypoxia. Figure 2, A–D, includes examples of activated cells (Fos-IR, pseudocolored red) in brain stem sections containing the CVLM from animals that were exposed to 3 h of normoxia (21% O2) or different intensities of hypoxia (12%, 10%, or 8% inspired O2). In control animals, there were very few Fos-IR cells in the CVLM, suggesting a low level of acute activation under normoxic conditions. Increasing the intensity of hypoxia augmented the number of Fos-IR cells. Mean data showing the increase in total number of activated cells in the CVLM are depicted in Figure 2E (left). Significant activation of CVLM cells was first apparent after breathing 12% O2. Greater intensities of hypoxia increased the number of Fos-IR neurons in the CVLM, with the greatest activation occurring following exposure to 8% O2. Evaluation of the caudal-to-rostral extent of the CVLM indicated that activation of cells was evenly distributed in all hypothalamic regions, with an intensity-dependent effect of hypoxia evident throughout (Fig. 2E, right).

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hypoxia. The greatest number of activated catecholaminergic cells was reached at 10% and 8% inspired O2. Analysis of activation of catecholamine cells through the caudal-rostral extent of the CVLM indicated a main effect of hypoxia intensity but no effect on the caudal-to-rostral level. Thus, catecholaminergic cells throughout the CVLM were activated by hypoxia with no distinct recruitment pattern (Fig. 3E, right).

The percentage of CVLM catecholaminergic cells that were activated by hypoxia is shown in Figure 3F and indicates that increasing hypoxia produced greater activation of TH-IR neurons. Furthermore, a considerable proportion of TH-IR cells (71 ± 4%) was activated at the highest intensity of hypoxia studied. Similar to absolute numbers (Fig. 3E), examination of the caudal-to-rostral distribution as a percentage of catecholamine cells activated (data not shown) indicated an intensity-dependent effect but no distinct caudal-to-rostral pattern of activation.

On the basis of their caudal location within the medulla, we would expect the majority of catecholaminergic cells evaluated to be noradrenergic (A1) rather than adrenergic (C1) neurons. Nevertheless, examination of TH immunoreactivity does not allow us to unequivocally distinguish between these two subgroups. Therefore, in a subgroup (n = 3) of animals exposed to 10% O2, we also evaluated PNMT immunoreactivity, which would be observed only in C1 neurons. At the caudal-rostral levels of the medulla examined in the current study, only 1.7 ± 1.7% of the TH-IR neurons also were PNMT-IR. Thus, ~98% of the catecholaminergic neurons examined in the current studies belong to the A1 cell group. Furthermore, none of the activated TH-IR cells at these levels expressed PNMT.

Hypoxia activates PVN-projecting CVLM cells. Representative pseudocolored photomicrographs of FG- and Fos-IR colabeled cells within the CVLM of animals that were exposed to normoxia (21% O2) and increasing intensity of acute hypoxia are shown in Figure 4, A–D. Very few PVN-projecting cells exhibited Fos immunoreactivity after exposure to normoxia, but activation of FG-labeled cells became apparent at more severe hypoxic intensities. Mean data showing activation of PVN-projecting CVLM cells in response to acute hypoxia are shown in Figure 4, E and F. Whether evaluated as an absolute...
number or as a percentage of FG-labeled cells, breathing 12% O₂ did not activate PVN-projecting cells compared with normoxia. However, the number of PVN-projecting CVLM cells that coexpressed Fos was significantly increased at 10% and 8% hypoxia, with 8% O₂ producing the greatest activation of these cells. Interestingly, 8% O₂ activated more than half of all PVN-projecting cells (Fig. 4F).

With respect to the caudal-to-rostral extent of Fos immunoreactivity in the CVLM, two-way ANOVA revealed a significant main effect of hypoxia (Fig. 4E, right). As with the total number (Fig. 4E, left), there was no significant effect of exposure to 12% O₂, but a progressive increase was observed at 10% and 8% O₂. There was a trend (P = 0.08) for more colabeled cells to be localized caudally (Fig. 4E, right). In addition, because the overall distribution of PVN-projecting CVLM cells was such that there were fewer in the most caudal CVLM sections analyzed (Fig. 1C), the percentage of PVN-projecting cells activated caudally was significantly more prominent at caudal levels (−15.00 through −14.64) compared with more rostral regions (14.10, −14.28, and −14.46) relative to bregma.

### Hypoxia preferentially activates catecholaminergic PVN-projecting cells in the CVLM

Of the activated PVN-projecting CVLM cells, almost all were also catecholaminergic (Fos + FG + TH). This was independent of hypoxia severity, and on average, 89 ± 3% of activated projecting cells were also catecholaminergic. Therefore, we specifically examined the activation of PVN-projecting catecholaminergic cells. Photomicrographs illustrating activation of PVN-projecting catecholaminergic neurons are presented in Figure 5 for normoxia (A) and all hypoxia intensities (B–D). Although there was little activation of these cells following normoxia or exposure to 12% O₂, increasing severity of hypoxia produced substantially more Fos immunoreactivity in PVN-projecting catecholaminergic neurons (Fig. 5, C and D). The mean number of triple-labeled (Fos-, TH- and FG-colabeled) cells (Fig. 5E, left) was not significantly altered in response to 12% inspired O₂. However, labeling was significantly greater at 10% and 8% hypoxia compared with both 21% and 12% O₂ levels. Independent of caudal-to-rostral distribution, within the CVLM (Fig. 5E, right) activation was greater in animals exposed to 10% and 8% O₂. Similar to what

![Image](https://example.com/image1.png)  
**Fig. 4.** Increasing intensities of hypoxia progressively activated PVN-projecting CVLM neurons. Representative merged photomicrographs showing Fos-IR (red) and FG labeling (pseudocolored blue) in the CVLM from individual animals exposed to normoxia (A) and different intensities of hypoxia (B–D). Higher magnification of boxed areas shows colabeling of Fos and FG. E: total number of PVN-projecting CVLM cells (presented in Fig. 4A–D) and all hypoxia intensities (left) and caudal-rostral distribution of activated PVN-projecting CVLM cells for each intensity of acute hypoxia (right). Two-way repeated measures ANOVA indicated a significant main effect of hypoxic intensity but not caudal-rostral level. F: percentage of PVN-projecting CVLM cells that were activated following exposure to normoxia or increasing intensities of acute hypoxia: [(Fos + FG)/(FG) × 100] followed a similar pattern. In E and F, *P ≤ 0.05 vs. 21% O₂; **P ≤ 0.01 vs. 21% and 12% O₂; ***P ≤ 0.001 vs. 21%, 12%, and 10% O₂. Scale bars = 250 μm.
is stated above, because there were fewer PVN-projecting catecholaminergic CVLM cells in the most caudal CVLM sections analyzed (Fig. 1D), the percentage of PVN-projecting catecholaminergic cells activated was significantly more prominent at the caudal levels –15.00 through –14.64 compared with rostral levels –14.10 and –14.46 relative to bregma. Up to 88% of PVN-projecting catecholaminergic neurons were activated in the most caudal sections of the CVLM after inspiration of 8% O₂.

We also directly compared the activation of PVN-projecting CVLM neurons with respect to their phenotype (catecholaminergic or noncatecholaminergic; Fig. 5F). The percentage of catecholaminergic PVN-projecting neurons (TH- and FG-cotabeled, solid bars) activated by exposure to 12% inspired O₂ was similar to that in normoxia. However, there was substantial activation of these neurons in response to breathing 10% or 8% O₂. A large proportion (71 ± 7%) of PVN-projecting catecholaminergic cells was activated at the most intense hypoxic stimulus. In contrast, compared with the substantial number of catecholamine neurons activated, hypoxia-induced Fos expression was less in noncatecholaminergic PVN-projecting CVLM neurons following exposure to 12%, 10%, and 8% O₂ (Fig. 5F, striped bars). Fos immunoreactivity in the noncatecholaminergic PVN-projecting neurons was not significantly different from that in normoxia after exposure to any intensity of hypoxia.

**Hypoxia Activates Nonphenotyped CVLM Neurons**

Hypoxia also activated cells in the CVLM of unknown phenotype (Fos-IR-only cells). There was a significant increase in the number of these cells at 10% and 8% hypoxia (10% O₂, 60 ± 8; 8% O₂, 91 ± 6) compared with normoxia (11 ± 3) and 12% hypoxia (21 ± 2). Thus a substantial proportion of Fos-IR CVLM neurons was neither catecholaminergic nor projected to the PVN.

**Proportional Activation of CVLM Cells Remains Constant with Increasing Intensities of Hypoxia**

We also considered the entire activated cell population in the CVLM and evaluated the proportion of the total number of activated cells made up by each phenotype. Interestingly,
although the total number of activated CVLM cells increased with increasing severity of hypoxia (Fig. 2E), the proportion of this total number comprising each phenotype remained the same. For example, about 50% of all activated CVLM cells were catecholaminergic, independent of hypoxia severity (12% O₂, 55 ± 5%; 10% O₂, 49 ± 6%; 8% O₂, 43 ± 2%). Thus although the number of activated catecholaminergic cells was augmented with each increase in hypoxia, the proportion of the overall number of Fos-IR cells that were TH-positive was similar, independent of hypoxia intensity. In addition, of all activated CVLM cells, ~30% projected to the PVN, and this percentage also was not influenced by intensity of hypoxia (12% O₂, 30 ± 4%; 10% O₂, 25 ± 6%; 8% O₂, 30 ± 5%). Remarkably, of these activated PVN-projecting cells, almost all were catecholaminergic (12% O₂, 94 ± 4%; 10% O₂, 95 ± 4%; 8% O₂, 81 ± 8%) at all intensities of hypoxia. Finally, a similar percentage of all Fos-IR cells were unphenotyped (neither catecholaminergic nor PVN-projecting), again independent of hypoxia severity (12% O₂, 44 ± 5%; 10% O₂, 54 ± 10%; 8% O₂, 51 ± 4%). Thus although hypoxia activates progressively more cells with each increase in hypoxia intensity, at each intensity, a similar proportion of each phenotype was activated.

Activation of CVLM Neurons by Hypoxia Is Not Dependent on Changes in Arterial Pressure

Results indicate an increase in activation of PVN-projecting CVLM neurons after a hypoxic stimulus. It is well known that CVLM neurons respond to changes in blood pressure (6, 7, 63) and that some CVLM neurons are activated by decreases in blood pressure (6, 11, 12, 39). Previous studies indicate that acute hypoxia in conscious rats produces moderate depressor responses (35, 44) that could potentially alter Fos immunoreactivity in the CVLM. To evaluate the effect of arterial pressure on CVLM neuronal activation during hypoxia, in a separate group of animals PE was infused (10% O₂ + PE; 0.6 mg/ml, 0.1–0.5 ml/h iv) to hold MAP constant during hypoxia. Fos immunoreactivity in these animals was compared with that in the group of rats exposed to acute hypoxia only (10% O₂ group). In animals implanted with telemetry devices, exposure to 10% O₂ decreased MAP from 106 ± 3 to 87 ± 4 mmHg at 30 min, and the decrease in pressure was maintained throughout the hypoxic period (93 ± 2 mmHg at the end of hypoxia). Infusion of phenylephrine during exposure to hypoxia prevented the depressor response to hypoxia (108 ± 6 to 115 ± 7 mmHg at 30 min) and MAP was maintained throughout (106 ± 1 at the end of hypoxia). Figure 6 includes photomicrographs showing Fos and TH immunoreactivity from animals in which blood pressure was not controlled (Fig. 6A) or was held constant (Fig. 6B). Group data revealed that there was no significant difference in total number of cells activated (Fig. 6C) or caudal-to-rostral distribution (data not shown) of Fos immunoreactivity between groups. In addition, the number (Fig. 6D) and distribution (data not shown) of activated CVLM catecholaminergic cells also were similar whether MAP was allowed to decrease during hypoxia or was held constant. These results suggest that the modest decreases in MAP during 10% acute hypoxia did not alter the number of CVLM neurons activated.

DISCUSSION

Acute hypoxia activates peripheral chemoreceptors and elicits physiological responses to promote enhanced gas exchange, maintain cardiovascular function, and preserve overall homeostasis. The complex central nervous system pathways involved in the chemoreflex have not been fully delineated. The current studies show that hypoxia progressively activates CVLM neurons, and contrary to our initial hypothesis, activation occurred...
even at relatively low intensities of hypoxia (12% O2). Hypoxia activated a large proportion (up to 71%) of catecholaminergic CVLM neurons, suggesting an important role for these neurons in cardiorespiratory reflexes. Importantly, CVLM neurons that directly project to the PVN are also activated by hypoxia but require a more intense hypoxic stimulus to be recruited. The majority (89 ± 3%) of hypoxia-sensitive PVN-projecting CVLM neurons were catecholaminergic, providing an anatomic and phenotypic substrate for a role of the CVLM-PVN pathway in chemoreflex responses. Finally, we found that hypoxia-induced decreases in blood pressure did not alter the number of activated CVLM neurons.

Classically, the CVLM has been shown to contribute to the arterial baroreflex pathway via its inhibition of presympathetic RVLM neurons (63). However, the role of the CVLM in peripheral chemoreflex function has been equivocal. Although CVLM inhibition does not alter the tonic sympathoexcitatory pressor response induced by hypoxia (37), others have shown that CVLM neurons exhibit hypoxia-induced activation (18, 70) and contribute to the respiratory coupling of RVLM neuronal activity and sympathetic nerve activity during hypoxia (42, 47). Our data support and extend the concept that the CVLM may contribute to chemoreflex function, even at low intensities of hypoxia. The mildest stimulus examined (12% O2) produced Fos immunoreactivity in the CVLM, and increasing intensities of hypoxia progressively activated CVLM cells, which parallels previously observed decreases in oxygen saturation and increases in ventilation (35).

Interestingly, a substantial proportion of hypoxia-activated CVLM neurons were catecholaminergic, with severe hypoxia activating ~70% of these TH-IR neurons. Collectively, the observations that CVLM neurons, and specifically catecholaminergic CVLM cells, are activated early in hypoxia and are further activated by more intense stimuli, suggest that these neurons may fulfill a significant functional role in mediating hypoxic-related responses. Interestingly, CVLM catecholaminergic neurons may also be important for long-term adaptations to hypoxia and in respiratory diseases. Partial acclimatization to long-term hypoxia is associated with TH upregulation in the ventrolateral medulla (56), and chronic intermittent hypoxia (an experimental model of obstructive sleep apnea) increases noradrenergic terminal densities in brain regions that receive input from A1 neurons in the CVLM (48, 57). Thus increased noradrenergic input from A1 neurons to other regions of the brain may contribute to hypertension and augmented cardiorespiratory reflexes in chronic intermittent hypoxia. Furthermore, impairment of the transcription factor Phox2b, which is expressed largely in noradrenergic neurons (30, 74), has been linked to several respiratory pathologies characterized by hypoventilation and impaired responses to hypoxia and hypercapnia, suggesting that catecholaminergic cells are critical to both central and peripheral chemoreception (31, 68, 71). Thus CVLM catecholamine neurons may be essential for not only acute, but also chronic respiratory system challenges.

Chemoreflex activation increases sympathetic and respiratory activity through multisynaptic central pathways that originate in the nTS. Although projections from the nTS to the RVLM contribute to chemoreflex function (24), the central pathways that elicit the multifactorial response to hypoxia remain poorly understood. The PVN plays an important role in control of breathing, sympathetic nerve activity, and neuroendocrine function, and contributes to their modulation in response to chemoreflex activation (8, 41, 54). Activation (16, 75) or disinhibition (61) of the PVN increases respiratory rate, MAP, and heart rate. Stimulation of peripheral chemoreceptors induces Fos immunoreactivity within PVN neurons (5), including magnocellular (66) and preautonomic neurons (9). In contrast, lesion or blockade of the PVN depresses ventilation and attenuates arterial chemoreceptor-mediated responses including increased arterial pressure, and sympathetic and phrenic nerve activity (49, 54). PVN projections to the pituitary gland also participate in vasopressin and ACTH release due to hypoxia (19, 40). Thus activation of the PVN contributes to the robust and varied cardiorespiratory response to hypoxia.

The PVN receives perhaps the most dense catecholaminergic innervation of any region in the central nervous system (20). Functionally, blockade of adrenergic receptors in the PVN blunts cardiovascular responses and vasopressin release due to carotid chemoreceptor stimulation (38), suggesting that catecholaminergic inputs to the PVN are important in eliciting chemoreceptor-induced pressor responses. Given that a large proportion of catecholaminergic innervation to the PVN arises from the noradrenergic cell group of the CVLM (59, 69), we evaluated whether these cells exhibit hypoxia-induced Fos immunoreactivity. An important and novel finding in the present study is that a relatively large proportion of PVN-projecting CVLM neurons were activated by hypoxia, suggesting that the CVLM is an important source of chemoreflex-related input to the PVN. In contrast to PVN-projecting neurons from the nTS, which were activated after breathing 12% O2, the hypoxic threshold for activation of PVN-projecting CVLM neurons was greater (10% O2). Thus the CVLM-to-PVN pathway may be less sensitive to hypoxic stimulation and a more intense stimulus is needed to recruit this pathway, perhaps via activation of the nTS-to-CVLM pathway. However, once activated, PVN-projecting CVLM neurons are recruited further as severity of hypoxia increases. Functionally, the combination of these two hypoxia-sensitive pathways to the PVN could increase the responsiveness of the chemoreflex over a wide range of hypoxic stimuli. For example, more severe hypoxia intensities (7–9% O2) are required to increase plasma vasopressin (52), suggesting that a relatively strong chemoreflex stimulus is needed to initiate vasopressin secretion. The caudal CVLM has strong projections to magnocellular cells in the PVN (10) and lesions encompassing this region of the CVLM reduce activation of vasopressin and oxytocin PVN cell types following hypoxia (22, 66). Thus recruitment of PVN-projecting CVLM neurons in response to a strong hypoxic stimulus, as shown in the current experiments, could be an important mechanism for promoting vasopressin secretion during intense hypoxia.

Of all PVN-projecting neurons, there was a specific sensitivity of catecholaminergic PVN-projecting neurons to increasing severity of hypoxia. In fact, almost all hypoxia-activated PVN-projecting cells were catecholaminergic, independent of hypoxic intensity, indicating that hypoxia preferentially activates catecholaminergic rather than noncatecholaminergic PVN-projecting CVLM cells. As such, we might expect that a larger proportion of all activated cells would be catecholaminergic. However, when all activated cells in the CVLM were considered, progressively severe hypoxia recruited a greater number of each cell type (e.g., catecholaminergic or PVN-projecting) in proportion to the overall number of CVLM cells.
activated. Thus rather than sequentially recruiting different phenotypes of cells, increasing intensity of hypoxia recruited proportionally more of each phenotype. This is an interesting finding, suggesting that the approach to counter more severe external hypoxia stimuli is to recruit more cells overall, rather than different neuronal phenotypes.

Independent of hypoxia severity, approximately half of all CVLM cells activated in response to hypoxia were neither catecholaminergic nor PVN-projecting. Some of these neurons may be involved in chemoreflex function but could relay chemoreceptor-related information to brain regions other than the PVN. For example, GABAergic barosensitive CVLM neurons respond to hypoxia and likely project to the RVLM (42) to influence presym pathetic RVLM neurons (47) and respiratory related changes in sympathetic activity (43). Pontine groups that play a role in respiratory rhythmogenesis also receive projections from cells in the CVLM (23, 25). The CVLM has projections to the thoracic spinal cord that terminate on intercostal, sympathetic preganglionic, and phrenic motor neurons (17, 26, 73), supporting their involvement in respiratory control. Taken together, these data suggest that CVLM neurons are a heterogeneous population with the ability to influence a variety of other brain regions and physiological responses to hypoxia.

Acute hypoxia in conscious rats produces moderate depressor responses. In addition, CVLM cells receive convergent baroreceptor and chemoreceptor inputs (40, 43), and CVLM neurons respond to decreases in blood pressure (6, 11, 12). We therefore evaluated the effect of decreases in arterial pressure during hypoxia on CVLM neuronal activation. Because overall CVLM activation and activation of catecholaminergic neurons following hypoxia were similar whether MAP was allowed to decrease or was held constant, we propose that recruitment of CVLM neurons in response to acute hypoxia was predominantly due to chemoreflex activation and not the modest decrease in MAP. We cannot eliminate the alternative that in the same CVLM neurons hypoxia and changes in blood pressure resulted in a different magnitude of cell activation compared with hypoxia alone, because Fos immunoreactivity indicates activation, but not the magnitude of activation. Nevertheless, the similarity in results for the effects of hypoxia alone and hypoxia with blood pressure held constant on activation of CVLM neurons indicates that the decrease in blood pressure during hypoxia in the current experiments did not activate additional neuronal populations.

Perspectives and Significance

One of the most fundamental functions of the brain is the control of breathing. The central nervous system respiratory network helps regulate oxygen supply to the body and responds to acute, chronic, and intermittent decreases in blood oxygen levels. Given the evidence that chemoreflex function contributes to acclimatization to high altitude (55), exercise (67), and autonomic imbalances in various diseases (33, 51, 64), it is becoming increasingly important to fully understand the central nervous system pathways and mechanisms involved during hypoxia. Because the CVLM-to-PVN pathway projects both to paraventricular and magnocellular regions of the PVN (10), the present study suggests that this pathway may be important to all facets of the chemoreflex: cardiopulmonary, sympathetic nerve activity, and neuroendocrine responses to acute hypoxia. Moreover, the CVLM-PVN pathway may provide a robust influence given the large proportion of this pathway activated by hypoxia compared with other pathways known to be activated by cardiorespiratory stimuli. For example, only 10–15% of nTS cells that display Fos immunoreactivity in response to hypoxia also project to the RVLM (36), 12–13% of CVLM-projecting nTS neurons express Fos in response to arterial baroreceptor activation (72), and ~15% of RVLM-projecting PVN neurons are activated by osmotic challenges (32). Overall, the relatively large proportion of catecholaminergic PVN-projecting cells in the CVLM that are activated by hypoxia suggests these neurons might be critical in relaying chemoreflex afferent information to the PVN. They are also ideally situated for facilitating the autonomic and humoral responses during hypoxia because they not only receive input from regions with direct input from chemoreflex afferents (such as the nTS), but they also contain projection neurons that densely innervate the PVN. As a major integrative center, the PVN may have a significant role in receiving and sending hypoxic-related signals from and to other central pathways, especially in response to more severe hypoxia. Although the CVLM has been studied extensively for its role in the arterial baroreflex via projections to the RVLM, given the present data, the CVLM should be considered for its important role in the chemoreflex as well, through projections that include the PVN.

GRANTS

This study was supported by National Heart, Lung, and Blood Institute Grant HL-98602 to E. M. Hasser, C. M. Heesch, and D. D. Kline.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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