Carotid body responses to O₂ and CO₂ in hypoxia-tolerant naked mole rats

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Abstract
Aim: Naked mole rats (NMRs) exhibit blunted hypoxic (HVR) and hypercapnic ventilatory responses (HCVR). The mechanism(s) underlying these responses are largely unknown. We hypothesized that attenuated carotid body (CB) sensitivity to hypoxia and hypercapnia contributes to the near absence of ventilatory responses to hypoxia and CO₂ in NMRs.

Methods: We measured ex vivo CB sensory nerve activity, phrenic nerve activity (an estimation of ventilation), and blood gases in urethane-anesthetized NMRs and C57BL/6 mice breathing normoxic, hypoxic, or hypercapnic gases. CB morphology, carbon monoxide, and H₂S levels were also determined.

Results: Relative to mice, NMRs had blunted CB and HVR. Morphologically, NMRs have larger CBs, which contained more glomus cells than in mice. Furthermore, NMR glomus cells form a dispersed pattern compared to a clustered pattern in mice. Hemeoxygenase (HO)-1 mRNA was elevated in NMR CBs, and an HO inhibitor increased CB sensitivity to hypoxia in NMRs. This increase was blocked by an H₂S synthesis inhibitor, suggesting that interrupted gas messenger signaling contributes to the blunted CB responses and HVR in NMRs. Regarding hypercapnia, CB and ventilatory responses to CO₂ in NMRs were larger than in mice. Carbonic anhydrase (CA)-2 mRNA is elevated in NMR CBs, and a CA inhibitor blocked the augmented CB response to CO₂ in NMRs, indicating CA activity regulates augmented CB response to CO₂.

Conclusions: Consistent with our hypothesis, impaired CB responses to hypoxia contribute in part to the blunted HVR in NMRs. Conversely, the HCVR and CB are more sensitive to CO₂ in NMRs.

KEYWORDS
carbon monoxide, carbonic anhydrase, heme oxygenase, hydrogen sulfide, hypercapnia, hypercapnic ventilatory response, hypoxia, hypoxic ventilatory response
Naked mole rats (NMRs; *Heterocephalus glaber*) are subterranean and eusocial rodents, who reside in crowded underground burrows. A notable feature of NMRs is their remarkable tolerance to hypoxia, as they can survive for hours and weeks at 3% and 8% O\(_2\), respectively, and up to 18 min in an anoxic environment. NMRs are also highly tolerant to environmental hypercapnia. For example, NMRs survive several hours breathing 80% CO\(_2\), which is lethal to mice within minutes. Moreover, NMRs do not exhibit metabolic, thermoregulatory, or behavioral changes to hypercapnia (<10% CO\(_2\)). Conversely, acute hypoxia suppresses the metabolic rate of NMRs, which is in part due to modest reductions in physical activity and a total cessation of thermoregulation, resulting in decreased body temperature to near ambient levels in hypoxia.

Unlike NMRs, most adult mammals do not produce robust suppression of metabolic rate during hypoxia. Instead, they rely more so on the hypoxic ventilatory response (HVR, i.e., a reflex increase in ventilation in hypoxia) to enhance the delivery of O\(_2\) to tissues when environmental O\(_2\) is limited. Similarly, most adult mammals respond to increased ventilation by CO\(_2\) (hypercapnic ventilatory response or HCVR). Intriguingly, whereas most mammals respond to hypoxia or hypercapnia with an increase in ventilation, NMRs manifest a blunted HVR (7% inspired O\(_2\)) and also a blunted HCVR. The mechanism(s) underlying the attenuated HVR and HCVR in NMRs are not known.

Carotid bodies (CBs) are the major sensory organs for monitoring the chemical composition of arterial blood, particularly in hypoxemia and to a lesser extent hypercarbia. The chemoreceptor tissue in CBs is composed of O\(_2\)-sensitive glomus cells and supporting type II cells. Hypoxemia stimulates carotid sinus nerve (CSN) activity, triggering a reflex increase in breathing (i.e., the HVR). On the other hand, the CB chemoreflex underlies ~20%–40% of the HCVR in mammals, while the remaining 60%–80% is mediated by the central chemoreceptor(s) located in the brainstem. Given that the CB plays a major role in mediating the HVR, and to a lesser extent the HCVR, we hypothesized that impaired CB responses to hypoxia and hypercapnia contribute in part to the blunted HVR and HCVR in NMRs. We tested this hypothesis by monitoring CSN and efferent phrenic nerve responses to hypoxia and hypercapnia in urethane-anesthetized NMRs and C57BL/6 (BL6) mice, which we have previously studied.

Baseline phrenic nerve activity was recorded in urethane-anesthetized animals breathing room air. Animals were challenged with 100% O\(_2\) (hyperoxia) for 30 s. Changes in phrenic nerve activity were analyzed during the last 20 s of the hyperoxic challenge. The initial 10 s were excluded to account for the dead space in the breathing circuit. Brief hypoxia depressed breathing in mice, but not in NMRs (Figure 1A,B; \(p < 0.01; \ n = 7\) animals for each species).

Examples of CSN responses to hypoxia in a mouse and an NMR are shown in Figure 1C. Overall, CSN responses to hypoxia were blunted in NMRs compared to mice. Specifically, CSN responses to severe (Po\(_2\) ~ 40 mmHg) and moderate hypoxia (Po\(_2\) ~ 56 mmHg) were significantly attenuated in NMRs compared to mice (Figure 1D; \(p < 0.01; \ n = 9\) CBs from 5 mice and 12 CBs from 6 NMRs).

CB O\(_2\) sensitivity in humans and rodents, and second by directly measuring CSN activity in an ex vivo CB preparation. We chose the ex vivo CB preparation to exclude confounding influences from blood pressure changes on CSN activity, which is commonly encountered in intact anesthetized animal preparations.
Phrenic burst frequency and tidal phrenic amplitude increased in mice, whereas these effects were nearly absent in NMRs (Figure 2A,B). Specifically, baseline respiratory rate (RR, phrenic bursts/min) and minute neural respiration (MNR) were significantly less in NMRs compared to mice (Table 1, mice vs. NMR, \( p < 0.01 \); †). Because the baseline breathing was different between NMRs and mice, we analyzed the HVR as a percent of baseline breathing in animals breathing 100% O2 (Figure 2C–E). Compared to mice, NMRs manifested an attenuated HVR in 10% O2, which was due to lesser increase in tidal phrenic amplitude (i.e., tidal volume) and minute neural respiration (MNR) than mice (Figure 2D,E; \( p < 0.01 \); † †; \( n = 7 \) animals for each species).

Arterial blood gases were also measured in NMRs and mice breathing while breathing either room air (21% O2) or hypoxic gas (10% O2) (Table 2, BL6 vs. NMR, \( p < 0.05 \)).

### 2.3 CB and breathing responses to CO2 in NMRs

#### 2.3.1 CB response to CO2

CSN responses to hypercapnia were examined in NMRs and mice. Figure 3A,B depict representative examples of CSN responses to CO2 in a mouse and an NMR and average data with graded hypercapnia is presented in Figure 3C. Overall, NMRs had greater CSN responses to CO2 than BL6 mice (\( p < 0.01 \); \( n = 7 \) CBs in each species; 4 animals for each species).
2.3.2 | HCVR

Representative examples of phrenic nerve responses to 10% CO₂ in a mouse and an NMR are shown in Figure 4A,B. Average data of absolute values of phrenic nerve responses are presented in Table 3 and HCVR data analyzed as a percent of baseline ventilation while breathing 90% O₂ is shown in Figure 4C–E. The magnitude of the hypercapnia-mediated increase in minute neural respiration (MNR) was higher in NMRs than mice, which was due to a greater increase in respiratory rate (i.e., phrenic burst frequency) (Figure 4C,E; mice vs. NMR $p < 0.01; n = 7$ mice; 6 NMRs).

2.4 | CB morphology

To assess CB morphology, CB sections from NMRs and mice were stained with anti-TH and ant-CGA antibodies,
which are established markers of glomus cells,\textsuperscript{14,22} and morphometric analysis was performed as described in the “Methods” section. CBs were bigger in NMRs than mice, as indicated by a greater CB volume (~230\%) in NMRs compared to CBs (Figure 5A; Table 4, mice vs. NMR, \( p < 0.05; n = 4 \) each species). The number of TH and CGA positive cells was higher in NMR CBs but the ratio of TH or CGA positive cells to the CB volume was comparable between NMRs and BL6 (Table 4; BL6 vs. NMR, \( p > 0.05 \)).

NMR glomus cells formed a dispersed pattern compared to a clustered pattern in BL6 mice CBs (Figure 5B).

2.5 CO-H\(_2\)S signaling is interrupted in NMR CBs

Emerging evidence suggests that the regulation of CB sensitivity to hypoxia involves carbon monoxide (CO) and H\(_2\)S gas messenger signaling pathways.\textsuperscript{23} Therefore, we next examined whether altered CO-H\(_2\)S signaling contributes to the blunted CB response to hypoxia in NMRs. Endogenous CO is produced by hemeoxygenase (HO)-1 and HO-2, and CSE is a major H\(_2\)S producing enzyme in the CB.\textsuperscript{23} Therefore, \( Hmox1 \), \( Hmox2 \), and \( CTH \) mRNAs encoding HO-1, 2, and CSE, respectively, were determined in CBs of NMRs and mice. Transcript abundance was normalized to \( 18S \) mRNA. \( Hmox1 \), which encodes HO-1, was ~6-fold higher in NMR than mouse CBs, whereas \( Hmox2 \) and \( CTH \) abundances were comparable between CBs of both species (Figure 6A; \( p < 0.05; n = 4 \) animals for each species). Technical difficulties with antibodies precluded the analysis of HO-1, HO-2, and CSE proteins in NMR CBs.

CO inhibits CSE and reduces H\(_2\)S in the CB.\textsuperscript{23,24} Hypoxia inactivates HO-2 and reduces CO production thereby releasing inhibition on CSE. This leads to increased H\(_2\)S production, which in turn stimulates CSN activity.\textsuperscript{24} On the other hand, hypoxia has no effect on CO produced from HO-1, because it lacks O\(_2\)-sensitive heme regulatory motifs (HRM).\textsuperscript{25} Because NMR CBs have elevated \( Hmox-1 \), which encodes O\(_2\)-insensitive HO-1, we hypothesized that hypoxia would not effectively alter CO levels in NMRs. Testing this possibility using biochemical assays requires pooling of several CB tissues from numerous animals, which was not possible in our study due to the limited availability of NMRs. We have previously used rat pheochromocytoma (PC)-12 cells as a substitute for CB glomus cells.\textsuperscript{26} However, we are not aware that PC12 cells express HO isoforms and CSE. Interestingly, liver tissue expresses a high abundance of HO-2\textsuperscript{27} and CSE.\textsuperscript{28} In CBs, hypoxia reduces CO production by directly inhibiting HO-2 and increases H\(_2\)S levels\textsuperscript{24} and in liver homogenates in response to chronic intermittent hypoxia through ROS mechanisms.\textsuperscript{29} Together, these findings indicate similar
interactions between CO and H\textsubscript{2}S in liver as in the CB. Therefore, we utilized liver tissues from NMRs and mice to assess the effects of hypoxia on CO and H\textsubscript{2}S signaling. In this analysis, we found that hypoxia reduced CO and increased H\textsubscript{2}S in mice but not in NMR liver (Figure 6B,C; p < 0.01; n = 6 NMRs and mice each).
2.6 | An HO inhibitor improves CB response to hypoxia and HVR in NMRs

We next assessed whether increasing H$_2$S with an HO inhibitor improves CB response to hypoxia in NMRs. To test this, NMRs were treated with chromium (III) mesoporphyrin IX chloride (CrM459; 5 mg/kg; i.p), a pan HO inhibitor, either alone or in combination with L-propargyl glycine (L-PAG; 30 mg/kg; i.p.), an inhibitor of H$_2$S synthesis from CSE. CBs were harvested 1 h after administration of these compounds and CSN responses to hypoxia were determined. The CSN response to hypoxia was markedly improved in HO inhibitor-treated NMRs compared to vehicle-treated controls, and this effect was blocked with L-PAG (Figure 7A,B; $p < 0.01$; see Figure 7 for number of animals in each group).

Consistent with this improved CB response to hypoxia, HO inhibitor enhanced the HVR in NMRs (Figure 8A,B; absolute values of phrenic nerve activity in Table 5). The improved HVR was due to increased respiratory rate (i.e., phrenic burst frequency) and tidal phrenic amplitude (Figure 8C–E and Table 5).

2.7 | Carbonic anhydrase is involved in augmented CB response to CO$_2$ in NMRs

Carbonic anhydrases (CAs) are zinc-containing enzymes that catalyze the conversion of CO$_2$ to bicarbonate and hydrogen ions. The CB response to CO$_2$ involves CA. We next examined whether CA contributes to the augmented CB response to CO$_2$ in NMRs. mRNA encoding CA2 was analyzed in CBs from NMRs and mice. CA2 abundance was higher in NMR compared to mice CBs (Figure 9A; $p < 0.05$; $n = 4$ each species). Non-specific staining with commercially available anti-CA-2 antibodies precluded analysis of CA-2 protein in NMR CBs. However, methazolamide (30 μM), a membrane-permeable CA inhibitor, reduced the augmented CSN response to hypercapnia in NMRs but not in mice compared to vehicle-treated controls (Figure 9B,C; Vehicle vs. methazolamide NMRs $p < 0.01$; mice $p > 0.05$; $n = 5$ for mice and 6 for NMRs).

3 | DISCUSSION

Previous studies have reported that NMRs manifest a blunted HVR and HCVR; however, the mechanisms

TABLE 4  Morphometric analysis of the carotid body (CB) in mice and NMRs

|          | CB volume ($\times 10^4 \mu m^3$) | TH volume ($\times 10^4 \mu m^3$) | CGA volume ($\times 10^4 \mu m^3$) | TH/CB (%) | CGA/CB (%) |
|----------|----------------------------------|----------------------------------|----------------------------------|-----------|------------|
| Mice     | 2.1 ± 0.14                       | 4.3 ± 0.28                       | 2.8 ± 0.1                        | 20.2 ± 0.3| 15.7 ± 0.9 |
| NMRs     | 4.9 ± 0.7*                       | 9.1 ± 1.1†                      | 6.8 ± 0.98*                      | 19 ± 0.4* | 13.9 ± 0.2* |

Note: Data were presented as Mean ± SEM. $N = 4$ each species.
Abbreviations: CGA, chromogranin A; TH, tyrosine hydroxylase.

*p < 0.05.
†p < 0.01.
*"p > 0.05, not significant, mice vs. NMRs, Mann–Whitney test.
underlying this phenomenon are largely unknown. The current study addressed this knowledge gap by testing the hypothesis that attenuated hypoxic and CO₂ sensing by the CB chemoreceptor contributes, in part, to the blunted HVR and HCVR in NMRs. Our study had several salient findings. First, and consistent with our hypothesis, the CB response to hypoxia was attenuated in NMRs compared to mice, indicating that the blunted HVR in NMRs is partially due to reduced CB sensitivity to hypoxemia. We further found that the mechanism underlying...
this blunted response involves HO signaling because a pan-HO-inhibitor improved CB sensitivity to hypoxia and HVR became detectable in NMRs. On the other hand, HCVR and CB response to CO\textsubscript{2} was augmented in NMRs compared to mice, which may be due to altered CA signaling.

### 3.1 The CB response to hypoxia

The following findings demonstrate that NMRs exhibit impaired CB responses to hypoxia: first, NMR ventilation was less inhibited by brief hyperoxia than in mice (Dejourn’s test), which is an indirect measure of CB sensitivity to O\textsubscript{2}.
Second, the CSN response to graded hypoxia was attenuated in NMRs. Consistent with earlier reports, we also found a near absence of HVR in NMRs. This blunted HVR may be partially due to reduced body temperature since conscious NMRs cease thermoregulation and reduce their body temperature to near ambient temperatures in hypoxia. However, our study suggests that this is unlikely because we determined the HVR in anesthetized animals, while maintaining body temperature at 33°C in NMRs and 38°C in mice, which are their respective body temperatures. On the other hand, the blunted HVR is likely not due to anesthesia because previous studies reported similar impairment of the HVR in un-anesthetized NMRs. Given that the CB chemoreflex is a major driver of the HVR, it is therefore likely that the attenuated hypoxic sensitivity of the CB in part contributes to the near absence of HVR in NMRs. Whether NMRs also have impaired processing of CB sensory nerve information in the central nervous system remains to be investigated.

Being burrow-dwelling animals, hypoxic lifestyle of NMRs may have impacted the morphological phenotype of their CBs. Indeed, prolonged exposure to hypoxia increases CB size in animals as well as in humans. Consistent with this, CBs were bigger, and the number of glomus cells was higher in NMRs compared to mice (Figure 5). Interestingly, NMR glomus cells displayed a dispersed pattern, as opposed to the clustered pattern in mice CBs, which is typical of most adult mammalian CBs. Glomus cells are of secretory phenotype and are connected via gap junctions, allowing electrical coupling at rest. Hypoxia uncouples glomus cells and increases coupling resistance. The dispersed pattern of glomus cells in NMRs might reflect the impact of hypoxia resulting from life in crowded underground burrows. Interestingly, although the number of glomus cells is higher in NMR CBs, the ratio of TH to the CB volume was similar in NMR and mice, suggesting that the blunted hypoxic sensitivity of NMR CBs is likely due to defective hypoxia sensing at glomus cells (see below) and not fewer chemosensitive cells in NMRs per se.

### 3.2 Cellular mechanisms underlying the blunted CB response to hypoxia in NMRs

Recent studies suggest that CB sensory nerve excitation by hypoxia involves O₂-dependent interactions between CO and H₂S in glomus cells. HO-2 and CSE are the major enzymes that produce CO and H₂S, respectively, in CB glomus cells. While CO is a physiological inhibitor of the CSN response to hypoxia, H₂S, like hypoxia, stimulates
Biochemical assays showed the absence of the effects of mRNA encoding HO-1 was higher in NMR CBs than mice. Pamenter et al. 2019. Conversely, whereas two previous studies showed the absence of the effects of hypoxia on CO and H2S levels in NMR compared to mice. Notwithstanding the limitation of using liver tissue for biochemical assay, these results indicate O2-insensitive CO generation from HO-1 by inhibiting CSE reduces H2S production in NMR CB. The reduced H2S production might account for the blunt CSN excitation by hypoxia in NMRs. Such a possibility was supported by the finding that a pan HO-inhibitor markedly improved CB hypoxic response in NMRs and 1-PAG, a CSE inhibitor blocked the effect of the HO inhibitor (Figure 7). The improved CB hypoxic response was associated with enhanced HVR in NMRs treated with HO inhibitor (Figure 8). It should be noted that Hmox-1 upregulation in NMR CB was not associated with increased baseline CO levels (Figure 6B), which might be due to incomplete translation of the Hmox-1 gene to HO-1 protein, a possibility that requires further investigation.

3.3 The HCVR and CB responses to CO2 in NMRs

It is well established that burrowing rodents have relatively higher resting PaCO2 levels and attenuated HCVRs. Interestingly, we found PaCO2 was not elevated in NMRs, which is consistent with a previous study (see Table 1 in Pamenter et al. 2019). Conversely, whereas two previous studies in whole animals have reported that NMRs have a blunted HCVR that does not manifest below 10% inhaled CO2 whereas we report an augmented HVR in NMRs. This discrepancy may be due to differences in the duration of hypercapnia exposure. For example, a previous study examined breathing responses in NMRs to 1 h of hypercapnia, whereas in the present study we tested the effects of 5 min of hypercapnia on ventilation (i.e., on phrenic nerve activity). In addition, this previous study used awake animals whereas our preparation was anesthetized. The other earlier study also employed anesthetized animals but only measured breathing empirically via remote observation, which prevented this investigation from reporting tidal volume changes with hypercapnia. Thus, it is not possible to directly compare this study to our present findings.

In other species, acute hypercapnia increases ventilation for a few minutes but prolonged hypercapnia depresses ventilation. It is likely the depressed HCVR reported in an earlier study in awake NMRs is due to prolonged hypercapnic challenge (1 h). NMRs likely experience hypoxia and hypercapnia simultaneously in their natural burrowing environment. The augmented HCVR likely increases O2 delivery to the lungs through a left-shifted O2-Hb dissociation curve, thereby reducing the impact of hypoxia. The enhanced HCVR may be an important contributing factor to the hypoxia tolerance of NMRs.

Hypercarbia (i.e., elevated arterial blood CO2) stimulates CSN activity, albeit to a lesser extent than hypoxemia in most adult mammals. We report that, unlike hypoxia, the CB response to CO2 is augmented in NMRs compared to mice. This is important because it demonstrates that NMR CBs exhibit selective impairment of their ability to detect hypoxia, but not CO2. Unlike the response to hypoxia, the CB response to CO2 is relatively underexplored. However, the available information suggests CO2 activation involves CA activity. Intriguingly, NMR CBs have higher CA-2 mRNA abundance than mice, suggesting divergence in the function of this pathway between species. Unfortunately, technical problems with antibodies precluded the analysis of CA2 protein in CBs; however, methazolamide, a membrane-permeable CA inhibitor reduced the enhanced CB response to CO2 in NMR CB preparations. Therefore, we propose that elevated CA-2 leads to greater hydration of CO2, resulting in an accumulation of H+. H+ in turn depolarize glomus cells by inhibiting either TASK-like K+ channels or acid-sensing ion channels (ASICs), and thereby contribute to the augmented CO2 response of the NMR CB. However, further studies are needed to test this possibility by evaluating whether NMR glomus cells express TASK or ASIC channels and whether they contribute to the augmented CB CO2 response.

In summary, our results support the hypothesis that the blunted HVR in NMRs is associated with attenuated CB sensitivity to hypoxia. On the other hand, and contrary to our hypothesis, the HCVR and CB CO2 sensitivity are augmented in NMRs. As a largely subterranean species, NMRs likely experience prolonged periodic hypoxia and hypercapnia in their natural burrows. Although much is known about the impact of intermittent hypoxia, such as that experienced with obstructive sleep apnea on CB function and the HVR, little is known regarding the physiological consequences of long-term exposures to a combination of periodic hypoxia and hypercapnia such
as that experienced in burrowing animals. NMRs are relatively resistant to aging, neurodegeneration, and devastating diseases such as cancer, all of which have been linked to derangements in cellular oxygen handling. Future studies on experimental animals treated with long-term periodic hypoxia and hypercapnia simulating the burrowing environment may thus provide mechanistic insights on how and why NMR are less susceptible to such diseases and pathologies.

4 | METHODS

4.1 | Preparation of animals

Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago (Protocol # ACUP 71811, approved on February 27, 2019). Experiments were performed on adult male NMRs (males; body weight 45.2 ± 1.6 g, and 1–3 years old; bred at the University of Ottawa, and reared at the University of Chicago, Animal Resource Center). BL6 mice (males; 28.4 ± 0.8 g, 4–6 months old, purchased from Charles River Laboratories, USA).

4.2 | Measurements of phrenic nerve activity

Animals were anesthetized with intraperitoneal injections of urethane (1.2 g/kg). Supplemental doses (10% of the initial dose of anesthetic) were given when corneal reflexes or responses to toe pinches were observed. Animals were placed on a warm surgical board. The trachea was cannulated, and animals were allowed to breathe spontaneously. Core body temperature was monitored by a rectal thermistor probe and maintained at 38°C (mice) or 33°C (NMR) by a heating pad (RightTemp, Kent Scientific, Torrington, CT). The phrenic nerve was isolated unilaterally at the level of the C3 and C4 spinal segments, cut distally, and placed on bipolar stainless-steel electrodes. Integrated efferent phrenic nerve activity was monitored as an index of respiratory neuronal output. The electrical activity was filtered (band pass, 30 Hz –10 kHz), amplified (P511K, Grass Instrument, West Warwick, RI), and passed through Paynter filters (time constant of 100 ms; CWE Inc.) to obtain a moving average signal. Data were collected and stored in a computer for further analysis (PowerLab/8P, AD Instruments Pty Ltd, Australia). Phrenic nerve activity (burst frequency, an index of respiratory rate, bursts/min); tidal phrenic nerve activity (arbitrary units, a.u.); and minute neuronal respiration (MNR = RR × tidal phrenic nerve activity) were analyzed. The effects of different O2 levels (21% or 10% O2-balanced N2), and hypercapnia (3%, 5%, or 10% CO2-balanced O2) on phrenic nerve activity were determined. Gases were administered through a needle placed in the tracheal cannula and gas flow was controlled by a flow meter. To examine the responses to different O2 levels, baseline phrenic nerve activity was monitored while animals breathed 100% O2 for 3 min. Subsequently, inspired gas was switched to 21% or 10% O2 for 3 min. The duration of 3 min for hypoxia was chosen because a longer duration of hypoxic exposure (>5 min) in anesthetized mice leads to hypotension which confounds the interpretation of results. For hypercapnic responses, 5 min of 3%, 5%, or 10% CO2-balanced O2 was preceded with exposure to 100% O2 for 3 min. At the end of the experiment, animals were killed by overdose of urethane (>3.6 g/kg, i.p.).

4.3 | Measurements of arterial blood gases

Arterial blood (0.1 ml) was collected in the anesthetized animals at the end of 3 or 5 min of gas challenges via a catheter (PE-10) inserted into the femoral artery. Blood gases (PaO2, PaCO2, and pH) were determined by a blood gas analyzer (ABL-80, Radiometer, Copenhagen, Denmark). Blood gas analyzer provides data corrected at 37°C as well as normal body temperature of the animals. Two to four blood samples were collected in each experiment.

4.4 | Recording of CB sensory nerve (CSN) activity

The CSN activity was recorded from CBs ex vivo as described previously. Briefly, CBs (two CBs from a given animal) along with the sinus nerves were harvested from anesthetized animals, placed in a recording chamber (volume, 250 μl), and superfused with warm bicarbonate-based physiological saline (35°C) at a rate of 3 ml/min. The composition of the medium was (in mM): NaCl, 125; KCl, 5; CaCl2, 1.8; MgSO4, 2; NaH2PO4, 1.2; NaHCO3, 25; d-glucose, 10; Sucrose, 5. The solution was bubbled with 21% O2/5% CO2. Hypoxic challenges were achieved by switching the perfusate to physiological saline equilibrated with the desired levels of O2. Oxygen levels in the medium were continuously monitored by a platinum electrode placed next to the CB using a polarographic amplifier (Model 1900, A-M Systems, Sequim, WA). To facilitate the recording of clearly identifiable action potentials, the sinus nerve was treated with 0.1% collagenase for 5 min. Action potentials (1–3 active units) were recorded from one of the nerve bundles with a suction electrode and stored in a computer via a data acquisition system.
4.5 | CB morphology

CBs were harvested from anesthetized animals (urethane 1.2 g/kg, i.p.) perfused with heparinized saline followed by 4% paraformaldehyde. The protocols for fixation of CBs were essentially the same as described previously. Specimens were frozen in Tissue Tek (OCT; VWR Scientific), sectioned at 8 μm, and mounted on collagen-coated coverslips. Mouse CB usually yields 7–8 sections. However, 4–5 sections representing the middle of each CB were chosen for morphometric analysis (4–5 sections per CB; 2CBs from each animal; n = 4 animals for each species). Sections were blocked in PBS containing 1% normal goat serum and 0.2% Triton X-100, and then incubated with anti-chromogranin A (CGA, 1:1000; AB Cam) or with anti-tyrosine hydroxylase (TH, 1:300; Sigma) antibody in PBS with 1% NGS and 0.2% Triton X-100 at 4°C for 16 h. After washing in PBS, sections were incubated with Texas red-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (1:250; Molecular Probes) in PBS with 1% NGS and 0.2% Triton X-100 at room temperature for 1 h. After washing with PBS, sections were mounted in DAPI-containing media and visualized using a fluorescent microscope (Eclipse E600; Nikon). Carotid body morphology, glomic volume, and glomus cell numbers were analyzed in adjacent sections using ImageJ (NIH) as described previously. Briefly, serial sections (minimum 4–5 sections from each CB) were imaged individually. For each image, the CB area was measured manually by tracing the periphery of the carotid body and glomus cell area was calculated by tracing the periphery of glomus cells stained with TH or CGA (marker proteins). CB and glomic cell volumes were calculated by sum of each area multiplied by thickness and number of sections as described.

4.6 | Measurements of mRNAs

Real-time RT-PCR was performed using a MiniOpticon system (Bio-Rad Laboratories) with SYBR GreenER two-step qRT-PCR kit (#11764–100, Invitrogen). Briefly, RNA was extracted from CBs using TRIZOL and was reverse transcribed using superscript III reverse transcriptase. Primer sequences for real-time RT-PCR amplification were as follows: CA2 forward: CAC CAA GTT GGC GGG AGC CTA T; CA2 reverse: TCT CCA TTG GCA ATG GGG AAG TCC; CTH forward: TGC TTG GAA AAA GCA GTG GC; CTH reverse: CCT CTA GCA ATT TGG TTT TGG A; Hmax -1 forward: GGA GCA GGA CAT GAC CTT CT; Hmax -1 reverse: AGG TCA CCC AGG TAG CGG GT; Hmax -2 forward: TGA AGG AAG GGA CCA AGG AAG; Hmax -2 reverse: GTG GTC CTT GTT GCG GTC C; 18s forward: CGC CGC TAG AGG TGA AAT TC; 18s reverse: CGA ACC TCC GAC TTT CGT TCT. Relative mRNA quantification was calculated using the comparative threshold (CT) method using the formula “2−ΔΔCT” where ΔΔCT is the difference between the threshold cycle of the given target cDNA between BL6 and NMRs. The CT value was taken as a fractional cycle number at which the emitted fluorescence of the sample passes a fixed threshold above the baseline. Values were compared with an internal standard gene 18S. Purity and specificity of all products were confirmed by omitting the template and by performing a standard melting curve analysis.

4.7 | Measurements of H2S production

H2S levels in the livers were determined as described previously. Briefly, liver homogenates were prepared in 100 mM potassium phosphate buffer (pH 7.4). The enzyme reaction was carried out in sealed tubes flushed with either 100% N2 or 21% O2. The PO2 of the reaction medium was determined by a blood gas analyzer (ABL-80). The assay mixture in a total volume of 500μl contained (in final concentration) 800μM L-cysteine, 80μM pyridoxal 5’-phosphate, 100 mM potassium phosphate buffer (pH 7.4), and tissue homogenate (10 μg of liver protein). The reaction mixture was incubated at 37°C for 1 h and at the end of the reaction alkaline zinc acetate (1% wt/vol; 250μl) and trichloroacetic acid (10% vol/vol) were added sequentially to trap H2S generated and to stop the reaction, respectively. The zinc sulfide formed was reacted sequentially with acidic N,N-dimethyl-p-phenylenediamine sulfate (20μM) and ferric chloride (30μM) and the absorbance was measured at 670nm using a microplate reader. A standard curve relating the concentration of Na2S and absorbance was used to calculate H2S concentration and expressed as nanomoles of H2S formed per hour per milligram protein.

4.8 | Measurements of CO production

CO abundance was measured in the livers using a spectrophotometric procedure as previously described.
Reaction mixtures containing 10 μg liver protein, NADPH (1 mM), hemin (25 μM), and NADPH Regenerating System Solution (BD Biosciences) were equilibrated to 21% O₂ or 100% N₂ at 37°C in sealed tubes. CO generated in the reaction was trapped in a reaction mixture containing 25μM leuco crystal violet, 200μM palladate, and 4 μM iodate. CO concentrations were calculated from a standard curve relating CORM-2 concentration to absorbance of 620 nm light.

4.9 | Data analysis

In anesthetized animals, the following respiratory variables were analyzed: respiratory rate (phrenic bursts per minute), tidal amplitude of the integrated phrenic nerve activity (a.u., arbitrary units), and minute neural respiration (MNR, number of phrenic bursts per min, RR×tidal amplitude of the integrated phrenic nerve activity, a.u.). In a given animal, absolute values of phrenic variables the response to hypoxia or hypercapnia as well as normalized data as the percentage of the phrenic nerve activity while breathing 100% O₂ were analyzed. CSN activity (discharge from “single” units) was averaged during 3 min of baseline and during the entire 3 min of hypoxic or 5 min of hypercapnic challenge and expressed as impulses per second. Each data point represents the average of two trials in each animal for a given gas challenge. Average data are presented as mean ± SEM. Statistical significance was assessed by t-test, or Mann–Whitney test, or One-Way or Two-Way ANOVA followed by a post hoc test, or Two-Way ANOVA with repeated measures followed by a post hoc test using SigmaPlot (version 11). \( p \) Values < 0.05 were considered significant.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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