Heme oxygenase-1-dependent central cardiorespiratory adaptations to chronic hypoxia in mice

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Sunderram J, Semmlow J, Thakker-Varia S, Bhaumik M, Le-Hoang O, Neubauer JA. Heme oxygenase-1-dependent central cardiorespiratory adaptations to chronic hypoxia in mice. Am J Physiol Regul Integr Comp Physiol 297: R300–R312, 2009.—Adaptations to chronic hypoxia (CH) could reflect cellular changes within the cardiorespiratory regions of the rostral ventrolateral medulla (RVLM), the C1 region, and the pre-Bo¨tzinger complex (pre-BötC). Previous studies have shown that the hypoxic chemosensitivity of these regions are heme oxygenase (HO) dependent and that CH induces HO-1. To determine the time course of HO-1 induction within these regions and explore its relevance to the respiratory and sympathetic responses during CH, the expression of HO-1 mRNA and protein in the RVLM and measures of respiration, sigh frequency, and sympathetic activity (spectral analysis of heart rate) were examined during 10 days of CH. Respiratory and sympathetic responses to acute hypoxia were obtained in chronically instrumented awake wild-type (WT) and HO-1 null mice. After 4 days of CH, there was a significant induction of HO-1 within the C1 region and pre-BötC. WT mice acclimated to CH by increasing peak diaphragm EMG after 10 days of CH but had no change in the respiratory response to acute hypoxia. There were no significant differences between WT and HO-1 null mice. In WT mice, hypoxic sigh frequency and hypoxic sensitivity of sympathetic activity initially declined before returning toward baseline after 5 days of CH, correlating with the induction of HO-1. In contrast, HO-1 null mice had a persistent decline in hypoxic sigh frequency and hypoxic sensitivity of sympathetic activity. We conclude that induction of HO-1 in these RVLM cardiorespiratory regions may be important for the hypoxic sensitivity of sighs and sympathetic activity during CH.

pre-Bötzinger complex; C1 sympathoexcitatory region; sighs; spectral analysis of heart rate variability

CHRONIC HYPOXEMIA IS A PHYSIOLOGICAL result of ascent to high altitude and a common clinical consequence of a number of respiratory and cardiovascular disorders, including chronic obstructive pulmonary disease, hypoventilation syndromes, and congestive heart failure. The cardiorespiratory adaptations that develop during chronic hypoxia coordinate increases in ventilation (1, 10, 13, 14) and sympathetic activation of cardiovascular function (5, 8, 37) and represent an important strategy for maintaining oxygen delivery to tissues. Much work has been done demonstrating that cellular and biochemical adaptations within the peripheral chemoreceptors are essential for respiratory and sympathetic acclimatization during chronic hypoxia (33). However, work done by us and others also suggests that, in addition to the peripheral chemoreceptors, there are central hypoxia-sensitive cardiorespiratory sites that may also be important for the cardiorespiratory adaptations to chronic hypoxia (30, 33, 44). Two of these brain regions are within the rostral ventrolateral medulla (RVLM), the C1 sympathoexcitatory region, and the pre-Bo¨tzinger complex (pre-BötC). The C1 region contains neurons that are important for the generation of tonic vasomotor tone and the integration of reflex changes in blood pressure (34), whereas the pre-BötC is the site of rhythm generation responsible for the generation of eupnea, sighs, and gasps (38). Brief exposure of these regions to local hypoxia produces increases in sympathetic and augmented inspiratory activity (sighs and gasps), respectively (29, 39–42). In addition, the sensitivity of RVLM neurons to hypoxia is enhanced after 4–5 days of chronic hypoxia (31). Thus, adaptations to chronic hypoxia could reflect cellular and molecular changes within the central hypoxia-sensitive sites.

An important oxygen-sensing molecule and a potential molecular target for adaptation to chronic hypoxia is heme oxygenase (HO) (45, 46). HO isoforms HO-2 and HO-3 are expressed constitutively, while the inducible isoform HO-1 is expressed in response to hypoxia, oxidative stress, and various other stimuli (7, 27). A hypoxia-sensing function for HO-2 has been found in the carotid body (45), and we have recently shown that HO is necessary for the oxygen-sensing function of the cardiorespiratory regions of the RVLM (9). In a primary cell culture preparation of RVLM neurons, excitation of these neurons by either sodium cyanide hypoxia or hypoxic hypoxia was blocked by tin protoporphyrin IX, an HO blocker, and those neurons that were hypoxia excited were immunoreactive for HO-2 (9). Since hypoxia is a trigger for inducing the expression of HO-1 and our prior work has shown that chronic hypoxia induces expression of HO-1 in the C1 and pre-BötC regions (26), we hypothesized that induction of HO-1 within these medullary regions may be important for their oxygen-sensing function during chronic hypoxia. Because the C1 region is the sympathoexcitatory site and the pre-BötC is the site for the generation of sighs (24), the frequency of which are sensitive to local hypoxia, we were specifically interested in examining how chronic hypoxia altered the sigh-frequency responses to acute hypoxia (as a window to the pre-BötC) and sympathetic activity (as an index of changes in the C1 region). Thus, we examined the time-dependent changes in the expression of HO-1 mRNA and protein in the RVLM during exposure to 10 days of chronic hypoxia in wild-type mice and correlated them with the time-dependent changes in sigh and sympathetic responses to hypoxia in both wild-type and HO-1 null mice.

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METHODS

All studies were performed in adult mice (18–23 wk old) and all experimental procedures were approved by the UMDNJ-Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

Expression of HO-1 in the RVLM During Chronic Hypoxia

Expression of HO-1 in the RVLM During Chronic Hypoxia

Exposure to chronic hypoxia. Adult Balb/c mice (Jackson Labs, Bar Harbor, ME) were placed in four identical air-tight chambers (45 cm × 37.5 cm × 45 cm) and exposed to hypoxia (10% O₂:90% N₂) at ambient pressure or room air (21% O₂). Chronic hypoxia was produced using the Turnkey Oxygen Profile System ( Biospherix) to introduce nitrogen to the chambers sufficient to reduce the FIO₂, to 10%. This system monitors the percent of oxygen and carbon dioxide (CO₂) via a computer, and CO₂ levels were maintained at < 10,000 ppm. Animals were fed ad libitum and maintained in a 12:12-h light-dark cycle.

Real-time PCR assay for HO-1. Mice were exposed to either 10% O₂ (n = 32) or room air (n = 32) for 1, 3, 4, 5, 6, and 10 days. At the end of the timed exposure, mice were overanesthetized with pentobarbital sodium (100 mg/kg ip) and the brain quickly dissected. To assess whether placement in the chamber alone induced HO-1 expression, the brain stems of nine naïve mice (unexposed) were also processed for HO-1 expression. The brain stem was removed, and the RVLM was dissected as previously described (25). In brief, the brain stem was removed, and the RVLM was dissected as previously described (25). In brief, the medulla from the obex to the pontomedullary border was isolated using a stereoscopic dissecting microscope (×40). The medulla was then transversely sectioned, and the RVLM containing the C1 and pre-BötC regions was dissected. The tissue was flash frozen and homogenized in guanylate isothiocyanate buffer. RNA was purified using cesium chloride gradients, and then cDNA was synthesized. cDNA (100 μl) was synthesized from 2 μg RNA obtained from control/room air or hypoxia-exposed mice by using random primers and Superscript II reverse transcriptase. Then 25-μl PCR reactions were carried out using gene-specific primers for HO-1 designed by Primer Express software (7000 Sequence Detection System; Applied Biosystems, Foster City, CA) and SYBR Green master mix. Duplicate wells were included for each condition and primer pair. Housekeeping gene GAPDH was used as an internal control. All samples were first normalized to GAPDH and then represented as a ratio to room air controls. Data analysis was performed according to the protocol provided by Applied Biosystems.

Immunocytochemical localization of HO-1 in the C1 and pre-BötC.

Since the real-time PCR data showed a significant increase in HO-1 mRNA expression in the RVLM after 4 days of exposure to chronic hypoxia, we determined whether this mRNA expression was associated with an expression of HO-1 protein specifically within the hypoxia-sensitive regions of the C1 and pre-BötC. Thus, we determined HO-1 expression using immunocytochemistry in a separate group of mice at 1, 3, 4, and 10 days of exposure to either 10% O₂ (n = 23) or room air (n = 23). At the end of the exposure period, mice were overanesthetized with pentobarbital sodium (100 mg/kg ip) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde (paraformaldehyde in PBS, pH 7.4; Sigma). After perfusing the mouse with 20 ml of heparinized saline (15 min), the perfusate solution was switched to a 4% paraformaldehyde solution and the mouse was perfused for an additional 15 min (20 ml). After the perfusion was complete, the brain was removed and placed in 4% paraformaldehyde overnight at 51°C. The tissue was then cryoprotected in 30% sucrose for 72 h at 51°C. The brain was frozen on dry ice, coated with embedding matrix (Lipshaw) and sectioned (40 μm) by using a cryostat microtome.

Sections were then processed for localization of HO-1 in the C1 and pre-BötC regions. Since catecholaminergic neurons within the C1 region are immunoreactive for tyrosine hydroxylase (TH) and respiratory-related neurons within the pre-BötC are immunoreactive for the neurokinin-1 receptor (NK-1R), immunocytochemistry was performed using antibodies against TH and NK-1R to anatomically define these regions within the RVLM. Brain sections were placed in 12-well culture trays (Falcon) and incubated in 4% paraformaldehyde for 45 min at room temperature, followed by incubation with 3% Triton X-100 (Sigma) for 45 min and with 10% normal goat serum in 0.1 M PBS (blocking buffer) for 1 h. After being washed with PBS (pH 7.4), sections were incubated with a monoclonal mouse anti-HO-1 primary antibody (1:500; Stress Gen) for 24 h at room temperature. Following incubation with the primary antibody, sections were incubated with secondary antibody and biotinylated goat anti-mouse IgG (1:400; Vector) for 2 h followed by Streptavidin Alexa Fluor 488 (1:1,000; Vector) for 1 h at room temperature. Double labeling was then achieved by processing the sections with either rabbit anti-TH (1:300; Chemicon) or guinea pig anti-NK-1R (1:1,000; Chemicon) overnight at room temperature, followed by an incubation with secondary antibodies (1:100 goat anti-rabbit, Texas Red for TH or 1:100 goat anti-guinea pig, Texas Red for NK-1R). Sections were washed in distilled water and mounted onto gelatin-coated glass microscope slides (Fisher) with cytoseal (Stephens Scientific). Control sections for background staining and antibody specificity were processed in parallel. Sections were then visualized for immunoreactivity, and images were digitally captured (SPOT Camera) by using an inverted microscope with fluorescent optics (Nikon). An LSM510 Meta confocal laser scanning microscope (Zeiss) was used to determine colocalization of HO-1 neurons with TH or NK-1R immunoreactive neurons. Serial sections not processed for HO-1, TH, or NK-1R were directly mounted on gelatin-coated glass slides after cryosectioning and stained with neutral red to visualize cell bodies for anatomical localization of nuclei. Cell nuclei were identified according to stereotactic anatomy as described by Franklin and Paxinos (15).

Physiological Studies

Breeding and genotyping of wild-type and HO-1 null mice. Physiological studies were performed on adult wild-type (n = 6) and HO-1 null mice (n = 6). Wild-type and HO-1 null mice were generated from a breeding pair of HO-1−/− mice currently in F10 generation of a Balb/c and 129 SvJ genetic background, which were generously provided by Dr. Yet (46). To identify the HO-1 gene by PCR, primers were generated from exon 2, E2F (5′-TGG CTG AGG CCT TGA AGG AG-3′) and exon 3 E3R (5′-CCA GAG TCT GCA TTC GAG CA-3′) to amplify a 1,500-bp fragment. To identify the mutated allele, a reverse primer was generated for the neomycin gene NeoR2 (5′-TCC TCC TGC AGT TCA TTC GAG CA-3′) to amplify a 1,000-bp fragment. PCR reaction was performed on genomic DNA prepared from tail snips in 20 mM Tris·Cl, 50 mM KCl, 2 mM MgCl₂, 0.5 mM dNTPs, 1 μM each primers, 2.5 units of Taq DNA polymerase at 94°C/3 min, 94°C/3 min, 54°C/30 s, 72°C/45 s for 35 cycles followed by 72°C/10 min. Samples were analyzed on 1% agarose gel 1× Tris-acetate-EDTA buffer at 100 volts for 3 h. PCR amplified product using primer pairs E2F and E3R resulted in a 1,500-bp fragment identifying the wild-type mouse. The HO-1 null mouse was identified by the PCR-amplified product resulting from a primer pair of E2F and Neo R2 in a 1,000-bp fragment.

Surgical implantation of EKG and diaphragmatic electrodes. Wild-type and HO-1 null mice were anesthetized with Avertin (0.9% solution, 135 mg/kg ip) and supplemented with 25 mg/kg as needed. Mice were kept warm by placing them on a thermal barrier (model V21; Vetko). Adequacy of anesthesia was verified by regular tail pinches. Under aseptic technique, a right subcostal incision was made and the abdominal cavity was exposed. The liver was pushed down, and the diaphragm electrodes were positioned on the diaphragm and glued in place (Super Glue; Ross). The diaphragm electrodes consist of hooked miniature platinum iridium bipolar electrode wires (0.0050 in diameter) insulated with quad Teflon (Cooner Wire Specialty, Chatsworth, CA) positioned in a silicon plug (medical adhesive
silicone type A; Dow Corning) that was molded into a small, flat, round wafer (~3–4 mm in diameter). The electrode lead wires were secured in position by suturing them to the abdominal muscles, and then tunneled subcutaneously to the back of the neck where they were exteriorized. Two unpolarized electrodes similarly made from the same wire and positioned in a silicon plug were placed subcutaneously on either side of the thorax through the tunnel and glued into place to record an EKG. The incisions were closed in layers. A disposable skin staple (model DS-5; 3M, St. Paul, MN) was placed between the ears and acted as a reference. The animals were allowed to recover for at least 7 days prior to participating in a study.

**Experimental protocol.** Based on the results of the real-time PCR and immunocytochemistry showing the time of induction of HO-1 mRNA and protein, diaphragm EMG (EMGDi) and EKG recordings were obtained on day 0 (prior to chronic hypoxia exposure) and on 2 days prior to HO-1 induction and 3 days after HO-1 induction. Specifically, EMGDi and EKG were measured on days 1, 3, 5, 7, and 10 of chronic hypoxia exposure in both wild-type and HO-1 null mice.

On each study day at about the same time of day, mice were removed from the exposure chamber and placed in a mouse warming restrainer chamber (Kent Scientific) and continuous recordings of EKG and EMGDi activity were obtained. The mice were allowed to acclimate to the restrainer chamber for 20 min under room air conditions, prior to each recording. Respiratory, sigh, and heart rate responses to acute exposures to hypoxia (10% O2 for 10 min), hyperoxia (100% O2 for 10 min), and hypocapnia (8% CO2 balance O2 for 10 min) were determined. Gases were introduced into the restrainer chamber at the nose piece of the chamber at a flow rate of 4 l/min using a flow meter. Since the volume of the restrainer chamber was 80 ml, gases equilibrated within the chamber in about 1 s.

**Analysis of respiratory and sigh activity.** Changes in respiratory and sigh activity were assessed from the EMGDi signal. The EMGDi signal was amplified (10 K; model 1700; AM Systems), filtered (100–20,000 Hz), and full-wave rectified. A moving time average of the EMGDi signal was obtained using a time constant of 50 ms. Signals were recorded simultaneously on an oscilloscope (model 1604; Gould) and polygraph recorder (model K2G: Grass) for visual evaluation and online by digitizing the signals using computerized multichannel, digital data acquisition package (CODAS, DATAQ) with a sampling rate of 1,000 Hz/channel for subsequent analysis. Peak and valley detection was used to analyze breath-by-breath EMGDi activity and Tn (inspiratory time), Tc (expiratory time), Ttot (Tc + Tn), peak EMGDi (baseline to peak of moving time average EMGDi activity) were calculated. Respiratory parameters were analyzed over the last minute of the acute exposures to hypoxia, hypocapnia, and hypoxia. In addition, since peak respiratory responses to acute hypoxia were observed at the end of 1 min of acute hypoxia, the last few breaths (8–15 breaths) at the end of 1 min of acute hypoxia were used to obtain peak respiratory activity (breathing frequency, peak EMGDi and minute EMGDi). Instantaneous breathing frequency was obtained as 1/Ttot. Peak EMGDi was normalized to the mean peak sigh response and expressed as a percent of mean peak sigh response. Minute EMGDi was calculated as the product of respiratory frequency and peak EMGDi. Sighs were characterized as augmented breaths with amplitude that was at least 150% of mean peak EMGDi. Sighs were further characterized by the presence of a prolongation of Tc (150% of baseline) and/or prolongation of Tn, due to the presence of a post-sigh apnea. The total number of sighs was recorded and sigh frequency was determined for each condition. Sniffs, identified as rapid breaths with an increase in tonic EMGDi, and artifact due to movement were eliminated from the analysis.

**Autoregressive spectral analysis of heart rate variability.** Indirect analysis of sympathetic activity was determined from the spectral analysis of heart rate variability (HRV) from the EKG recording. Instantaneous heart rate depends on the interaction between sympathetic and parasympathetic (vagal) efferent activities and spectral analysis of HRV provides a means of distinguishing the relative influences of sympathetic and parasympathetic activity (2). The EKG signal was amplified (20 K) and filtered (100–10,000 Hz). The signal was recorded simultaneously on an oscilloscope and polygraph recorder for visual inspection and online by digitizing the signals using CODAS at a sampling rate of 1,000 Hz/channel. The peak and valley detection was used to analyze peaks of QRS complexes and determine RR intervals from the last 5 min of each condition. Premature beats, artifacts resulting from noise, oversensing, and drop outs were manually corrected. The spectral analysis technique requires that the data be represented as an evenly spaced time series of the instantaneous RR interval. The series of RR intervals obtained from the recordings are not evenly spaced in time and thus need to be converted to an evenly spaced time series. To convert these data to an evenly sampled time series, a linearly increasing sequence of numbers was constructed representing time sampled at even intervals. This sequence increased in 50-ms increments, reflecting an assumed sample frequency of 20 Hz and spanned the time frame of the raw data. The raw RR interval data were then linearly interpolated onto these evenly sampled time data points to give an evenly spaced time series of instantaneous RR intervals. The spectral frequencies of HRV were determined from the time interval data using autoregressive analysis to obtain spectra with improved low-frequency characteristics. In this technique, the resultant spectra are constrained to be that of a linear system of specified order (in autoregressive analysis, the transfer function of the model contains only denominator terms and is referred to in systems analysis as an “all-pole” model). Before spectral analysis, the time interval data were detrended using the MATLAB routine detrend. This operation removes the best straight-line fit linear trend from the data—reducing artifacts due to long-term drift in the data acquisition hardware. To provide adequate spectral detail, a 10th-order autoregressive model was applied to the detrended data using the modified covariance method implemented in the MATLAB Signal Processing Toolbox.

To study changes in the spectrum that may occur over time, the time interval data were divided into 60-s time segments, and each of these segments were analyzed separately using the autoregressive model. The resulting spectra were plotted separately. Mean values of spectral power of the 60-s bins were then obtained for the frequency ranges described below. Mean heart rate and standard deviation of mean heart rate were also determined.

**Spectra** were divided into four frequency ranges: 1) low frequency (LF1) 0.08–0.4 Hz; 2) low frequency (LF2) 0.4–1.0 Hz; 3) mid-frequency (MF) 1–3 Hz; and 4) high frequency (HF) 3–10 Hz (21). Spectral power was log transformed. Janssen et. al. (21) have demonstrated that decreases in spectral power in the frequency range of 0.4 to 3 Hz (LF+MF) reflects an increase in sympathetic outflow, while an increase in spectral power in the 0.08 to 0.4 Hz (LF) range indicates an increase in parasympathetic outflow in mice.

**Statistical analysis of the data.** All statistical analysis was performed using SAS version 9.1 software (SAS Institute). For the analysis of real-time PCR, because of different numbers of observations in groups (unbalanced design), we used SAS PROC GLM (General Linear Model) statement for two-way ANOVA while comparing HO-1 mRNA expression over time and between room-air controls and hypoxia-exposed mice. If a significant effect of time and hypoxia exposure was uncovered, then post hoc comparisons of least square (Bonferroni’s test) were used to identify any significant differences between day 0 (baseline) and later days. Results are presented as means ± SE of fold increase in HO-1 mRNA. A P value < 0.05 was considered significant.

**Repeated-measures ANOVA** were conducted to examine whether there were baseline (day 0) differences in sigh frequency, LF1, and LF2+MF across each condition compared with normoxia within each genotype. If a significant effect of condition was uncovered, then post hoc comparisons of least square (Bonferroni’s test) were used to identify any significant differences between normoxia and hypocapnia, hypoxia, or hyperoxia.

Repeated-measures ANOVA were performed to examine differences in baseline respiratory responses, peak hypoxic respiratory responses, sigh responses, and LF1 and LF2+MF prior to and follow-
HO-1 mRNA levels were observed on mice exposed to room air at the same time points. Significant increases in days. Fold increases were determined from a comparison of values obtained in mice exposed to room air at the same time points. Values are means ± SE. A significant increase in HO-1 mRNA within the RVLM was first noted after 4 days of chronic hypoxia and persisted until 10 days of chronic hypoxia. Immunocytochemical localization and time course of HO-1 expression in the RVLM of wild-type mice. Since a significant increase in HO-1 mRNA within the RVLM was first noted after 4 days of chronic hypoxia and persisted until 10 days of chronic hypoxia, immunofluorescent techniques were used to determine when induction of HO-1 protein expression was first detectable within the C1 and pre-Boč regions of the RVLM.

**Table 1. Colocalization of HO-1 with NK1-R (pre-Boč region) and TH (C1 region) during 10 days of exposure to room air or chronic hypoxia**

<table>
<thead>
<tr>
<th></th>
<th>NK1-R</th>
<th>HO-1</th>
<th>HO-1 + NK1-R</th>
<th>TH</th>
<th>HO-1</th>
<th>HO-1 + TH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Room air</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>193 (3)</td>
<td>0</td>
<td>0</td>
<td>377 (4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>day 4</td>
<td>183 (3)</td>
<td>0</td>
<td>0</td>
<td>324 (4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>day 10</td>
<td>213 (2)</td>
<td>0</td>
<td>0</td>
<td>515 (4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Chronic hypoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>93 (1)</td>
<td>0</td>
<td>0</td>
<td>401 (4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>day 4</td>
<td>359 (4)</td>
<td>259</td>
<td>242</td>
<td>470 (4)</td>
<td>270</td>
<td>19</td>
</tr>
<tr>
<td>day 10</td>
<td>191 (2)</td>
<td>173</td>
<td>156</td>
<td>764 (4)</td>
<td>626</td>
<td>176</td>
</tr>
</tbody>
</table>

Values are number of positive cells; number within parentheses is the number of mice. NK1-R, neurokinin-1 receptor; HO-1, heme oxygenase-1; TH, tyrosine hydroxylase.
Respiratory Responses in Wild-Type and HO-1 Null Mice Exposed to Chronic Hypoxia

Normoxic respiratory parameters after exposure to chronic hypoxia in wild-type and HO-1 null mice. To determine whether induction of HO-1 resulted in a change in the respiratory response to chronic hypoxia, breathing frequency and peak EMG_{Di} were examined in both wild-type and HO-1 null mice. Exposure to chronic hypoxia resulted in changes in respiratory parameters while inspiring normoxia that were similar in both wild-type and HO-1 null mice. In wild-type mice, chronic hypoxia caused a significant increase in breathing frequency on day 1 and an increase in the peak EMG_{Di} after 10 days of CH (Fig. 4), resulting in a significant increase in minute respiration at day 10 of exposure to chronic hypoxia (Fig. 5). There were no significant differences in the responses between wild-type and HO-1 null mice. These results suggest that the acclimatization of respiration with exposure to chronic hypoxia is not dependent on induction of HO-1 in these mice.

Respiratory response to acute hypoxia after exposure to chronic hypoxia in wild-type and HO-1 null mice. To determine whether induction of HO-1 resulted in a change in the respiratory sensitivity to hypoxia during chronic hypoxia, the respiratory response to acute hypoxia was examined in both wild-type and HO-1 null mice. Prior to exposure to chronic hypoxia, acute hypoxia caused an increase in hypoxia to
normoxia ratios of both frequency (Hx/Nx = 1.6 ± 0.1) and peak EMGDi (Hx/Nx = 1.2 ± 0.2). Exposure to chronic hypoxia had no significant effect on the respiratory response to acute hypoxia in either wild-type or HO-1 null mice (Fig. 6). Thus, induction of HO-1 had no effect on the hypoxic sensitivity of breathing frequency or amplitude of EMGDi during exposure to chronic hypoxia. Presumably, the lack of correlation between the induction of HO-1 in the RVLM in the overall sensitivity of the respiratory response to acute hypoxia is because the peripheral carotid chemoreceptors are the primary afferent sensors of the respiratory response to hypoxia.

Baseline sigh frequency and responses to acute hypoxia, hypercapnia, and hyperoxia in the wild-type and HO-1 null mice. Spontaneous sighs were observed under all of the experimental conditions in both wild-type (n = 6) and HO-1 null (n = 6) mice (Fig. 7). Prior to exposure to chronic hypoxia, the sigh-frequency responses to chemical stimuli were the same in both wild-type and HO-1 null mice (Fig. 8). Specifically, while both wild-type and HO-1 null mice had no significant change in their sigh-frequency response to normoxia, hyperoxia, or hypercapnia, both wild-type and HO-1 null mice responded to acute episodes of hypoxia with about a threefold increase in sigh frequency. Thus, sigh frequency was significantly in-

Fig. 3. Confocal laser photomicrographs of the C1 region following 3, 4, and 10 days of CH showing HO-1 immunoreactivity with green Alexa Fluor 488 (left), tyrosine hydroxylase (TH) immunoreactivity with Texas Red (middle), and an overlay to show colocalization (yellow) of HO-1 and TH (right). A: after 3 days of CH, there is an absence of HO-1-positive neurons in the region containing TH-immunoreactive neurons. B: after 4 days of CH, HO-1-positive neurons are observed; however, the majority of these HO-1-expressing neurons do not colocalize with the TH-immunoreactive neurons. C: after 10 days of CH, HO-1-positive neurons are still present in this TH-immunoreactive region. Scale bar = 50 μM.
increased in response to acute episodes of hypoxia, making it a useful index for hypoxic sensitivity of the pre-Bo\textsuperscript{\textregistered}C, the neural site linked to the generation of sighs.

**Sigh responses following exposure to chronic hypoxia.** Exposure to chronic hypoxia did not alter sigh-frequency responses to acute episodes of normoxia, hyperoxia, or hypercapnia in either wild-type or HO-1 null mice (Table 2). However, the response to acute episodes of hypoxia was significantly altered by chronic hypoxia and the changes in sigh frequency over time differed significantly between wild-type and HO-1 null mice (Fig. 9, Table 3). Following 1 day of chronic hypoxia, there was a significant loss of the sigh-frequency response to acute hypoxia in both wild-type and HO-1 null mice compared with baseline responses prior to chronic hypoxia (Fig. 9A, Table 3), which was not significantly different between wild-type and HO-1 null mice. In wild-type mice, after the initial day 1 loss of a sigh-frequency response to acute hypoxia, the sigh response returned toward baseline with significant increases on days 5, 7, and 10, compared with day 1. In contrast, in HO-1 null mice, following the significant decline in sigh frequency at day 1, there was no recovery of sigh response toward baseline for the duration of the chronic hypoxia exposure (Fig. 9, Table 3). A comparison of the changes in sigh-frequency responses to acute hypoxia from 1 day of chronic hypoxia and the later exposure days found that there were significant differences in the changes in sigh-frequency response to acute hypoxia from day 1 to days 7 and 10 between wild-type and HO-1 null mice (Table 3).

Since the recovery of the sigh-frequency response to acute hypoxia is absent in HO-1 null mice, and the recovery of the response in wild-type mice correlated with the time course of induction of HO-1 in the pre-Bo\textsuperscript{\textregistered}C, these findings suggest that induction of HO-1 in the pre-Bo\textsuperscript{\textregistered}C during chronic hypoxia is important for the increase in the hypoxic sensitivity of the sigh-frequency response with continued exposure to chronic hypoxia.

**Sympathetic and Parasympathetic Responses in Wild-Type and HO-1 Null Mice Exposed to Chronic Hypoxia**

Baseline parasympathetic and sympathetic activity and responses to acute hypoxia, hypercapnia, and hyperoxia in wild-type and HO-1 null mice prior to exposure to chronic hypoxia. Parasympathetic and sympathetic activity were assessed using autoregressive analysis of HRV, whereby an increase in LF\textsubscript{1} reflects an increase in parasympathetic activity and a decrease in LF\textsubscript{2}+MF reflects an increase in sympathetic activity (21). The spectral power of LF\textsubscript{1} and LF\textsubscript{2}+MF during normoxia, hypoxia, hypercapnia, and hyperoxia prior to exposure to chronic hypoxia for both wild-type and HO-1 null mice are shown in Fig. 10. Prior to exposure to chronic hypoxia, exposure to acute hypoxia caused a significant decrease in LF\textsubscript{1} in both wild-type and HO-1 null mice with a concomitant decrease in LF\textsubscript{2}+MF in wild-type mice and HO-1 null mice reflecting a decrease in parasympathetic and increase in sympathetic activity, respectively. Acute episodes of hyperoxia and hypercapnia caused significant increases in LF\textsubscript{1} and LF\textsubscript{2}+MF in both wild-type and HO-1 null mice, indicating an increase in parasympathetic and decrease in sympathetic activity during these conditions. Thus, prior to exposure to chronic hypoxia, the responses to chemical stimuli were similar in wild-type and HO-1 null mice, such that acute hypoxia increased sympathetic activity with a concomitant decrease in parasympathetic activity, while hyperoxia and hypercapnia resulted in increased parasympathetic activity with a concomitant decrease in sympathetic activity.

**Sympathetic and parasympathetic responses following exposure to chronic hypoxia.** Spectral analysis of HRV were obtained prior to and on days 1, 3, 5, 7, and 10 of exposure to
chronic hypoxia in both wild-type and HO-1 null mice as a measure of changes in sympathetic and parasympathetic activity. Increases in LF1 reflected relative increases parasympathetic activity, and decreases in LF2 + MF were interpreted as a relative increase in sympathetic activity. Figure 11 plots the effect of chronic hypoxia on the changes in baseline (normoxic) levels of parasympathetic and sympathetic power in wild-type and HO-1 null mice. Exposure to chronic hypoxia produced an increase in the relative parasympathetic power and decrease in relative sympathetic power during normoxia in both wild-type and HO-1 null mice beginning at 1 day of chronic hypoxia and sustained throughout the chronic hypoxia exposure. There were no significant differences in the temporal changes in baseline (normoxic) parasympathetic or sympathetic power during chronic hypoxia between wild-type and HO-1 null mice.

Exposure to chronic hypoxia altered the changes in parasympathetic and sympathetic power in response to acute challenges of hypoxia in both wild-type and HO-1 null mice (Fig. 12). However, although the initial responses were similar, there were differences in time course of changes in parasympathetic and sympathetic power between wild-type and HO-1 null mice after 5 days of chronic hypoxia. The initial response to acute hypoxia in both wild-type and HO-1 null mice was an increase in parasympathetic and a decrease in sympathetic power at days 1 and 3 of chronic hypoxia. After 3 days of chronic hypoxia, wild-type mice began to show a decline in parasympathetic power with a concomitant increase in sympathetic power. In contrast, in HO-1 null mice, the initial change in parasympathetic and sympathetic power persisted throughout the 10 days of chronic hypoxia. Thus, the sympathetic and parasympathetic responses to acute hypoxia differed signifi-
Values are means ± SE of sigh/min; n = 6 mice. Values were compared using 2-way repeated-measures ANOVA. No statistical differences were found across genotype, condition, or time.

### Table 2. Effect of chronic hypoxia on sigh frequency during normoxia, hypercapnia, and hyperoxia in wild-type and HO-1 null mice

<table>
<thead>
<tr>
<th>Day</th>
<th>Normoxia (Sighs/min)</th>
<th>Hypercapnia (Sighs/min)</th>
<th>Hyperoxia (Sighs/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.73±0.16</td>
<td>0.92±0.12</td>
<td>0.68±0.15</td>
</tr>
<tr>
<td>1</td>
<td>0.56±0.09</td>
<td>0.65±0.09</td>
<td>0.41±0.08</td>
</tr>
<tr>
<td>3</td>
<td>0.6±0.07</td>
<td>0.85±0.17</td>
<td>0.52±0.09</td>
</tr>
<tr>
<td>5</td>
<td>0.76±0.09</td>
<td>0.78±0.1</td>
<td>0.6±0.07</td>
</tr>
<tr>
<td>7</td>
<td>0.71±0.07</td>
<td>0.9±0.08</td>
<td>0.61±0.06</td>
</tr>
<tr>
<td>10</td>
<td>0.73±0.06</td>
<td>0.8±0.06</td>
<td>0.61±0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normoxia (Sighs/min)</td>
<td>Hypercapnia (Sighs/min)</td>
<td>Hyperoxia (Sighs/min)</td>
</tr>
<tr>
<td>0</td>
<td>0.72±0.1</td>
<td>1.06±0.17</td>
<td>0.55±0.08</td>
</tr>
<tr>
<td>1</td>
<td>0.61±0.12</td>
<td>0.9±0.13</td>
<td>0.53±0.06</td>
</tr>
<tr>
<td>3</td>
<td>0.74±0.08</td>
<td>1.08±0.2</td>
<td>0.53±0.07</td>
</tr>
<tr>
<td>5</td>
<td>0.71±0.08</td>
<td>0.99±0.06</td>
<td>0.54±0.07</td>
</tr>
<tr>
<td>7</td>
<td>0.66±0.07</td>
<td>0.99±0.18</td>
<td>0.44±0.03</td>
</tr>
<tr>
<td>10</td>
<td>0.72±0.06</td>
<td>0.89±0.09</td>
<td>0.53±0.04</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The present study extends our previous work demonstrating that HO is an important mediator in the sensitivity of the medullary hypoxia-sensitive regions by showing that: 1) induction of HO-1 in the pre-BötC and C1 regions of the medulla occurs after 4 days of chronic hypoxia and persists for the 10 days of exposure to chronic hypoxia; and 2) these temporal changes in HO-1 induction correlate with time-dependent changes in the sigh-frequency responses to acute hypoxia and sympathetic responses in wild-type mice but are absent in HO-1 null mice.

In contrast to the dependence of the temporal changes in sigh frequency and sympathetic responses on HO-1 induction, the overall respiratory response and hypoxic sensitivity of respiration during chronic hypoxia were not influenced by the induction of HO-1 in the RVLM. Both wild-type and HO-1 null mice acclimated to chronic hypoxia by increasing peak EMGDi after 10 days of chronic hypoxia consistent with other studies in mice (21). There were, however, no changes in the hypoxic respiratory responses with exposure to chronic hypoxia in either wild-type or HO-1 null mice. This suggests that the increase in the sigh response to hypoxia was not the result of a generalized increase in respiratory sensitivity to hypoxia and that the correlation with induction of HO-1 in the RVLM is unique to the hypoxic sensitivity of the neural network generating sighs.

**Hemoxygenase** is necessary for excitatory responses of RVLM neurons to local hypoxia (9). Electrophysiological studies of RVLM neurons reveal that these neurons are excited by NaCN and low PO2. The excitatory response of these neurons is blocked by the HO blocker tin protophorphyrin IX. In addition, the neurons that are excited by hypoxia are immunoreactive for HO-2. These findings suggest that HO is involved in the oxygen-sensing mechanism of these neurons. Our present study has shown that sigh responses and sympathetic responses to chronic hypoxia are significantly decreased initially prior to induction of HO-1 in wild-type mice, but partially restored after 5 days of chronic hypoxia when HO-1 has been induced, suggesting that the continued chemosensitive function of these neurons may be dependent on the expression of HO-1. In contrast, HO-1 null mice fail to demonstrate any recovery in sigh or sympathetic responses to hypoxia.
HO-1 is not expressed in the brain under normoxic conditions (7, 26, 43), but its expression is induced in selective brain regions by chronic hypoxia including the hypoxia-sensitive regions of the RVLM (26, 43). The selective induction of HO-1 in these hypoxia-sensitive regions and the persistent reduction in the sigh response to acute hypoxia in HO-1 null mice suggests that HO-1 may be involved in the oxygen-sensing function of these hypoxia-sensitive regions. There is other evidence in the literature that has shown the dependence of a vascular response on a shift in the relative expression of HO isoforms during chronic hypoxia. For example, a shift from HO-2 to HO-1 has been demonstrated in the cerebral vasculature where HO-2 has been shown to provide short-term protection against postictal cerebral vascular dysfunction, but is inadequate for long-term protection, while the induction of HO-2 provides long-term protection (32).

The temporal profile of the induction of HO-1 by chronic hypoxia has not been examined previously. In our study we found that HO-1 mRNA levels in the RVLM increased significantly after 4 days of chronic hypoxia and remained elevated for the 10 days of exposure to chronic hypoxia. This induction of mRNA is temporally correlated with the expression of HO-1 protein at day 4 within both the pre-BötC and C1 regions. The time for the induction of HO-1, while seemingly long, is consistent with other observations where the induction of HO-1 in the lungs and vasculature following exposure of chronic hypoxia appear to vary from 1 day to a few days depending on the tissue examined (6, 22). There are several mechanisms that could be responsible for induction of HO-1 during chronic hypoxia. HO-1 has been linked to the transcription factors hypoxia-inducible factor 1 and activator protein-1 (19, 23). Other studies in the rat heart, endothelial cells, and vascular smooth muscle cells have shown that induction of HO-1 is stimulated by nitric oxide (12, 18, 20). There has also been some suggestion that the increase in HO-1 mRNA with chronic hypoxia is a result of downregulation of HO-2 (11).

The present study also provided some additional insight into which neurons within these medullary regions express HO by demonstrating that within the pre-BötC, HO-1 was induced within the NK-1R expressing neurons (e.g., respiratory-related neurons), while within the C1 region, HO-1 was induced primarily in cells in proximity to the TH-expressing catecholaminergic neurons. These findings are consistent with the evidence in the literature that has shown the dependence of a vascular response on a shift in the relative expression of HO isoforms during chronic hypoxia.

Table 3. Change in sigh frequency following exposure to chronic hypoxia between baseline (day 0) and day 1 and between day 1 and the following days for wild-type and HO-1 null mice

<table>
<thead>
<tr>
<th></th>
<th>Day 0 to 1</th>
<th>Day 1 to 3</th>
<th>Day 1 to 5</th>
<th>Day 1 to 7</th>
<th>Day 1 to 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>−2.13±0.40*</td>
<td>0.45±0.37</td>
<td>1.36±0.49*</td>
<td>1.10±0.21*</td>
<td>1.15±0.26*</td>
</tr>
<tr>
<td>HO-1 null</td>
<td>−1.47±0.40*</td>
<td>0.09±0.37</td>
<td>−0.03±0.49</td>
<td>−0.10±0.21</td>
<td>−0.10±0.26</td>
</tr>
<tr>
<td>WT vs. HO-1 null</td>
<td>0.66±0.56</td>
<td>−0.36±0.53</td>
<td>−1.40±0.69</td>
<td>−1.20±0.30*</td>
<td>−1.25±0.36*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data were compared using 2-way repeated-measures ANOVA. *P < 0.05.
power (sympathetic power) increases the frequency of these augmented breaths (3, 16), a breathing in all mammals (4). It is well known that hypoxia intervals between eupneic breaths, are a normal feature of which are augmented breaths that occur spontaneously at and when stimulated, can generate sighs and gasps (17). Sighs, sary for a normal rhythm generation (inspiratory pacemaker), previously been shown to express the NK-1 receptor, are neces-

vious for additional insights with regard to the phenotype of these hypoxia-sensitive cardiorespiratory neurons.

The respiratory-related pre-BoëT neurons, which have previously been shown to express the NK-1 receptor, are necessary for a normal rhythm generation (inspiratory pacemaker), and when stimulated, can generate sighs and gasps (17). Sighs, which are augmented breaths that occur spontaneously at intervals between eupneic breaths, are a normal feature of breathing in all mammals (4). It is well known that hypoxia increases the frequency of these augmented breaths (3, 16), a phenomenon that persists even after sectioning the carotid sinus nerves. In addition, local hypoxia within the pre-BoëT produces augmented breaths (e.g., sighs and gasps) (39, 40) a finding that has been replicated in vitro in brain stem slices (24). The observation that the sigh-frequency response to acute hypoxia is lost after 1 day of chronic hypoxia and increased after induction of HO-1 in pre-BoëT of wild-type mice and was not observed in HO-1 null mice suggests that induction of HO-1 may be important for the hypoxic sensitivity of sighs. Furthermore, we propose that the sigh response to acute hypoxia may be good bioassay for changes in the hypoxic sensitivity of the pre-BoëT.

The C1 sympahtoexcitatory region of the RVLM is the location of the catecholaminergic bulbospinal neurons (TH-immunoreactive), which provide the main supraspinal excitatory input to sympathetic vasomotor preganglionic neurons (35). The C1 region also responds directly to local hypoxia with an increase in sympathetic activity (41), and destruction of the C1 region using the immunotoxin antidopamine β-hydroxy-
ylase-saporin abolishes the response to local hypoxia (36). The question addressed in the present study was how chronic hypoxia affects the sensitivity of this region to acute hypoxia and whether induction of HO-1 is important for the hypoxic sensitivity of sympathetic activity. To accomplish this in conscious mice, we used the indirect technique of spectral analysis of HRV to assess changes in sympathetic activity. Mice have high respiratory rates, which impacts the high-frequency spectrum, making it difficult to use the usual LF-to-HF ratio as an index of sympathetic activity. However, a comprehensive study by Janssen et al. (21) has described a methodology that can be used to accurately assess autonomic tone in mice using spectral analysis of HRV using the low- and mid-frequency ranges. Using this technique, we found that chronic hypoxia had two major effects. First, it caused a decrease in the baseline sympathetic activity (activity during normoxia), which is independent of HO-1 induction, suggesting that there are other factors that set the balance of baseline sympathetic activity when mice are subjected to chronic hypoxia. Second, chronic hypoxia altered the sensitivity to acute hypoxic challenges, which was dependent on the induction of HO-1 as indicated by an increase in sympathetic activity after 5 days of chronic hypoxia and a failure to increase sympathetic activity in HO-1 null mice. These observations support the hypothesis that HO-1 induction is also important for the hypoxic sensitivity of sympathetic activity with continued exposure to chronic hypoxia. Of interest is that, unlike the pre-BoëT, where the induction of HO-1 colocalized with the respiratory-related-neurons, we found that in the majority of the neurons, HO-1 was not found within the TH-immunoreactive neurons but rather in neurons that were in close proximity to the bulbospinal neurons. Hence, it is likely that the hypoxic stimulation of the C1 region may be mediated by a distinct population of hypoxia-sensitive neurons within this region.

The initial loss of sigh and sympathetic responses to acute hypoxia after 1–3 days of chronic hypoxia suggests that the constitutive form, HO-2, accounts for the hypoxic sensitivity under normal baseline conditions but is unable to sustain the function in the presence of chronic hypoxia. The likely expla-

Fig. 11. Temporal changes in baseline (normoxic) sympathetic and parasympathetic power during exposure to CH in WT and HO-1 null mice. Note that in WT mice, there is an increase in parasympathetic (●) and decrease in sympathetic power (■) throughout CH exposure. HO-1 null mice show a similar baseline increase in parasympathetic (○) and decrease in sympathetic power (△) with exposure to CH. Changes over time between WT and HO-1 null mice were not statistically significantly different at any specific time point. Values are means ± SE. *P < 0.05 compared with day 0.

Fig. 12. Temporal changes in acute hypoxic responses of sympathetic and parasympathetic power following exposure to CH in WT and HO-1 null mice. Note that in WT mice there is an initial increase in parasympathetic (●) power and a decrease in sympathetic power (■), with a subsequent return toward baseline of parasympathetic and sympathetic power by day 5. In contrast, in the HO-1 null mice, parasympathetic power (○) increases and sympathetic power (△) decreases and that these changes in power persist throughout the 10-day exposure to CH. Significant differences were seen between WT and HO-1 null mice on days 7 and 10. Values are means ± SE. *P < 0.05 compared with day 0. †P < 0.05 comparing WT and HO-1 null mice.

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nation for this phenomenon is a difference in the oxygen affinity of the two isoforms, which requires an isoform with a high affinity for oxygen when tissue oxygen levels are kept low for a prolonged period of time.

In summary, following exposure to chronic hypoxia, HO-1 is induced in the C1 and pre-Bo”tC regions of the RVLM after 4 days of exposure. This induction of HO-1 is important for the continued hypoxia chemosensitivity of the pre-Bo”tC region and the C1 sympathoexcitatory neurons of the RVLM during chronic hypoxia.

**Perspectives and Significance**

The present study demonstrates the role of HO-1 in the central cardiorespiratory adaptations to chronic hypoxia. This study shows that in the presence of chronic hypoxia the chemosensitivity of the central cardiorespiratory controllers, the pre-Bo”tC and the C1 sympathoexcitatory neurons, is correlated with the induction of HO-1. This study also shows that during the initial period of chronic hypoxia, there is a brief loss of chemosensitivity of these regions. Since recent studies have shown that loss of pre-Bo”tC neurons can result in central sleep apnea (28), the impaired chemosensitivity of these neurons early during chronic hypoxia could mean that periodic breathing seen initially at high altitude may in part be as a result of this impaired function. Furthermore, polymorphisms in the HO-1 gene that result in reduced HO activity could result in persistent impairment of chemosensitive function of these regions, resulting in persistent breathing instability during chronic hypoxia.

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