Adaptation to hypobaric hypoxia involves GABA<sub>A</sub> receptors in the pons

Yee-Hsee Hsieh, Thomas E. Dick, and Ruth E. Siegel

Department of Pharmacology, Division of Pulmonary and Critical Care Medicine, Department of Medicine, and Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio

Submitted 14 May 2007; accepted in final form 30 November 2007

Hsieh Y-H, Dick TE, Siegel RE. Adaptation to hypobaric hypoxia involves GABA<sub>A</sub> receptors in the pons. Am J Physiol Regul Integr Comp Physiol 294: R549–R557, 2008. First published December 5, 2007; doi:10.1152/ajpregu.00339.2007.—Survival in low-oxygen environments requires adaptation of sympathorespiratory control networks located in the brain stem. The molecular mechanisms underlying adaptation are unclear. In naïve animals, acute hypoxia evokes increases in phrenic (respiratory) and splanchnic (sympathetic) nerve activities that persist after repeated challenges (long-term facilitation, LTF). In contrast, our studies show that conditioning rats to chronic hypoxia (CHH), an environment characteristic of living at high altitude, diminishes the response to hypoxia and attenuates LTF in a time-dependent manner. Phrenic LTF decreases following 7 days of CHH, and both sympathetic and phrenic LTF disappear following 14 days of CHH. Previous studies demonstrated that GABA is released in the brain stem during hypoxia and depresses respiratory activity. Furthermore, the sensitivity of brain stem neurons to GABA is increased following prolonged hypoxia. In this study, we demonstrate that GABA<sub>A</sub> receptor expression changes along with the CHH-induced physiological changes. Expression of the GABA<sub>A</sub> receptor α4 subunit mRNA increases two-fold in animals conditioned to CHH for 7 days. In addition, de novo expression of δ and α6, a subunit normally found exclusively in the cerebellum, is observed after 14 days. Consistent with these changes, diazepam-insensitive binding sites, characteristic of GABA<sub>A</sub> receptors containing α4 and α6 subunits, increase in the pons. Immunohistochemistry revealed that CHH-induced GABA<sub>A</sub> receptor subunit expression is localized in regions of sympathorespiratory control within the pons. Our findings suggest that a GABA<sub>A</sub> receptor mediated-mechanism participates in adaptation of the sympathorespiratory system to hypobaric hypoxia.

NEURAL NETWORKS THAT CONTROL homeostasis are plastic. Adaptation to reduced oxygen at high altitude requires days-to-weeks of continuous exposure (2, 24, 29, 30, 38). This adaptation involves both peripheral and central control mechanisms that maintain homeostasis. Central sympathorespiratory control networks express short-term plasticities elicited by brief (45 s) hypoxic challenges. These plasticities result in different sympathorespiratory motor output after challenges are terminated. Two types of behaviors are evoked depending on the pattern of acute hypoxic challenges. A single challenge elicits a transient (min) decrease in respiratory frequency termed posthypoxic frequency decline (PHFD) and increases respiratory effects on sympathetic nerve activity (9, 12). Multiple challenges elicit a sustained (h) increase in sympathorespiratory motor output termed long-term facilitation (LTF) (1, 18). Exposure to chronic hypoxia (days) attenuates PHFD, whereas its effect on LTF is unknown (16, 18). Prolonged hypoxic exposures also increase tyrosine hydroxylase expression in brain stem catecholaminergic cells (14, 28, 34, 35). Although these changes in sympathorespiratory motor patterns are mediated by brain stem nuclei, the neurotransmitters involved and the interplay among these nuclei are not fully identified.

The pons, a brain stem region, contains nuclei that participate in the expression of PHFD and LTF. We previously demonstrated that cells in the ventrolateral (vl) pons mediate PHFD. Disrupting activity in this region blocked PHFD without affecting the respiratory response during acute hypoxia (9, 20). Furthermore, stimulating the A5 neuronal population increased splanchnic sympathetic nerve discharge (17). Cells in the ventromedial pons (caudal raphé) project to sympathorespiratory motor nuclei and mediate LTF (25, 26, 37). The contribution of these pontine nuclei to the adaptation to chronic hypobaric hypoxia (CHH) has not been investigated.

Many findings suggest that the response and adaptation to hypoxia depend on the GABAergic system. During hypoxia, GABA is released in brain stem regions involved in the control of homeostasis (32). Furthermore, bilateral microinjections of a GABA<sub>A</sub> receptor agonist, muscimol, in the vl pons blocks PHFD (9). In contrast, injection of a GABA<sub>A</sub> receptor antagonist, bicuculline, alters the hypoxic response (16). Finally, we previously found that the mRNAs encoding the GABA<sub>A</sub> receptor α6 and δ subunits are expressed de novo and exclusively in the pons after 2 wk of CHH (16).

To delineate the role of GABAergic system in the physiological adaptation to CHH, we compared the temporal relationship between changes in activity-dependent plasticity (LTF) and GABA<sub>A</sub> receptor expression. We report that CHH-elicited attenuation of LTF is associated with induction of the expression of select GABA<sub>A</sub> receptor subunit mRNAs in pontine nuclei involved in sympathorespiratory control. These changes include an increase in the α4 subunit and de novo expression of the α6 and δ subunits. The upregulation of subunit mRNA expression is associated with an increase in GABA<sub>A</sub> receptor number in the pons. In addition, the α6 subunit polypeptide was expressed only by cells in the raphé and vl pons. These findings suggest that GABA<sub>A</sub> receptor plasticity contributes to adaptation to hypobaric hypoxia.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley (Zivic Miller, Zelienople, PA) rats (280–320 g) were maintained in hypoxic (n = 56) or normoxic conditions (n = 12), or left in naïve state (n = 12). The hypoxic group was placed in a hypobaric chamber simulating hypoxia at high altitudes (0.5 atm) for 3.7, or 14 days. Normoxic animals were placed in the chamber without hypoxic exposure for similar times.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Animals were placed on a circadian light-dark cycle, had free access to food and water, and were inspected daily. The hypobaric chamber was opened 2 or 3 times a week to clean cages and to replenish food and water; during this period of about 15 min, the animals were at the normal barometric pressure (~1 atm). Naive animals were never placed in the chamber.

**Electrophysiology.** The hypoxic response and long-term facilitation (LTF) in the anesthetized in vivo rodent preparation were assessed as previously described (9, 16). All surgical and experimental protocols followed National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee. In brief, immediately after hypobaric hypoxic conditioning, animals were anesthetized with equithesin (30 and 133 mg/kg pentobarbital sodium and chloral hydrate, respectively), and the anesthetic level was evaluated by assessing the withdrawal reflex before neuromuscular blockade and by assessing the cardiorespiratory response after neuromuscular blockade (pancuronium bromide ~0.1 mg/100 g body wt). If responses were evoked by nociceptive stimuli, then anesthesia was supplemented by intravenously administering one-tenth of the initial dose. Neuromuscular blockade was supplemented hourly also by intravenously administering one-tenth of the initial dose. The femoral artery and vein were cannulated to monitor blood pressure and dose. Neuromuscular blockade was supplemented hourly also by intravenously administering one-tenth of the initial dose. The femoral artery and vein were cannulated to monitor blood pressure and dose. Neuromuscular blockade was supplemented hourly also by intravenously administering one-tenth of the initial dose. The femoral artery and vein were cannulated to monitor blood pressure and dose.

**Quantitative analysis of respiratory and sympathetic responses to hypoxia.** Breathing pattern was measured by assessing phrenic nerve activity (PNA). Onset of PNA was the point on the positive slope of the integrated PNA signal that was 10% above the interburst value. Offset of PNA was identified on the negative slope of the signal that was 25% below the peak value. The end of the cycle was identified as the integrated PNA signal that was 10% above the interburst value. The exposures were poikilocapnic and end-tidal PCO2 decreased less than 2 mmHg during this brief hypoxic exposure.

**Quantitative analysis of respiratory and sympathetic responses to hypoxia.** Breathing pattern was measured by assessing phrenic nerve activity (PNA). Onset of PNA was the point on the positive slope of the integrated PNA signal that was 10% above the interburst value. Offset of PNA was identified on the negative slope of the signal that was 25% below the peak value. The end of the cycle was identified as the onset of the next breath. The peak activity of PNA was averaged for 10 cycles immediately before starting the 10 cycles of intermittent hypoxia, in the 5-min recovery period between the 9th and 10th acute hypoxic stimuli and 1 h after the 10th acute hypoxic stimulus.

Quantitative analysis of splanchnic sympathetic nerve activity (sSNA) was based on cycle-triggered averages (CTA) constructed to compare the coupling patterns between PNA and sSNA. Averaging increased the signal-to-noise ratio of sSNA that was time-locked to the respiratory cycle (12). For averaging, the analog signal of sSNA was rectified, integrated (CWE, Wood Dale, IL; Paynter Filter, 50-ms time constant), sampled at 200 Hz, and summed (National Instruments, Analog-to-Digital board, Austin, TX). The reference point (time 0) for CTAs was the phase transition between inspiration and expiration.

The average amplitude under the curves was calculated for PNA and sSNA. The inspiratory and expiratory portions of the sSNA cycle-triggered averages were divided in half, thereby allowing direct comparison of the magnitude of sSNA. The significance of observed differences in values proceeding and following repeated acute hypoxic exposures were determined by two-way ANOVA for repeated measures. The factors for the two-way repeated-measures ANOVA were (1) subject (the animal), (2) days of conditioning, and (3) time point (before the acute hypoxic exposure, 5 min between the 9th and 10th exposures, and 60 min after the 10th acute hypoxic exposure). The variables, area of integrated PNA, and average amplitude of integrated sSNA in the first and second halves of inspiration and of expiration were ranked and transformed to pass the normality test. Significant differences within variables were identified by the Student-Newman-Keuls test. To identify significant correlations among sSNA and PNA, linear regression analysis during the first and second halves of each phase was performed and values of P ≤ 0.05 were taken as significant. Data are presented as means ± SE as indicated.

**RNA isolation and RT-PCR.** Relative levels of GABA<sub>A</sub> receptor subunit mRNAs in normoxic and hypoxia-exposed animals were determined using a semiquantitative RT-PCR protocol essentially, as previously described (3, 4, 33). In brief, the pons, medulla, thalamus, and cerebellum were dissected from brains of euthanized animals after the indicated exposure to CHH (naive rats were housed in the chamber with airflow but not hypobaric pressure). Pontine tissue was dissected using the following landmarks: rostral-caudal to the inferior colliculi (dorsal) and rostral to the pontine gray (ventral); caudal-adjacent to origins of cranial nerves V, VI, VII, and VIII (the inferior border of the pontine prominence). RNA was extracted from all samples using TRIzol, according to manufacturer’s (Invitrogen, Carlsbad, CA) protocol, reverse transcribed, and processed for PCR using GABA<sub>A</sub> receptor subunit-specific primers in buffer containing [α-<sup>32</sup>P]dCTP. The expression of GAPDH and 18S RNAs were also quantified as internal controls. All experiments were performed at least six times using tissue prepared from different animals.

The PCR products were separated on 8% nondenaturing polyacrylamide gels, which were dried and detected with a Molecular Dynamics Phosphorimager. The band intensities were quantified using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA). To compare mRNA expression in the different experimental conditions, the intensities of the receptor subunit mRNAs were normalized to the density of either the GAPDH or 18S bands in the samples. Significant differences in receptor subunit mRNA levels in naïve vs. hypoxia-conditioned animals were assessed by applying a two-way ANOVA (Sigma Stat 2.03). Specific differences were then identified using Student-Newman-Keuls post hoc test. Primer sequences are shown in Table 1.

**Ligand binding assay.** Membranes from the pons, medulla, thalamus, and cerebellum of hypoxia-conditioned and normoxic rats were harvested, and binding assays were performed using the benzodiazepine partial inverse agonist [1H]Ro-15-4513 (Perkin-Elmer, Waltham, MA), a ligand that recognizes diazepam-insensitive sites characteristic of GABA<sub>A</sub> receptors containing α6, α4, and ß subunits. Naïve and CHH-conditioned rats were euthanized by exposure to hypercapnic hypoxia. Protein extraction was performed as previously described (39). Samples (50–300 μg of protein) were incubated with

<table>
<thead>
<tr>
<th>Table 1. Primer sequence</th>
<th>GABA&lt;sub&gt;A&lt;/sub&gt; Receptor Subunit</th>
<th>Positive 5′-3′</th>
<th>Negative 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>GCGACACCGCAGATGAATGTT</td>
<td>GAGCCGATAAAAAGAGAACGG</td>
<td></td>
</tr>
<tr>
<td>α4</td>
<td>CTAGGGCAGCAGTGGCTCC</td>
<td>GGGAGTCGGAGCAGGAAGAG</td>
<td></td>
</tr>
<tr>
<td>α6</td>
<td>GCGAGAAACACCGCAGCTGATATT</td>
<td>GACATTAGTTCTGCGAAGGAC</td>
<td></td>
</tr>
<tr>
<td>β2</td>
<td>CTTGAGAAGCTCAAGGCTGAGGGGAGC</td>
<td>TGGCTCAAGATGCGCAGAATTTA</td>
<td></td>
</tr>
<tr>
<td>δ</td>
<td>CCTAGACACCTCCATTTGGATGTTGC</td>
<td>ACGCTGCGGCGTGAGGCGGATAA</td>
<td></td>
</tr>
<tr>
<td>ε</td>
<td>AAGAGGCTAGTACATGTTGTTCT</td>
<td>GAAATTCTTCTGTGGAGAACC</td>
<td></td>
</tr>
<tr>
<td>γ2</td>
<td>GGCTGATAGAAGCGCTGAGGAAATT</td>
<td>CACAGATGTCAGGCGAGACG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGGTGTGAACACCTGAGAAGAATA</td>
<td>GGTTGTCAGGAGGAGAGTT</td>
<td></td>
</tr>
</tbody>
</table>

**AJP-Regul Integr Comp Physiol** • VOL 294 • FEBRUARY 2008 • www.ajpregu.org
saturating concentrations of 10 μM diazepam and the indicated concentrations of [3H]Ro15–4513 for 1 h at 4°C and harvested on nitrocellulose membranes using 50 mM Tris-HCl, pH 6.9 and counted. GraphPad Prism (3.0) software was used to fit the data and to a one-site binding hyperbola. The data represent values obtained from three assays in five different animals from each hypoxic exposure period.

**Immunohistochemistry.** For immunostaining, control and hypoxia-exposed rats were euthanized and immediately perfused via cannulation through the left ventricle with 100 ml heparinized (20 units/ml) 10 mM saline followed by 200 ml 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The brains were removed, postfixed at 4°C overnight, transferred to 30% sucrose in PBS at 4°C, and then stored at −80°C until use.

Coronal sections (15 μm) were cut through the entire brain stem and mounted on poly-L-lysine slides. The sections were permeabilized in PBS containing 0.3% Triton X-100 and 5% BSA for 1 h. Sections were then incubated in dilution buffer containing the antibodies against the α6 subunit at 1:200 (Chemicon, Temecula, CA) and tryptophan hydroxylase at 1:1,000 (Chemicon) for 3 h at room temperature. Western blot analysis demonstrated that the α6 antibody recognizes a single band of the appropriate molecular weight in the cerebellum of naïve rats, supporting its specificity. After incubation, the sections were rinsed three times in PBS and incubated with species-specific secondary antibodies at 1:500 (Jackson ImmunoResearch Laboratories, Bar Harbor, ME) for 1.5 h at room temperature. After washing three times for 10 min in PBS, sections were mounted and examined with a Nikon FX microscope. Background staining was determined by incubating adjacent sections with a secondary antibody alone. Images were collected from experimental and control samples prepared from at least four different animals.

**RESULTS**

**CHH induces alterations in the sympatho-respiratory response to acute hypoxia.** Exposure to brief hypoxic stimuli elicited changes in sympatho-respiratory activity. In naïve rats, repeated acute hypoxic challenges (n = 10) augmented both sSNA and PNA during the stimulus period (Fig. 1A, compare left to middle panel). The elevation in sSNA and PNA remained 1 h after cessation of the hypoxic challenges, indicative of LTF (Fig. 1A, right).

Conditioning to CHH altered the sympatho-respiratory response to acute hypoxic challenges in a time-dependent manner. After 3 days of conditioning, acute hypoxia increased sSNA as in naïve animals (Fig. 1B, middle); in contrast, PNA did not increase. Both motor outputs, however, augmented progressively following cessation of the hypoxic challenges (Fig. 1B, right) and thus exhibited LTF. In contrast, after 7 days of CHH, sSNA and PNA were affected differently by acute hypoxic stimuli; LTF persisted in sSNA, but was absent in PNA (Fig. 1C). After 14 days of CHH, the repetitive acute

![Fig. 1. Conditioning with chronic hypobaric hypoxia (CHH) reshaped the sympatho-respiratory response to acute hypoxia in a time-dependent manner. A–D: representative tracings of integrated splanchnic sympathetic (sSNA) and phrenic nerve (PNA) activities are shown before (left), during (middle), and following a 1-h recovery period after acute repetitive hypoxic challenges (right). Recruitment of sSNA and PNA by repetitive hypoxic exposures (10 challenges of 8% O2, 45 s, separated by 5 min of recovery) increased after 3 days of conditioning (B), whereas PNA was attenuated by 7 days (C). By 14 days, both sSNA and PNA LTF were blunted (D). Thus, CHH affects both sympathetic and respiratory response to hypoxia over different time courses.](http://ajpregu.physiology.org/)
hypoxic stimuli failed to augment either sSNA or PNA, and as a result, LTF of both activities (evident 1 h after cessation of acute hypoxic challenges) was not evoked (Fig. 1D).

Sympathetic and respiratory hypoxic responses were quantified by examining the effects of CHH on the average amplitude of integrated sympathetic activity and the peak amplitude of integrated phrenic nerve activity. Analysis of sSNA was based on the respiratory pattern. In naïve animals, the amplitude of sSNA was elevated most notably during the first half of expiration (sE-1), and this increase persisted 1 h after cessation of repetitive acute hypoxic challenges (Fig. 2A, top). The rise in sSNA during sE-1 roughly paralleled increased PNA (Fig. 2B, top). After 3 days of CHH conditioning, sympathetic activity increased uniformly across the respiratory cycle and exhibited an enhanced LTF (Fig. 2A, second panel). In contrast to the individual animal shown in Fig. 1, group data showed that PNA did increase immediately after the acute hypoxic challenges after 3 days of CHH conditioning. Similar to the individual animal’s tracing, group data showed robust LTF after 1 h of recovery (Fig. 2B, second panel). After conditioning for 7 days, sSNA was recruited primarily in the second half of inspiration (sI-2) during hypoxic challenge (Fig. 2A, third panel). Further, expression of LTF within PNA was abrogated completely (Fig. 2B, third panel). After 14 days of CHH, acute hypoxic challenges failed to elicit increases in sympathetic and phrenic activities, either during the challenges or after 1 h of recovery (Fig. 2, A and B, bottom), at which time LTF was clearly evident in naïve rats. This analysis clearly demonstrates that CHH conditioning differentially affects the temporal changes in the patterns of sSNA and PNA. In addition, 14 days of CHH conditioning was required to abolish LTF completely in both classes of motor activity.

Altered pontine GABA<sub>A</sub> receptor subunit expression accompanies CHH-induced changes in the sympathorespiratory response. To determine whether GABA<sub>A</sub> receptor expression in the brain stem could contribute to the CHH-induced plasticity of sympathorespiratory responses, we measured receptor sub-
unit mRNAs in regions involved in their control. GAPDH mRNA expression in the brain stem tissue remained consistent throughout the hypoxic period. Within 3 days of CHH and parallel with the onset of the delay of LTF in PNA, the level mRNA expression for the GABA_A receptor α6 subunit rose twofold in the pons; its level remained elevated throughout the experimental period (Fig. 3A). After 14 days of CHH and coinciding with attenuation of the sympathetic response to hypoxia (Figs. 1 and 2), robust de novo expression of the mRNAs encoding the α6 and δ subunits in the pons; neither subunit was detectable in naïve or normoxic (not shown) animals. In contrast, expression of the β2 subunit was upregulated at least twofold to plateau levels by 3 days of CHH. Similar patterns were observed in six independent experiments. B: CHH failed to elicit similar changes in GABA_A receptor subunit expression in the medulla. GAPDH, which remained at a constant level, was used to normalize data. Cb, cerebellum; Th, thalamus.

To show that elevated expression of subunit mRNAs was associated with increased GABA_A receptor number, receptor levels were measured by radioligand binding. Because the α4 and α6 subunits contribute to receptors that are insensitive to diazepam, binding was assessed with [3H]Ro 15–4513, a ligand selective for these sites. These studies demonstrated that the diazepam-insensitive sites rose progressively within the pons during CHH. [3H]Ro 15–4513 binding was unaltered after 3 days of hypoxia but then increased by 250% and 500% over the control level after 7 and 14 days of CHH (Fig. 4A). The K_d of Ro 15–4513 binding was the same in animals exposed to all periods of CHH (Fig. 4B and Table 2), indicating that receptor affinity is unaltered.

The CHH-induced increase in receptor concentration was restricted to the pons. No changes in diazepam-insensitive binding were seen in the medulla, in agreement with the lack of detectable changes in subunit mRNA expression in this region (data not shown). In addition, CHH did not alter receptor binding in the cerebellum, the only brain region in which the α6 subunit is expressed in naïve animals. The concentrations of diazepam-sensitive receptors in the pons, medulla, and cerebellum were likewise unaffected by CHH (data not shown), underscoring the selectivity of the CHH-induced elevations of pontine Ro 15–4513 binding.

Finding GABA_A receptor α6 subunit mRNA expression in the pons is novel and surprising. To localize expression of this subunit, α6 polypeptide distribution was examined immunohistochemically. As expected, α6 subunit staining was not detected in the pons of naïve animals (Fig. 5B) or in animals exposed 3 and 7 days to CHH (data not shown). In contrast, subunit-expressing cells were found after 14 days CHH when the subunit mRNA was first detected (Fig. 3). At this time, α6-subunit-positive cells were found in an area in the vl pons containing A5 adrenergic neurons (Fig. 5, C and D), cells involved in sympatho-respiratory control. In contrast, staining was absent from the pontine reticular formation and the medulla (not shown). As expected, α6 subunit expression in cerebellar granule neurons was unaltered by CHH (Fig. 5, E and F).

Additional studies demonstrated that the α6 subunit was expressed in ventromedial pons. Following 14 days of CHH, positive cells were detected in the raphé pallidus (RPa; Fig. 6A). The staining was colocalized with serotonergic cells containing trypto-phan hydroxylase. Previous studies have shown that this region contains elements of the respiratory neural control network. Together, these findings suggest that GABA_A receptors containing the α6 subunit participate in reshaping LTF in response to repetitive hypoxic challenges.

**Fig. 3.** CHH induces the expression of select GABA_A receptor subunit mRNAs within the pons. A: autoradiograph of a representative RT-PCR experiment shows that the mRNAs encoding the GABA_A receptor α6 and δ were induced in the pons after 14 days CHH. β2 subunit expression was constant and ε subunit expression was absent. In contrast, expression of the α4 subunit was upregulated at least twofold to plateau levels by 3 days of CHH. Similar patterns were observed in six independent experiments. B: CHH failed to elicit similar changes in GABA_A receptor subunit expression in the medulla. GAPDH, which remained at a constant level, was used to normalize data. Cb, cerebellum; Th, thalamus.

**Fig. 4.** CHH increases the concentrations of GABA_A receptors recognized by Ro 15–4513 in pontine tissue. A: saturation binding assays show that binding levels are very low in naïve (○) and 3 d CHH (λ) rats but increase 250% after 7 days (■) and 500% after 14 d CHH (○). B: Scatchard analysis indicates that Ro 15–4513 binding affinities (K_d) are unaltered by CHH conditioning.
Table 2. Pharmacologic properties of Ro 15-4513 in pons of naïve and conditioned animals

<table>
<thead>
<tr>
<th>Condition</th>
<th>( K_d, \text{nM} )</th>
<th>( B_{max}, \text{fmol} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>3.75 ± 0.3*</td>
<td>340 ± 25*</td>
</tr>
<tr>
<td>3 days</td>
<td>3.60 ± 0.4</td>
<td>350 ± 20</td>
</tr>
<tr>
<td>7 days</td>
<td>4.00 ± 0.5</td>
<td>860 ± 70</td>
</tr>
<tr>
<td>14 days</td>
<td>3.90 ± 0.3</td>
<td>1500 ± 160</td>
</tr>
</tbody>
</table>

*Values represent means ± SE (n = 3 in independent experiments). Data for equilibrium binding (dissociation constant \( K_d \)) and maximum binding (\( B_{max} \)) were measured as described in MATERIALS AND METHODS.

**DISCUSSION**

The association of de novo GABA\(_A\) receptor subunit expression and the absence of plasticity in the motor activity is consistent with the GABAergic system partially mediating the physiological adaptation of adult rats to CHH. These studies demonstrate that the sympathorespiratory plasticity evoked by acute intermittent hypoxia depends on the number of days of conditioning in hypobaric hypoxia. Concurrent with these physiological changes, GABA\(_A\) receptor expression is upregulated in pontine nuclei that participate in sympathorespiratory control.

Our physiological results reveal that CHH attenuates sympathorespiratory LTF in a time-dependent manner. The loss of respiratory LTF (PNA) occurred progressively. Acute hypoxic challenges triggered an increase in PNA in naïve and 3-day CHH-conditioned animals; however, the onset of this change could be delayed in animals exposed 3 days to CHH (comparing Figs. 1 and 2). By 7 days, respiratory LTF was abolished, a change that remained at 14 days. In contrast, the change in sympathetic LTF was much slower, with no attenuation detected until 14 days of CHH. These temporal disparities in the effects of CHH suggest that sympathetic and respiratory LTF are differentially regulated.

In support of the role of GABAergic system in the adaptation to CHH, our studies demonstrate that expression of a subset of the GABA\(_A\) receptor subunits is upregulated or induced in pontine nuclei by this stress. Our findings suggest that GABA\(_A\) receptors containing the \( \alpha_4, \alpha_6, \) and \( \delta \) subunits are critical for adaptation due to their temporal and spatial patterns of expression. At 3 days, the increase in \( \alpha_4 \) subunit

Fig. 5. The GABA\(_A\) receptor \( \alpha_6 \) subunit is expressed in the vl pons after 14 days CHH. A: Cresyl violet staining of the vl pons shown for orientation. B–F: fluorescence micrographs of cells stained with an \( \alpha_6 \) subunit-specific antibody. B: expression of the \( \alpha_6 \) subunit is absent from the vl pontine neurons of naïve animals. C: cells positive for \( \alpha_6 \) subunit are prominent within the A5 region after 14 days of CHH. D: Higher magnification of boxed area shown in C. E: GABA\(_A\) receptor \( \alpha_6 \) subunit staining is present in the cerebellar granule cell neurons but not in the Purkinje cell layer in naïve rats, as expected. F: similar levels of expression in the cerebellum were observed after 14 days of CHH. SO, superior olive; 7n, facial nerve; tz, trapezoid body; rs, rubrospinal tract; G, cerebellar granule cell layer; and P, Purkinje cell layer. Scale bar = 100 \( \mu \)m.
mRNA expression coincides with the decrease in respiratory LTF. By 14 days, the de novo expression of the α6 and δ subunits mRNAs in the pons coincides with the absence of activity-evoked plasticities. Cells expressing the α6 polypeptide appeared exclusively in the raphe and vl pons, regions of sympathorespiratory control. This finding is novel because the α6 subunit is restricted to cerebellar granule neurons in naïve adult rodents. Finally, it is striking that no changes in subunit expression were observed in the medulla, the region containing the central respiratory pattern generator.

In agreement with changes in GABA<sub>A</sub> receptor subunit expression, GABA<sub>A</sub> receptor number was upregulated in the pons. Most importantly, the number of diazepam-insensitive GABA<sub>A</sub> receptors increased. This is consistent with the fact that the α4 and α6 subunits confer diazepam insensitivity. It is of interest that expression of the α6 and δ subunits was induced simultaneously. These two subunits have been shown to coexist in one GABA<sub>A</sub> receptor type (21, 27). The fact that the number of diazepam-sensitive sites did not change suggests that CHH induces the expression of a select type of GABA<sub>A</sub> receptor.

Both of our physiological and molecular findings implicate the importance of cells in the vl pons and raphe in mediating adaptation to CHH. Moreover, along with the anatomical and physiological findings of other investigators (8, 25, 36), our results support the importance of GABA and GABA<sub>A</sub> receptors in these regions for adaptation. In previous physiological studies, we demonstrated that bilaterally lesioning the vl pons and/or inhibiting activity in this region with injections of the GABA<sub>A</sub> agonist, muscimol, block PHFD (9) and attenuate the sympathetic response to hypoxia (22, 23). Conversely, stimulating the vl pons or blocking the GABA<sub>A</sub> receptor with the antagonist, bicuculline, increased the duration of expiration during hypoxia (16). Finally, recordings of vl pontine activity identified neurons that are activated during hypoxia and remain activated when the hypoxic stimulus is terminated (10).

It is surprising that a prolonged period (14 days) of CHH was required to induce α6 and δ subunit expression. We speculate that the delay in the expression of these subunits depends on the vital contribution of the peripheral mechanisms, such as the vascularization of carotid body and changes in circulating cerebrospinal fluid bicarbonate (28a). Furthermore, the delay may reflect the complex interactions between neurotransmitters (glycine, nitric oxide, glutamate, substance P, and GABA) that mediate the hypoxic response (5–7, 31).

Fig. 6. GABA<sub>A</sub> receptor α6 subunit expression was induced in rostral regions of the raphe pallidus nucleus after 14 days CHH. A: fluorescence photomicrograph of cells stained with an α6 subunit-specific antibody. B: cells in the same region positive for tryptophan hydroxylase (TrpOH) an enzyme identifying serotinergic cells. C: overlay of the α6 and TrpOH staining demonstrating colocalization within cells in the raphe pallidus. The small arrows indicates a neuron in the raphe pallidus expressing both α6 and TrpOH staining. py, pyramidal tracts; tz, trapezoid body; RPa, raphe pallidus; D, dorsal; Scale bar = 50 μm.

Fig. 7. Model of neural interactions after repetitive hypoxic exposures. A: In naïve animals, carotid body afferent input during repeated hypoxia activates pontine circuits and input to the medulla and spinal cord. This activation elicits a sustained increase in sympathetic and phrenic activity (LTF) even though afferent activity has returned to baseline. B: After 14 days of CHH, LTF is ablated, although afferent inputs from the carotid are present. This reflects an increased GABA<sub>A</sub> receptor number (Y) within the raphe and A5 nuclei. Activation of these GABA<sub>A</sub> receptors attenuates increases in raphe and A5 activity and thus prevents LTF. LTF, long-term facilitation; DI, diazepam-insensitive GABA<sub>A</sub> receptors; DS, diazepam-sensitive GABA<sub>A</sub> receptors; open arrows, baseline activity; black arrows, increased activity.
In conjunction with the findings of other investigators, our findings raise the possibility that the GABAergic and serotoninergic systems interact in the raphé to regulate LTF. Previous studies have shown that LTF is dependent on serotonin receptor activation and that systemic or intrathecal methysergide, a receptor antagonist, block LTF (11, 13, 15, 26). Moreover, the caudal raphé is the sole source of serotonin in the spinal cord, and serotoninergic neurons in the raphé magnus project directly to the phrenic motor nucleus. Recent work has shown that the serotoninergic and GABAergic systems interact in the raphé (8), a possibility supported by our immunohistochemical findings.

It is possible that the signaling in the raphé provides a central, rather than peripheral, control of sympatho-respiratory LTF motor activity. Our findings suggest that increased GABA A receptors in CHH animals decrease phrenic and sympathetic LTF by inhibiting activity in the caudal raphé.

On the basis of these findings, we present a model of the pathways involved in regulating LTF in naïve and CHH-conditioned animals (Fig. 7). In this model, pontine GABA A receptors play a key role in mediating the plasticity manifested by the sympatho-respiratory hypoxic response. In naïve animals, hypoxic conditions detected by the carotid body cause signaling to the nuclei of the solitary tract, and this is relayed to both the pons and ventrolateral medulla. These sensory and initial transmission phases of the response to hypoxia are mediated by glutamate (19, 32). The sensory input returns to baseline following acute hypoxic challenges and ceases to drive respiratory and sympathetic activities. In contrast, pontine neurons in the raphé and A5 areas that are activated during hypoxia remain active after the challenge and mediate the expression of LTF (Fig. 7A, gray solid arrow). The extent of raphé and A5 activation is regulated by GABA (Fig. 7A, solid broken arrow). Consequently, during long hypoxic exposures, GABA is released after the initial response and acts to decrease ventilation partially by inhibiting these modulatory nuclei.

In contrast, exposure to CHH leads to upregulation of the expression of select GABA A receptor subunits within the raphé and A5 areas. This may be due to either a prolonged release of GABA or the feedback inhibition to the excitatory glutameric input from the nTS. In any case, the change in GABA A receptor subunit expression alters the interplay between the GABAergic, serotoninergic (raphé), and adrenergic (A5) neurons within this region. Thus, while these pontine nuclei excite the sympatho-respiratory system following acute hypoxia in naïve animals, the enhanced GABA-mediated inhibition of these pontine subnuclei in animals exposed to CHH precludes the activation of PNA and sSNA, thus preventing LTF. Our studies predict that blocking expression of select GABA A receptor subunits within the pons prevents adaptation to CHH. Our study raises the possibility that the observed plasticity of GABA A receptor subunit expression within the pons represents a molecular basis of the neural mechanism for CHH-induced attenuation of both sympathetic and respiratory activities (Fig. 7B), an issue to be addressed in future studies using subunit-deficient animals. Furthermore, it is possible that with a return to normoxia, GABA A receptor subunit will be downregulated and the hypoxic response will return to that found in naïve animals. In the proposed model, the de novo expression of GABA A receptor subunits diminishes activation of neurons within the raphé and A5 area. We view this as an extension of the GABA-gain hypothesis, wherein both the neurophysiological properties and the plasticity of respiratory bursts is attenuated by increases in GABA-induced activity (40). Finally, we speculate that in pathophysiological states, including heart failure and obstructive sleep apnea, the accompanying sympatho-respiratory dysfunction may be due in part to concomitant alterations in the expression of GABA A receptor subtypes within the pons.

Perspectives and Significance

Our studies provide new insights into the neural mechanisms mediating the plasticity of the sympatho-respiratory neural network. Using both molecular and physiological approaches, we demonstrate that CHH exposure selectively alters GABA A receptor subunit expression in the pons and attenuates sympatho-respiratory LTF. On the basis of these findings, we present a conceptual model that defines the role of GABA A receptor in the blunted LTF found in CHH-exposed animals. Future studies need to identify the relationship between LTF and the expression of specific GABA A receptor subunits in the raphé, pontomedullary regions known to evoke LTF. For example, does blocking expression of specific GABA A receptor subunits rescue LTF and reestablish sympatho-respiratory plasticity in CHH conditioned animals? Furthermore, in CHH-conditioned animals, does a return to normoxia restore LTF and plasticity in the sympatho-respiratory network? If so, is this due to the downregulation of GABA A receptor subunits? Answers to these questions will provide further information concerning the role of GABA A receptor plasticity in mediating adaptation of the sympatho-respiratory system to chronic hypoxia.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health HL25830, HL080318, and NS43417. We gratefully acknowledge the technical assistance of Ning Wang, Rishi Dingra, and Mallika Padival and critical review of the manuscript by Drs. Jeffery Tatro and Martin Snider.

REFERENCES


