Effect of chronic hypoxia on cholinergic chemotransmission in rat carotid body

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He, L., B. Dinger, and S. Fidone. Effect of chronic hypoxia on cholinergic chemotransmission in rat carotid body. J Appl Physiol 98: 614–619, 2005; doi:10.1152/japplphysiol.00714.2004.—Current views suggest that oxygen sensing in the carotid body occurs in chemosensory type I cells, which excite synaptically apposed chemoafferent nerve terminals in the carotid sinus nerve (CSN). Prolonged exposure in a low-oxygen environment [i.e., chronic hypoxia (CH)] elicits an elevated stimulus-evoked discharge in chemoreceptor CSN fibers (i.e., increased chemosensitivity). In the present study, we evaluated cholinergic chemotransmission in the rat carotid body in an effort to test the hypothesis that CH enhances ACh-mediated synaptic activity between type I cells and chemoafferent nerve terminals. Animals were exposed in a hypoxic chamber (barometric pressure = 380 Torr) for 9–22 days before evaluation of chemoreceptor activity using an in vitro carotid body/CSN preparation. Nerve activity evoked by ACh was significantly larger (P < 0.01) after CH, suggesting increased expression of cholinergic receptors. Approximately 80% of the CSN impulse activity elicited by ACh (100- or 1,000-μg bolus) in both normal and CH preparations was blocked by the specific nicotinic receptor antagonist mecamylamine. A muscarinic receptor antagonist, atropine (10 μM), and a specific nicotinic receptor α7 subunit antagonist, methyllycaconitine (50 μM), blocked ~50% of the hypoxia-evoked activity in normal preparations but were ineffective after CH. Prolonged exposure to hypoxia appears to dramatically alter chemotransmission in the carotid body, and may induce alternative neurotransmitter mechanisms and/or electrical coupling between type I cells and chemoafferent nerve terminals.

carotid sinus nerve; low-oxygen environment; acetylcholine; hypercapnia

CHRONIC EXPOSURE IN A LOW-OXYGEN (O2) environment [i.e., chronic hypoxia (CH)] initiates a multitude of physiological adjustments that tend to mitigate the adverse effects of hypoxia. Among the most important of these adaptive changes is increased ventilation, evident as elevated tidal volume and/or frequency of breathing. CH, lasting for hours or days, initiates a time-dependent increase in breathing known as ventilatory acclimatization to hypoxia (VAH). In rats, VAH progresses over a 4- to 5-day period, and enhanced breathing persists for at least 14 days (25). Attempts to identify the structures that mediate VAH have focused principally on the carotid chemoreflex pathway, which consists of multiple components, including 1) chemoreceptor type I (glomus) cells in the carotid body; 2) chemoafferent neurons of the petrosal ganglion (PG), which innervate the type I cells via the carotid sinus nerve (CSN); 3) a complex circuit of brain stem neurons in proximity to the caudal nucleus tractus solitarius; and 4) respiratory phrenic motoneurons.

Chemoreceptor type I cells have been identified as chemo-transductive elements in the carotid body. Pharmacological assessments and direct measurements of putative neurotransmitters suggest that, in response to appropriate natural stimuli (e.g., low O2), these cells release a variety of neuroactive agents, including dopamine, acetylcholine (ACh), ATP, and neuropeptides (for reviews, see Refs. 15, 16). In view of the fact that CSN discharge is increased on exposure to CH (2, 3, 31), numerous studies have focused on elucidating adaptive changes in type I cells. Indeed, these efforts demonstrated adjustments in the functional properties of type I cells, including altered membrane currents, as well as changes in the metabolism and actions of endogenous neurotransmitters and neuropeptides (19–21, 29).

In the central nervous system, other investigations have focused on the brain stem neurons, which receive the projections from CSN chemoaffersents. In a series of studies (reviewed in Ref. 28), Pequignot and colleagues have shown that CH increases turnover of norepinephrine and upregulates tyrosine hydroxylase, the rate-limiting enzyme for catecholamine production, in a select subgroup of neurons in the caudal nucleus tractus solitarius. These neurochemical adjustments did not occur after acute hypoxia, suggesting that they are involved in the increased ventilatory response. Consistent with central readjustments, Powell et al. (26a) showed that CH facilitates the translation of chemoafferent input into ventilatory efferent output. Moreover, these findings were correlated with a decrease in the expression of the D2 subtype of dopamine receptors in the nucleus tractus solitarius, which have been shown to stimulate respiration (6).

Although type I cells and central neurons have been identified as potential contributors to VAH, the phenomenon nonetheless remains only partially understood. In fact, an important element in the chemoreflex pathway, namely, the chemoafferent PG neurons, has been largely ignored as possible adaptive components. Studies in other pathways have shown that certain primary sensory neurons undergo significant physiological adjustments in response to chronic stimulation. For example, chronic pain involving inflammation induces substantial changes in neuropeptide synthesis in neuron cell bodies and increases neurotransmitter release at peripheral and central projections (8, 17). Moreover, peripheral nerve injury or chronic inflammation induces
altered expression of neurotransmitter receptors by dorsal root ganglion neurons (24, 30).

Recent immunocytochemical studies have demonstrated the expression of nicotinic cholinergic receptors in PG neurons and afferent fiber terminals in the carotid body (27). Furthermore, cocultures of type I cells and PG neurons display synaptic properties consistent with cholinergic chemotransmission (34, 35). The possibility that CH induces significant adaptive change in the expression of nicotinic cholinergic receptors on PG neurons innervating rat carotid body chemoreceptors has come from recent studies in our laboratory that showed increased expression of α3- and α7-nicotinic receptor subunits after 9–14 days of hypobaric (380 Torr) hypoxia (13). These findings were correlated with increased antidromic nerve impulse traffic elicited in the CSN by the application of ACh to the PG after CH. In the present study, we have examined the hypothesis that CH-induced change in the expression of cholinergic receptors is correlated with enhanced cholinergic chemotransmission in the carotid body. Surprisingly, our findings suggest that CH results in a diminished contribution of cholinergic transmission at the chemosensory synapse between O2-sensitive type I cells and CSN afferent terminals.

MATERIALS AND METHODS

Animals and exposure to hypobaric hypoxia. Animal protocols were approved by the University of Utah Institutional Animal Care and Use Committee. Twenty-three adult male albino Sprague-Dawley rats (180–200 g) were housed in standard rodent cages with 24-h access to pellet food and water. Cages containing two to four rats were placed in a hypobaric chamber; pressure was decrementally lowered from ambient (~640 Torr at Salt Lake City, 1,400 m) over a 24- to 36-h period to 380 Torr (equivalent to 5,500 m). Because previous studies in our laboratory demonstrated that increased CSN hypoxic chemosensitivity plateaus after 9 days (10), animals were maintained in the hypobaric environment for 9–22 days. The chamber was opened briefly at 2-day intervals to replenish food and water. Age-matched control male rats were similarly housed at ambient pressure.

Electrophysiological recording of CSN activity. Rats were anesthetized with ketamine (10 mg/100 g im) plus xylazine (0.9 mg/100 g im), and the carotid artery bifurcations containing the carotid bodies were located and removed. The excised tissue was placed in a Lucite chamber containing 100% O2-equilibrated modified Tyrode solution at 0–4°C (in mM: 112 NaCl, 4.7 KCl, 2.2 CaCl2, 1.1 MgCl2, 42 sodium glutamate, 5 HEPES buffer, 5.6 glucose, pH = 7.4). Each carotid body along with its attached nerve was carefully dissected from the artery, cleaned of surrounding connective tissue, and placed in a conventional flow chamber, where the carotid body was continuously superfused (up to 4 h) with modified Tyrode solution maintained at 37°C and equilibrated with a selected gas mixture. For studies involving hypercapnic stimulation, preparations were maintained in bicarbonate-buffered solution consisting of (in mM) 116 NaCl, 24 NaHCO3, 5 KCl, 2 CaCl2, 1.1 MgCl2, 5.6 glucose, and 10 HEPES buffer. This solution was continuously bubbled with 5% CO2-95% O2 (pH 7.4 at 37°C). For isoflurane hypercapnic stimulation, the NaHCO3 concentration was increased to 96 mM (reduction in NaCl to 44 mM), and the solution was bubbled with 20% CO2-80% O2 resulting in a pH of 7.4 at 37°C.

The CSN was drawn up into the tip (~100-μm inner diameter) of a glass suction electrode for monopolar recording of chemoreceptor activity. Sufficient suction was applied to seal the electrode tip against connective tissue encircling the junction of the carotid body and CSN. The bath was grounded with an Ag-AgCl2 wire, and neural activity was led to an alternating current-coupled preamplifier, filtered, and transferred to a window discriminator and a frequency-to-voltage converter. Signals were processed by an analog-to-digital converter for display of frequency histograms on a personal computer monitor. Data were expressed as impulses per second and analyzed using ANOVA with Bonferroni multiple comparison posttests or paired t-tests. Bolus injections of 100 or 1,000 μg of ACh contained in 100 μl were delivered immediately upstream of the carotid body. Bath PO2 was measured with a Diamond General model 760 needle electrode, connected to a Harvard model 102 O2 electrode amplifier.

RESULTS

CH-induced increased sensitivity to ACh. Figure 1 shows CSN activity elicited by ACh application to the carotid body in normoxic vs. CH preparations. Basal (resting) activity in these superfused (nonvascular) preparations was established in solutions equilibrated with 100% O2, resulting in a bath PO2 of ~450 Torr and a mean basal nerve discharge rate of 11 ± 1.3 impulses/s (mean ± SE). In Fig. 1A, the application of a 1,000-μg bolus elicited a sharp increase in nerve activity, which gradually returned to the basal discharge rate. After 10–21 days of CH (Fig. 1B), application of 100 g or 1,000 μg of ACh also elicited an immediate response; however, in these preparations, the peak and total evoked activity were substantially larger than normal. Summary data for the evoked activity in these experiments are shown in Fig. 1C, which demonstrates that exposure to a 100- and 1,000-μg bolus of ACh elicited significantly larger total CSN responses in preparations from rats that had been exposed to CH.
Effect of mecamylamine on ACh-evoked CSN activity. The role of nicotinic cholinergic receptors in these responses was further investigated in experiments utilizing the specific nicotinic receptor antagonist mecamylamine. In these experiments, the application of mecamylamine did not alter basal nerve activity in either normal or CH preparations. However, the antagonist severely attenuated CSN activity evoked by ACh. Figure 2, A and B, shows that 100 μM mecamylamine blocked ~80% of CSN activity evoked by a 100-μg bolus of ACh in normal and CH preparations, respectively, confirming the involvement of nicotinic cholinergic receptors.

Effect of mecamylamine on hypoxia-evoked chemoreceptor activity. The possibility that CH elicits enhanced cholinergic chemotransmission between type I cells and afferent fiber terminals was tested by examining the effects of mecamylamine on CSN activity evoked by acute hypoxia in vitro. Figure 3, top, shows CSN activity evoked by a standard hypoxic stimulus (150 s at a Po2 of 120 Torr) in the presence or absence of the nicotinic antagonist. The mean basal (resting) discharge rate established at a Po2 of ~450 Torr in normal preparations was 9.35 ± 0.73 impulses/s. Hypoxia applied to a normal preparation (Fig. 3A, top) evoked a rapid increase in nerve discharge, which returned to the basal level of activity within a few seconds of reintroduction of oxygenated media. In the presence of 100 μM mecamylamine (superimposed trace), the basal discharge rate was not significantly altered, but the effect of hypoxia was dramatically reduced, consistent with nicotinic cholinergic chemotransmission. After a 20-min washin of drug-free oxygenated media, the hypoxic response is fully recovered. Summary data (Fig. 3A, bottom) indicate that, on average, mecamylamine blocks ~80% of the hypoxia-evoked nerve discharge in normal preparations. Figure 3B and C, shows that, after 9–22 days of CH, mecamylamine applied at concentrations of 100 μM (Fig. 3B) and 500 μM (Fig. 3C) is completely ineffective against either basal or hypoxia-evoked CSN activity, resulting in indistinguishable superimposed physiological records. In these CH preparations, the basal and hypoxia-evoked activity was significantly larger than normal (note different scales for Fig. 3A vs. Fig. 3B and C), as has been documented in our laboratory’s previous studies (10).

Effect of other cholinergic receptor antagonists on hypoxia-evoked nerve activity. Similar experiments compared the effects of an alternate nicotinic receptor antagonist, methyllycaconitine (50 nM), which binds to the α7-subunit of the receptor (12), and a general muscarinic antagonist, atropine (10 μM). Summary data presented in Fig. 4 show that these agents inhibit ~50% of hypoxia-evoked activity in normal preparations, but, like mecamylamine, they are completely ineffective after CH (11–15 days at 380 Torr). As with mecamylamine, methyllycaconitine and atropine did not significantly affect the resting nerve activity in normal or CH preparations. It is noteworthy that, at the concentration used here, atropine may interact with both muscarinic and nicotinic receptors (26).

Effect of mecamylamine on chemoreceptor activity evoked by hypercapnia. Figure 5 shows the effect of 14 days of CH on CSN responses evoked by isohydric hypercapnia (20% CO2, pH 7.4). In four normal preparations, this strong hypercapnic stimulus elicited an increase in nerve activity that was larger than responses elicited by moderate hypoxia. The hypercapnia-evoked CSN activity was nearly completely blocked by 100 μM mecamylamine (see Fig. 5, superimposed traces in top; summary data, bottom). In contrast, after CH (n = 4), hypercapnia-evoked CSN activity was blocked by mecamylamine in normal (left) and CH (right) preparations. Significant difference compared with activity evoked by ACh alone: **P < 0.01 and ***P < 0.001.
capnia elicited a rapid and markedly larger increase in CSN activity that was completely resistant to mecamylamine, resulting in indistinguishable, superimposed records of integrated nerve activity in the presence vs. absence of the drug. Thus, after CH, responses elicited by either hypoxia or hypercapnia are resistant to the nicotinic receptor antagonist.

**DISCUSSION**

Our previous experiments demonstrated increased ACh-evoked antidromic activity in the CSN after CH, indicating that PG neuron cell bodies express higher levels of cell surface cholinergic receptors in response to prolonged hypoxic stress (13). The present study extends these findings by showing increased nicotinic receptor-mediated responses in afferent axon fiber terminals that innervate glomus (type I) cells in the carotid body. Thus ACh applied to the carotid body after CH elicits a significantly larger response in the CSN, ~80% of which is blocked by the nicotinic receptor antagonist mecamylamine.

Multiple studies document increased chemosensitivity to hypoxia after CH, apparent both as elevated activity in the CSN and an enhanced hypoxic ventilatory response (1, 2, 4, 31). The present findings indicate that PG neurons express higher levels of nicotinic receptors after CH, which is consistent with the hypothesis that nicotinic receptors contribute to enhanced chemosensitivity. However, the failure of nicotinic and muscarinic blockers to depress the CSN discharge evoked by acute hypoxia after prolonged exposure to hypobaric hypoxia indicates that cholinergic chemotransmission is not involved in this adaptive phenomenon. In fact, our data indicate...
that, despite a substantial augmentation of the nicotinic receptor population, cholinergic synaptic activity is severely depressed. The present findings do not reveal whether this dramatic change in cholinergic activity may involve the failure of ACh synthesis and/or release. It is noteworthy that numerous pharmacological and immunocytochemical studies have supported cholinergic chemotransmission at the chemoreceptor synapse (see Refs. 15, 16, 27, 34, 35). Moreover, a recent study in rabbit carotid body demonstrated that hypercapnia evokes the release of ACh and that muscarinic receptors modulate this response (23). However, other important data call into question the expression of classical cholinergic properties in rat type I cells (18).

It is well established that CH induces enlargement of the carotid body, such that organ size is increased approximately threefold after 14 days of hypoxia (10). In our in vitro superfused preparations, increased size could conceivably create more severe internal hypoxia, which may render mecamylamine ineffective during CH. However, multiple observations suggest that greater organ mass does not explain mecamylamine resistance. First, steeper O2 diffusion gradients should result in elevated mecamylamine-sensitive basal nerve activity. Although the basal nerve activity was increased after CH in accord with our previous observations (10), it was completely insensitive to cholinergic antagonists. Second, switching the superfusate does not instantaneously lower Po2 in the bath; rather, Po2 is incrementally lowered through intermediate levels of hypoxia (see Figs. 5 and 6 in Ref. 13). Yet the presence of mecamylamine did not alter the onset of CSN activity during these transitions, resulting in indistinguishable physiological records in the presence of antagonists (Fig. 3). Finally, after CH, mecamylamine was also ineffective against CSN activity elicited by hypercapnia, a stimulus that has been demonstrated to release ACh from rabbit type I cells (23). Collectively, these data strongly suggest that cholinergic activity is severely downregulated after CH.

The loss of cholinergic synaptic activity suggests that an alternative mechanism may mediate chemotransmission after CH. Recent studies by Nurse and colleagues have demonstrated coparticipation of cholinergic and purinergic mechanisms in synapses formed between co-cultured type I cells and PG neurons (7, 34, 35). These studies have shown that PG neurons and their afferent terminals express P2X2 purinergic receptors, as well as nicotinic cholinergic receptors, and that a cocktail of specific nicotinic/purinergic antagonists completely blocks hypoxic chemotransmission. The current data are consistent with an expanded role for purinergic and/or other transmitter mechanisms at the chemoreceptor synapse after CH. However, previous studies of serotonin, dopamine, and various neuropeptides have not yielded compelling evidence of an essential role for these agents in excitatory synaptic transmission and/or increased chemosensitivity (5, 32). Noteworthy are recent studies in our laboratory indicating that endothelin (ET), a vasoactive peptide contained in type I cells, is a critical player in the elevated chemoreceptor discharge associated with CH (10). However, expression of ET receptors by PG neurons and chemosensitive terminals was not evident, and the actions of ET in the CH carotid body appears to be mediated via an autocrine/paracrine (nonsynaptic) mechanism involving specific ET A receptors located on type I cells.

An additional putative synaptic mechanism yet to be fully explored is electrical coupling between type I cells and chemosensitive terminals. Although chemical mechanisms may dominate synaptic activity in the normal carotid body, a recent study by Jiang and Eyzaguirre (22) suggests that electrical junctions also exist between type I cells and nerve terminals. Thus it is possible that our recent demonstration of increased expression of connexin proteins in type I cells and PG neurons after CH (11) correlates with newly formed gap junctions that could mediate afferent terminal depolarization via intercellular ionic currents. Such enhanced electrical coupling could provide an adaptive advantage by reducing the need for energet-
ically expensive neurotransmitter synthesis and release during periods of prolonged hypoxic stress. Finally, our data are consistent with the possibility that CH induces hypoxic sensitivity in chemosensory nerve terminals. In this regard, Campanucci et al. (9) have recently reported the existence of a subpopulation of PG neurons in normal rats that express O₂-sensitive K⁺ channels.

**REFERENCES**

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