Effect of chronic hypoxia on purinergic synaptic transmission in rat carotid body

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He, L., J. Chen, B. Dinger, L. Stensaas, and S. Fidone. Effect of chronic hypoxia on purinergic synaptic transmission in rat carotid body. J Appl Physiol 100: 157–162, 2006; doi:10.1152/japplphysiol.00859.2005.—Recent studies indicate that chemoafferent nerve fiber excitation in the rat carotid body is mediated by acetylcholine and ATP, acting at nicotinic cholinergic receptors and P2X2 purinoceptors, respectively. We previously demonstrated that, after a 10- to 14-day exposure to chronic hypoxia (CH), the nicotinic cholinergic receptor blocker mecamylamine no longer inhibits rat carotid sinus nerve (CSN) activity evoked by an acute hypoxic challenge. The present experiments examined the effects of CH (9–16 days at 380 Torr) on the expression of P2X2 purinoceptors in carotid body and chemoafferent neurons, as well as the effectiveness of P2X2 receptor blocking drugs on CSN activity evoked by hypoxia. In the normal carotid body, immunocytochemical studies demonstrated a dense plexus of P2X2-positive nerve fibers penetrating lobules of type I cells. In addition, type I cells were lightly stained, indicating P2X2 receptor expression. After CH, the intensity of P2X2 receptor immunostaining was maintained in chemosensory type I cells and in the soma of chemoafferent neurons. P2 receptor expression on type I cells was confirmed by demonstrations of ATP-evoked increased intracellular Ca2+; this response was modulated by simultaneous exposure to hypoxia. In normal preparations, CSN activity evoked by hypoxia in vitro was 65% inhibited in the presence of specific P2X2 receptor antagonists. However, unlike the absence of mecamylamine action after CH, P2X2 antagonists remained effective against hypoxia-evoked activity after CH. Our findings indicate that ATP acting at P2X2 receptors contributes to adjusted chemoreceptor activity after CH, indicating a possible role for purinergic mechanisms in the adaptation of the carotid body in a chronic low-O2 environment.

CHEMOTRANSDUCTION IN THE CAROTID BODY occurs in specialized O2-sensitive type I cells where hypoxia evokes a cascade of events, including membrane depolarization, Ca2+ influx, and neurotransmitter release. These highly specialized cells synthesize and store multiple biogenic amine [dopamine, norepinephrine, acetycholine (ACh)] and peptide (substance P, enkephalins, endothelins, atrial natriuretic peptide) neurotransmitters. Although morphological and pharmacological data support the existence of a chemical synapse between type I cells and the terminal endings of afferent fibers, the complex neurochemical makeup of type I cells has led to conflicting views regarding the identity of the excitatory neurotransmitter(s) (10, 16). In a recent study using a unique preparation of cocultured rat type I cells and chemoafferent neurons from the petrosal ganglion (PG), Zhang et al. (22) reported that excitatory postsynaptic potentials and action potentials are blocked by a combination of cholinergic and purinergic antagonists (i.e., mecamylamine plus suramin). These findings suggest that chemotransmission is primarily mediated by ACh and ATP, acting as depolarizing cotransmitters via nicotinic and purinergic receptors, respectively. Moreover, nicotinic and purinergic receptor antagonists completely blocked the hypoxia-evoked neural discharge in the carotid sinus nerve (CSN) in intact carotid bodies harvested from rat pups. Purinergic P2-receptors are broadly divided into two subclasses containing P2X ligand-gated ion channels and P2Y G protein-coupled receptors (19). In the rat carotid body, the existence of purinergic receptors was confirmed in immunohistochemical studies that localized P2X2 receptor protein in PG neurons and chemoafferent terminals (22).

Earlier studies in animals exposed to chronic hypoxia (CH) have shown that the carotid body undergoes a number of remarkable morphological, neurochemical, and physiological adjustments. Among the most significant of these is an increase in chemosensitivity, which has been demonstrated as an elevated hypoxia-evoked CSN response (3, 4, 20). In a recent study (11), our laboratory confirmed and extended the work of Zhang et al. (22) by showing that the nicotinic cholinergic antagonist mecamylamine blocks ~80% of CSN activity evoked by acute hypoxia in normal adult rat carotid bodies superfused in vitro. However, after CH (i.e., 10–14 days at 380 Torr), even very high mecamylamine concentrations (e.g., 500 μM) are ineffective against chemoreceptor responses elicited by hypoxia or hypercapnia. These findings suggest that cholinergic synaptic mechanisms in the carotid body are exquisitely sensitive to environmental conditions and that CH minimizes the involvement of ACh. The present study tests the hypothesis that, in the absence of cholinergic activity, CH elicits an enhancement of purinergic chemotransmission between type I cells and CSN afferent nerve fibers. Our studies focus on the expression of P2X2 receptor protein in chemoafferent neurons and type I cells and purinergic transmission at the chemosensory synapse.

METHODS

Animals and exposure to chronic hypoxia. Animal protocols were approved by the University of Utah Institutional Animal Care and Use Committee. Twenty-three adult male albino (Sprague-Dawley derived) 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, and pressure was decrementally reduced from ambient (~640 Torr at Salt Lake City, 1,400 m) over 24–36 h and then maintained at 380 Torr, equivalent to 5,500 m, for up to 16 days. The chamber was opened briefly at 2-day intervals to replenish food and water. Age-matched control male rats were simi-
larly housed at ambient pressure. In some experiments, the CSN was unilaterally transected 48 h before exposure in the hypobaric chamber. For these studies, animals were anesthetized with ketamine (10 mg/100 g im) plus xylazine (0.9 mg/100 g im) and the CSN was exposed and cut via a small ventral midline incision using sterile technique.

**Immunocytochemistry.** Detailed immunocytochemical methods have been published (4). Tissues were fixed by intracardiac perfusion of deeply anesthetized adult rats [ketamine (10 mg/100 g im) plus xylazine (0.9 mg/100 g im)] with ice-cold phosphate-buffered 4% paraformaldehyde, postfixed for 2 h, rinsed in 15% sucrose-PBS (2 h), and immersed overnight in 30% sucrose-PBS. Frozen sections (4–8 um) were exposed to avidin-biotin preblocking reagents (Vector), incubated overnight in primary antisera (anti-P2X2 receptor, Alomone Labs, Jerusalem, Israel), and then rinsed in PBS. Sections were exposed to biotinylated secondary antibodies for 2–4 h, and visible reaction products were formed at room temperature in avidin-biotinylated horseradish peroxidase complex, reacted with diaminobenzidine and H2O2. Intracellular [Ca2+]j measurements. Detailed methods for measuring Ca2+ concentration ([Ca2+]j) in dissociated type 1 cells have been published previously (12). Briefly, freshly dissociated type 1 cells attached to coverslips were incubated in F12 medium containing 0.5 uM Fura-2 AM for 10–15 min in a CO2 incubator at 36.5°C. Coverslips were placed in a flow chamber where they were superfused at 0.75 to 1.0 ml/min with modified Tyrodes solution equilibrated with air. The temperature was maintained at 35–36.5°C. The chamber was mounted on the stage of a Zeiss inverted microscope incorporated into a Zeiss/Attofluor workstation equipped with an excitation wavelength selector (filter changer) and an intensified charge-coupled device camera system. Fura-2 fluorescent emission was measured at 520 nm in response to alternating excitation wavelengths of 334 and 380 nm. Data were collected and analyzed by use of Attofluor Ratiovision software (version 6.0).

Recording of CSN activity in vitro. 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 Tyrodes solution at 0–4°C (in mM: 112 NaCl, 4.7 KCl, 2.2 CaCl2, 1.1 MgCl2, 42 sodium glutamate, 5 HEPES buffer, and 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 Tyrodes solution equilibrated with a selected gas mixture. The CSN was drawn into the tip (~100 μm ID) 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 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. The window discriminator was adjusted to obtain near-zero output at room temperature; basal and stimulus-evoked nerve impulse activity was recorded in solutions maintained at 37°C. Signals were processed by an analog-digital converter for display of frequency histograms on a personal computer. Data were expressed as impulses per second and analyzed by ANOVA with Bonferroni multiple-comparison posttests or paired t-tests. Bath PO2 was measured with a Diamond General model 760 needle electrode, connected to a Harvard model 102 oxygen electrode amplifier.

**Drugs.** Purinergic receptors were activated by using the classical agonist ATP, which is known to act at P2X and P2Y receptor subtypes. Three structurally diverse P2-selective antagonists were employed, including suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), and its isomer, pyridoxalphosphate-6-azo-phenyl-2′,5′-disulfonic acid (iso-PPADS). Each of these agents blocks P2X and P2Y receptors, but they exhibit different actions at other neurotransmitter and growth factor receptor subtypes (19).

**RESULTS**

Expression of P2X2 receptor protein in normal and CH petrosal ganglion and carotid body. In accord with results reported by Zhang et al. (22), our immunocytochemical data demonstrate differing levels of expression of P2X2 protein in the soma of sensory neurons of the rat PG. Although neurons immunopositive for P2X2 protein were distributed throughout the PG, the most intense staining occurred in cells near the distal pole, in association with the glossopharyngeal (IXth) nerve (Fig. 1A). The immunoperoxidase technique also revealed numerous P2X2-positive nerve fibers within the PG (Fig. 1C). Exposure to CH did not alter the intensity of P2X2 immunostaining, and a similar concentration of immunopositive cells occurred at the distal pole in the PG (Fig. 1B). Likewise, P2X2-immunopositive nerve fibers were common among neurons within the ganglion after CH (Fig. 1D).

In the carotid body, P2X2 receptor immunoreactivity revealed a plexus of positive afferent nerve terminals that appear...
to ramify within the parenchymal cell lobules and to contact type I cells (Fig. 2A). In these sections the majority of large ovoid type I cells also appear to express low levels of immunoreactivity. The darkly stained intralobular fiber plexus remained intact after a 14-day exposure to CH (Fig. 2B). Type I cell expression of the receptor appeared unchanged or marginally elevated after CH. Similar levels of P2X2 immunostaining were observed in type I cells 9 days after resection of the CSN in both normoxic (Fig. 2C) and CH (Fig. 2D) preparations. Some stained elements in the immunomicrographs are consistent with receptor expression in type II cells; however, because of limitations of the single-label technique and the small size of specific immunopositive profiles, it is not possible to identify all stained cells. Sections incubated in the absence of primary antibody lacked immunoreactivity in type I cells, nerve terminals, and PG neurons (not shown).

Effect of P2 receptor antagonists on CSN activity. Figure 3, top, presents representative physiological records of superfused carotid body and CSN preparations from normal and CH animals. Superimposed records of nerve activity show that a receptor-saturating concentration of the P2 antagonist iso-PPADS blocked a large portion of the CSN activity evoked by rapidly lowering bath \( \text{PO}_2 \) from 450 to \(~120 \) Torr. In these experiments, drug concentrations were chosen on the basis of published inhibitor constant values. Receptor saturation was indicated in experiments that showed that 10-fold higher drug concentrations did not evoke further inhibition of nerve activity (data not shown). After 9–16 days of CH (barometric pressure \(~380 \) Torr), basal and hypoxia-evoked CSN activity was significantly elevated (note different scales for physiological records) in agreement with our previous observations (4), which showed that the increased in chemosensitivity was stable after day 9 of CH exposure. In normal preparations, the basal discharge rate was \( 20.1 \pm 4.5 \) impulses/s (mean \( \pm \) SE), whereas after CH this value was elevated to \( 109.8 \pm 8.0 \) impulses/s. The averaged CSN activity evoked by the standard acute hypoxic challenge (2.5 min; \( \text{PO}_2 = 120 \) Torr) was \( 194.3 \pm 20.0 \) impulses/s in normal vs. CH preparations. Values in parentheses equal number of preparations. *** \( P < 0.001 \) vs. normal.

Fig. 3. Effect of P2 receptor antagonists on carotid sinus nerve (CSN) activity evoked by acute hypoxia. Top: 3 superimposed traces show nerve activity in the absence (control, recovery) and presence of 10 \( \mu \)M pyridoxalphosphate-6-azophenyl-2′,5′-disulfonic acid (iso-PPADS) in normal and 7-day CH preparations. A separate record indicated bath \( \text{PO}_2 \). Note different scales for nerve activity in normal vs. CH preparation. Bottom: summary data for effects of P2 antagonists on hypoxia-evoked CSN activity in normal and CH preparations. Values in parentheses equal number of preparations. *** \( P < 0.001 \) vs. normal.

Fig. 2. Carotid bodies from normoxic (A, C) rats have marginally lower levels of P2X2 receptor immunoreactivity than animals exposed to chronic hypoxia (CH) for 14 days (B, D). In innervated carotid bodies (A, B), highly immunoreactive axons outline and ramify within individual lobules containing lightly staining type I cells. Note the darker staining of axons after exposure to CH (B). Individual type I cells with differing levels of punctuate immunoreactivity are apparent 9 days after resection of the sinus nerve and degeneration of sensory afferent axon terminals (C, D). Approximately equivalent levels of P2X2 receptor immunoreactivity occur in most type I cells with a few more darkly staining elements after CH (D). Scale bar = 20 \( \mu \)m.
control response (absence of antagonist). The data for the three molecularly distinct P2 receptor antagonists indicate that suramin (100 μM), PPADS (30 μM), and iso-PPADS (10 μM) each blocked ~65–75% of CSN activity evoked by hypoxia in normal preparations. Basal CSN activity established at 450 Torr was not significantly affected (P > 0.05) by application of the P2 antagonists.

After CH, the P2 receptor antagonists remained effective blockers of a major portion of hypoxia-evoked CSN activity. However, the summary data (Fig. 3, bottom) show that, compared with effects in normal preparations, each of the P2 antagonists was significantly less effective after CH, if data are expressed as a percentage of the nerve activity evoked by hypoxia alone. Because CH induces an enhanced response to the acute hypoxic challenge, the absolute magnitude of the discharge blocked by drugs after CH is actually greater than in normal preparations. Moreover, the absolute discharge rate in the presence of the antagonists in CH preparations was not statistically different from the discharge in normal preparations in the absence of drug. Thus blocking P2 receptors after CH appears to eliminate the enhanced hypoxia-evoked CSN discharge usually observed after CH. The traces of CSN activity in Fig. 3 also indicate that the P2 antagonists had no effect on the elevated basal discharge in CH preparations; however, these agents markedly dampened the afterdischarge, further suggesting the participation of purinergic mechanisms in physiological adjustments induced by CH exposure.

**Involvement of P2X2 receptors in regulation of intracellular Ca\(^{2+}\) in type I cells.** We examined the effects of ATP on intracellular ([Ca\(^{2+}\)]\(_i\)) in isolated type I cells to test the possibility that ATP released by hypoxia or other chemosensory stimuli may participate in a feedback mechanism that modulates cell activity. Cells were first identified in which hypoxia at least doubled the basal level of [Ca\(^{2+}\)]\(_i\). Figure 4, A and B, shows that in these O\(_2\)-sensitive cells, 100 μM ATP markedly elevated [Ca\(^{2+}\)]\(_i\), whereas P2-receptor antagonists suramin (10 μM) and PPADS (10 μM) significantly attenuated this response. Similar results occurred in O\(_2\)-sensitive carotid body cells harvested from rats after 10–14 days of CH. In normal and CH preparations, 55.9 and 66.2% of O\(_2\)-sensitive cells, respectively, exhibited responses to 100 μM ATP (Fig. 4C). In all cases, these responses were inhibited by 10 μM suramin and 10 μM PPADS.

Separate experiments evaluated the interaction of ATP with hypoxia on [Ca\(^{2+}\)]\(_i\), in normal type I cells. The data illustrated in Fig. 5 demonstrate that although ATP potentiated the hypoxia-evoked Ca\(^{2+}\) response, the effects were not additive. In fact, the large increase in [Ca\(^{2+}\)]\(_i\), evoked by 100 μM ATP was more than 50% attenuated when ATP was presented in hypoxic media (PO\(_2\) ~ 20–24 Torr; P < 0.001). Thus hypoxia modulated Ca\(^{2+}\) responses evoked by ATP.

**DISCUSSION**

The present study indicates that purinergic chemotransmission in the rat carotid body is significantly altered by exposure to CH. In general agreement with the studies of Zhang et al.
(22), we find that the majority of primary sensory neurons in the PG express moderate levels of P2X2 receptor protein and that darkly stained neurons are clustered near the distal pole of the ganglion in both normal and CH animals. An earlier study by Finley et al. (7) also demonstrated a population of carotid body sensory neurons near the distal pole of the PG that expressed tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis, and DOPA decarboxylase, the synthetic enzyme for dopamine. Their ultrastructural studies further showed that these neurons give rise to axons that terminate in apposition to the O2-sensitive type I cells of the carotid body. A 14-day exposure to hypoxia does not alter the P2X2 receptor staining pattern in the PG, and the staining intensity in chemoafferent fibers of the carotid body is maintained consistent with continued purinergic synaptic transmission.

Zhang et al. (22) studied carotid bodies harvested from rat pups (postnatal 7–14 days) and reported that type I cells do not express P2X2 receptors. In contrast, our data from adult animals indicate the presence of P2X2 receptor expression in chemosensory type I cells in normal and CH carotid body. Recent pharmacological and immunocytochemical studies indicate that P2Y1 receptors occur on type II cells but not on type I cells (21). It is important to note that the epitope for the P2X2 antibodies used in our studies has no homology with other members of the large family of purinergic receptors (Ophra Gohar, Alamone Labs, personal communication). Moreover, our immunocytochemical findings are supported by a preliminary RT-PCR assay that confirms expression of P2X2 transcript in the normal and CH carotid body. Collectively, the results suggest that P2 receptor expression is developmentally regulated in neonatal vs. adult animals. Previous studies have demonstrated that expression of other type I cell-surface receptors, including dopaminergic D2 and adenosine A2, is likewise altered postnatally (2, 9).

The expression of functional purinergic receptors on type I cells was confirmed by the observation that ATP evokes increased [Ca2+]i in isolated O2-sensitive cells. Moreover, this response was blocked by the P2 antagonists suramin and PPADS. Similar results have been reported by Mokashi et al. (17) in a study of rat carotid body cells shown to express tyrosine hydroxylase, a signature marker of O2-sensitive type I cells. However, Xu et al. (21) reported that ATP failed to elicit increased [Ca2+]i in carotid body cells that secreted catecholamine in response to 50 mM KCl. In our study, the large increases in [Ca2+]i evoked by ATP were dampened when ATP and hypoxia were applied simultaneously. These findings are consistent with the hypoxia-evoked release of multiple excitatory and inhibitory agents, and the expression of receptors (dopamine D2, atrial natriuretic peptide-A, adenosine A2) on type I cells capable of modulating cellular activity (see Refs. 1, 10, 13). Mokashi et al. demonstrated that hypoxia-evoked CSN activity was inhibited when carotid bodies were first exposed to 100 μM ATP. These authors also showed that ATP enhanced K+ currents and hyperpolarized type I cells, presumably via the effect of increased [Ca2+]i on Ca2+-sensitive K+ channels (17).

In normal and CH carotid bodies, the majority of CSN activity was blocked by P2 receptor antagonists consistent with a purinergic component in chemotransmission between type I cells and PG primary afferent neurons. However, high (receptor saturating) concentrations of these drugs did not completely block hypoxia-evoked CSN activity. This result is expected in normal preparations because numerous classical and recent studies have confirmed the involvement of ACh in chemotransmission (6, 8, 15). However, in CH preparations our laboratory recently demonstrated that nicotinic and muscarinic antagonists have no effect on hypoxia-evoked CSN activity, even though these agents remain effective against activity evoked by exogenous ACh (11). Thus the failure of receptor-saturating concentrations of three P2 antagonists to fully block hypoxia-evoked activity suggests that CH induces an alternative mechanism that is resistant to P2 antagonists. Such a mechanism might involve an alternative neurotransmitter (e.g., 5-HT) or electrical coupling between type I cells and the afferent nerve terminals (14). In this regard, we have recently shown that CH upregulates the gap junction-forming protein connexin43 in rat carotid body (5).

Unlike cholinergic mechanisms, the role of purinergic activity appears to remain important in the carotid body after exposure to CH. In our preparations, P2 antagonists reduced CSN activity evoked by acute hypoxia to levels that are comparable to normal, and these agents partially blocked a prominent afterdischarge in the CSN. The latter phenomenon suggests that CH may induce changes in the regulation of neurotransmitter release mechanisms that result in a prolonged secretion of ATP after reestablishment of normal tissue PO2 levels. Interestingly, Olson and Dempsey (18) observed in rats after CH (1–14 days at ~450 Torr) that ventilation on return to normoxia remained 35–70% above control values, consistent with persistent hyperactivity in the carotid body. Overall, our immunocytochemical and pharmacological data indicate that ATP acting at P2X2 receptors remains intact after CH and, furthermore, that it may contribute to increased chemoreceptor activity, indicating an important role for purinergic mechanisms in the adaptation of the carotid body in a chronic low-O2 environment.

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REFERENCES