Enhanced adenosine A2b receptor signaling facilitates stimulus-induced catecholamine secretion in chronically hypoxic carotid body type I cells

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Submitted 13 May 2013; accepted in final form 18 July 2013

Livermore S, Nurse CA. Enhanced adenosine A2b receptor signaling facilitates stimulus-induced catecholamine secretion in chronically hypoxic carotid body type I cells. Am J Physiol Cell Physiol 305: C739–C750, 2013.—Chronic hypoxia (CHox) augments chemoaffective activity in sensory fibers innervating carotid body (CB) chemoreceptor type I cells; however, the underlying mechanisms are poorly understood. We tested the hypothesis that enhanced paracrine signaling via adenosine (Ado) A2b receptors is involved. Dissociated rat CB cultures were exposed for 24 h to normoxia (Nox, 21% O2) or CHox (2% O2) or treated with the hypoxia mimetic deferoxamine mesylate (DFX), and catecholamine secretion from type I cells was monitored by amperometry. Catecholamine secretion was more robust in CHox and DFX type I cells than Nox controls after acute exposure to acid hypercapnia (10% CO2, pH 7.1) and high K+ (75 mM). Exogenous Ado increased catecholamine secretion in a dose-dependent manner, and the EC50 was shifted to the right from ~21 μM Ado in Nox cells to ~78 μM in CHox cells. Ado-evoked secretion in Nox and CHox cells was markedly inhibited by MRS-1754, an A3a, receptor blocker, but was unaffected by SCH-58261, an A2a receptor blocker. Similarly, MRS-1754, but not SCH-58261, partially inhibited high-K+–evoked catecholamine secretion, suggesting a contribution from paracrine activation of A2b receptors by endogenous Ado. CB chemostimuli, acid hypercapnia, and hypoxia elicited a MRS-1754-sensitive rise in intracellular Ca2+ that was more robust in CHox and DFX than Nox cells. Taken together, these data suggest that paracrine Ado A2b receptor signaling contributes to stimulus-evoked catecholamine secretion in CHox and CHox CB chemoreceptors; however, the effects of Ado are more robust after CHox.

adenosine; A2 receptors; acid hypercapnia; hypoxia; carotid body chemoreceptor

THE MAMMALIAN CAROTID BODIES (CBs) are the primary arterial chemoreceptors located at the bifurcation of the common carotid arteries. These organs contain chemosensitive type I (glomus) cells, which respond acutely to decreases in Po2 (hypoxia) and increases in PCO2/H+ (acid hypercapnia) via membrane depolarization and Ca2+/H+–dependent release of excitatory and inhibitory neurotransmitters onto apposed affrent nerve endings (19, 33, 41). Activation of this reflex pathway increases impulse activity in the carotid sinus nerve (CSN), ultimately leading to compensatory increases in ventilation, so as to maintain blood homeostasis. Thus exposure to acute hypoxia initiates the hypoxic ventilatory response (HVR), which restores blood Po2. Sustained periods of hypoxia, for instance during sojourns to high altitude or obstructive lung disease, result in an augmentation of the HVR in a process known as ventilatory acclimatization to hypoxia (VAH) (44, 46, 58). During VAH, the CB chemoreceptor type I cells undergo plastic changes that sensitize their responses to acute bouts of hypoxia (44, 45). Although the cellular and molecular mechanisms of VAH are not completely understood, there is evidence for the involvement of changes in ion channel density (22, 52) and neurotransmitter and/or neuromodulatory functions (44, 46, 58) in the dopaminergic type I cells.

There is growing evidence that modifications in adenosine (Ado) signaling are critical for physiological adaptations to chronic hypoxia (CHox) (8, 12, 35, 50). For instance, Ado is involved in several systemic effects of hypoxia, such as vaso-dilation, angiogenesis, and vascular smooth muscle growth. Moreover, the hypoxic regulation of several Ado signaling-related proteins (e.g., adenosine kinase and 5′-econucleotidase) alters Ado dynamics during hypoxia, leading to an increase in Ado accumulation (11, 27). It is also noteworthy that CHox upregulates 5′-nucleotidase and that the genes encoding Ado A2b and A2a receptors contain hypoxia response elements, which allow for transcriptional regulation by hypoxia-inducible factors (HIF-1α and HIF-2α) (3, 28, 30, 53). Indeed, the Ado A2a receptor is upregulated by CHox in two O2-sensitive chromaffin-derived cell lines (PC-12 and MAH cells) (3, 26). In MAH cells, this upregulation is mediated via HIF-2α, which binds to the promoter region of the A2a receptor gene (3).

Interestingly, a nonspecific Ado receptor antagonist [8-(p-sulfophenyl)theophylline hydrate] was shown to blunt the increase in respiratory frequency elicited by acute hypoxia in CHox rats (56). Walsh et al. (56) concluded that the increased HVR in CHox rats was due to activation of A2 receptors by endogenous Ado released in the CB. Moreover, Ado A2a and A2b receptors are expressed in CB type I cells (10, 17), and Ado signaling contributes to chemoexcitation via presynaptic and postsynaptic mechanisms involving A2 receptors (9). Also, acute hypoxia leads to Ado accumulation (7), and exogenous Ado increases cAMP content (41) and stimulates catecholamine secretion (6) from whole CBs. In studies on isolated CB type I cells, Ado was found to inhibit voltage-dependent K+ channels (55), as well as background K+ channels, which led to membrane depolarization and a rise in intracellular Ca2+ ([Ca2+]i) (59). However, whether these presynaptic actions of Ado occur via the low-affinity A2b receptors or the high-affinity A2a receptors remains controversial. Furthermore, little is known regarding the actions of Ado on type I cells following CHox exposure and the respective roles of A2a vs. A2b receptors.

In the present study, we used carbon fiber amperometry and ratiometric Ca2+ imaging to test the hypothesis that Ado signaling via A2b receptors contributes to the enhancement of stimulus-evoked type I cell chemosensory responses following exposure to CHox. To avoid confounding variables in the circulation, we exposed dissociated rat CB cultures to 24 h of

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normoxia (Nox, 21% O₂) and CHox (2% O₂) in vitro. Also we tested whether the effects of CHox could be attributed to HIF stabilization by treating Nox cultures with the iron chelator and hypoxia mimetic deferoxamine mesylate (DFX) (57), which is known to enhance the HVR in vivo (39). Interestingly, we found that Ado signaling via type I cells is indeed enhanced in CHox and DFX cultures, and activation of the Ado A₂b receptor plays a key role.

**MATERIALS AND METHODS**

**Animal preparation and cell culture.** Dissociated rat CB cultures were prepared and grown according to procedures previously described (51), with slight modifications (34). All animal experiments were approved by the Animal Research and Ethics Board at McMaster University in accordance with the guidelines of the Canadian Council for Animal Care. Juvenile (11- to 12-day-old) Wistar rats (Charles River, Saint-Constant, PQ, Canada) were maintained under controlled conditions.

**Fig. 1.** Effects of acid hypercapnia and high (75 mM) K⁺ in type I cells exposed to normoxia (Nox), chronic hypoxia (CHox), and deferoxamine mesylate (DFX). A–C: representative amperometric recordings of acid hypercapnia-evoked quantal catecholamine secretion in Nox, CHox, and DFX (top traces) and cumulative catecholamine secretion corresponding to each cell (bottom traces). D–F: exemplar traces of high-K⁺-evoked catecholamine secretion in the same cells used in A–C in Nox, CHox, and DFX.

**Table 1.** *Stimulus-evoked catecholamine secretion in Nox, DFX, and CHox type I cells*

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>n</th>
<th>Frequency, events/min</th>
<th>Q, IC</th>
<th>Q₁/₂, IC₁/₂</th>
<th>Secretion Rate, fC/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% CO₂/pH 7.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nox</td>
<td>7</td>
<td>6.1 ± 1.5</td>
<td>22.6 ± 3.1</td>
<td>2.8 ± 0.1</td>
<td>153.3 ± 20.2</td>
</tr>
<tr>
<td>DFX</td>
<td>6</td>
<td>20.9 ± 7.2*</td>
<td>44.9 ± 9.6*</td>
<td>3.5 ± 0.2*</td>
<td>612.1 ± 235.3*</td>
</tr>
<tr>
<td>CHox</td>
<td>6</td>
<td>21.0 ± 5.9*</td>
<td>44.0 ± 9.8*</td>
<td>3.5 ± 0.2*</td>
<td>701.5 ± 201.4*</td>
</tr>
<tr>
<td>High K⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nox</td>
<td>7</td>
<td>13.5 ± 1.7</td>
<td>24.8 ± 1.5</td>
<td>2.7 ± 0.1</td>
<td>387.2 ± 61.2</td>
</tr>
<tr>
<td>DFX</td>
<td>6</td>
<td>98.6 ± 38.2*</td>
<td>31.5 ± 2.5*</td>
<td>3.0 ± 0.1*</td>
<td>2,390 ± 808.3*</td>
</tr>
<tr>
<td>CHox</td>
<td>6</td>
<td>81.0 ± 34.5</td>
<td>35.8 ± 2.7*</td>
<td>2.8 ± 0.1*</td>
<td>2,099 ± 72.6†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of cells. Nox, normoxia; CHox, chronic hypoxia; DFX, desferoxamine; Q, quantal charge. *P < 0.05, †P < 0.01 (by Kruskal-Wallis test).
lighting (12:12-h light-dark cycle) and temperature (22°C) with ad libitum access to food and water. Rats were rendered unconscious by a blow to the back of the head and then killed by decapitation. The carotid bifurcations were removed bilaterally and placed in ice-cooled L-15 medium containing penicillin-streptomycin. Whole CBs were isolated from the intact carotid bifurcations before digestion of the tissue in an enzymatic solution for 1 h. The enzyme was then replaced with prewarmed (37°C) modified F-12 medium (GIBCO), and CBs were teased apart with forceps, triturated 150 times, and then plated on modified Matrigel-coated (Collaborative Research) dishes with a modified F-12K medium. CB cultures were maintained in a humidified incubator at 37°C in 5% CO2-95% air for up to 7 days. At 24 h before experiments, the medium was replaced with a modified F-12K medium, and the cells were placed in an incubator under Nox (21% O2), CHox (2% O2), or Nox + DFX (100 μM), in the presence of 7% CO2. We found that these conditions increased our ability to detect stimulus-evoked catecholamine secretory events from type I cell clusters using amperometry and were similar to those recently used to study catecholamine secretion from cultured adult rat CB type I cells (34).

Carbon fiber amperometry: Carbon fiber amperometry was performed as previously described (32). Cell cultures were perfused under gravity using HCO3-buffered solutions (BBS; see below). A carbon fiber electrode (Dagan) polarized to +800 mV was lowered onto the surface of a type I cell cluster and then retracted slightly. The electrode was connected to a headstage (model CV203BU) and an amplifier (Axopatch 200B). The analog signal was acquired using a Digidata 1322A, sampled at 10 kHz, and analyzed using Clampex 9.2. Individual secretory events were rejected if they were <2.58 SDs (99% confidence interval) of the baseline noise (typically ~2 pA). The integrated area under individual quantal spikes is the quantal charge (Q), which is related to the number of oxidizable molecules by Faraday’s law. Since charge is the product of current and time, we measured peak current (Ipeak, the maximum current of each spike) and half-width (the duration of each spike at half of Ipeak) to quantify the exocytotic process. We also calculated the cube root of Q (Q1/3), which is proportional to vesicular radius, with the assumption that the vesicles are spherical and catecholamine concentration is constant (14). Mean Q and Q1/3 were calculated for each cell during stimulus application, as described previously (5); however, given the small number of individual amperometric spikes, data for Qpeak and half-width were pooled. Frequency was determined by calculation of the number of single-spike events per minute, and secretion rate was determined by summation of the charge obtained from successive individual quantal events over a given time period. Only cells with robust high-K+-evoked responses were considered healthy and were analyzed.

\[ \text{[Ca}^{2+}\text{]i}, \text{measurements} \] were measured as described in previous studies from this laboratory (43). Briefly, cultured cells were loaded with the fluorescent Ca2+ indicator fura 2-AM (2.5 μM) in BBS for 30 min at 37°C in an incubator with an atmosphere of 95% air-5% CO2. Cultures were placed on the stage of a Nikon Eclipse microscope (model TE2000-U) equipped with a digital charge-coupled device camera (ORCA-ET, Hamamatsu). Fluorescence was measured with the aid of an ultra-high-speed wavelength changer (Lambda DG-4, Sutter Instruments, Novato, CA) and a ×40 oil immersion objective lens (S-Fluor, Nikon). Data were recorded using Simple PCI software (version 5.3, Comipix), and absolute [Ca2+], was estimated using the equation of Grynkiewicz et al. (20a). The mean change in [Ca2+], \[ \Delta \text{[Ca}^{2+}\text{]} \], was calculated by measurement of the mean [Ca2+] during the stimulus and subtraction of the mean [Ca2+] during a 1-min period immediately preceding the stimulus. Cells were considered to be responsive if \[ \Delta \text{[Ca}^{2+}\text{]} \], was >20 nM and if they had a robust high K+ response.

Solutions: The standard BBS contained (in mM) 24 NaHCO3, 115 NaCl, 5 glucose, 17 sucrose, 5 KCl, 2 CaCl2, and 1 MgCl2 and was bubbled with 5% CO2-95% air to maintain the pH at 7.4. Hypercapnic solutions were obtained by bubbling BBS with 10% CO2-90% air; hypoxic solutions (~30 mmHg) were obtained by bubbling BBS with 95% N2-5% CO2. High-K+ (75 or 30 mM) solutions were kept at constant osmolality by equimolar substitution with NaCl. Ado, SCH-58261, MRS-1754, and DFX were purchased from Sigma-Aldrich.

Statistical analysis: Values are means ± SE. Data were analyzed using GraphPad Prism 5 and/or pClamp 9.2. Parametric data were compared using Student’s t-test or ANOVA; nonparametric data were analyzed using a Mann-Whitney U-test or Kruskal-Wallis test where indicated.

RESULTS

Augmented stimulus-evoked catecholamine secretion in CB type I cells exposed to 24 h of hypoxia or treated with DFX. Sister cultures containing CB type I cell clusters were exposed for 24 h to Nox (21% O2) or CHox (2% O2) or treated with an iron chelator (DFX, 100 μM, 21% O2), and catecholamine secretion was monitored using amperometry. To assess type I cell responses to CB chemostimuli, acid hypercapnia (10% CO2, pH 7.1) was used, because it is a common physiological stimulus and it evoked a more robust and consistent secretory response from single type I cells than hypoxia under the
different growth conditions used in the present study. As exemplified in Fig. 1A, switching solutions from normocapnia (5% CO₂, pH 7.4) to acid hypercapnia increased catecholamine secretion in type I cells grown under Nox. This increased secretion was reflected by an increase in frequency of quantal events (Fig. 1A, top trace) and total cumulative charge (Fig. 1A, bottom trace). After 24 h of CHox, type I cells exhibited a more robust secretory response to acid hypercapnia [Fig. 1B, top and bottom traces; note scale difference in cumulative charge (bottom traces) in Fig. 1, A and B]. Consistent with the involvement of the HIF pathway, treatment of Nox cultures with DFX (24 h), a hypoxia mimetic and HIF stabilizer (57), also resulted in a more robust secretory response to acid hypercapnia in type I cells than in control cultures without DFX (Fig. 1C). The increase in stimulus-evoked secretion in CHox and DFX type I cells was due to an increase in frequency of quantal events and mean \( Q \). As summarized in Table 1, acid hypercapnia produced an approximately three- to fourfold increase in quantal frequency in CHox (\( n = 6 \)) and DFX (\( n = 6 \)) cells compared with Nox (\( n = 7 \)) cells (\( P < 0.05 \) by Kruskal-Wallis test); the mean \( Q \) was nearly twofold greater in CHox and DFX cells than in Nox controls (\( P < 0.05 \) by Kruskal-Wallis test). Since secretion rate is the product of the mean \( Q \) and quantal frequency, the acid hypercapnia-evoked secretion rate was nearly fourfold larger in CHox and DFX cells than in Nox controls (\( P < 0.05 \); Table 1). We also compared the effects of the depolarizing stimulus high K⁺ on secretion in control Nox cells vs. CHox and DFX cells. Indeed, the secretory responses to high K⁺ were more robust in CHox (Fig. 1E) and DFX (Fig. 1F) than in Nox (Fig. 1D) cells. However, although the high-K⁺-evoked secretion rate was almost fivefold higher in CHox (\( n = 6 \)) and DFX (\( n = 6 \)) than Nox (\( n = 7 \)) cells (\( P < 0.05 \) by Kruskal-Wallis test), the effect on frequency was significantly greater (~6.5-fold increase, \( P < 0.05 \) by Kruskal-Wallis test), with a relatively minor effect (~1.3-fold increase) on mean \( Q \) (Table 1).
Analysis of amperometric spike parameters in Nox vs. CHox. Because CHox augmented stimulus-evoked mean Q in type I cells, we analyzed individual amperometric spikes to understand changes in the parameters that regulate exocytosis. Exemplar traces of amperometric spikes evoked by high K+ and acid hypercapnia in Nox vs. CHox cells are shown in Fig. 2, A and B, respectively. In general, peak oxidation currents (I_{peak}) were larger in spikes evoked in CHox cells by high K+ and acid hypercapnia than in spikes evoked in Nox cells. A cumulative frequency distribution of I_{peak} shows that acid hypercapnia-evoked (Fig. 2C) and high-K+-evoked (Fig. 2D) quantal spikes from CHox cells were rightward shifted (dotted curves) compared with those from Nox cells (solid curves), indicating a larger proportion of amperometric events with a larger oxidation current. In fact, the acid hypercapnia-evoked mean I_{peak} shifted from 19.6 ± 3.9 pA (n = 45 spikes) in Nox cells to 22.9 ± 1.3 pA (n = 196 spikes) in CHox cells (P < 0.001 by Mann-Whitney U-test). Similarly, the mean high-K+-evoked I_{peak} shifted from 16.2 ± 0.9 pA (n = 125 spikes) in Nox cells to 21.6 ± 0.9 pA (n = 429 spikes) in CHox cells (P < 0.001 by Mann-Whitney U-test). In contrast, the cumulative frequency distribution of spike half-widths overlaps significantly for acid hypercapnia-evoked (Fig. 2E) and high-K+-evoked (Fig. 2F) quantal events. During acid hypercapnia, the mean half-width was 1.5 ± 0.1 ms (n = 45 spikes) in Nox cells and 1.5 ± 0.1 ms (n = 196 spikes) in CHox cells, respectively (P = 0.9 by t-test). Similarly, high-K+-evoked quantal spikes showed no statistically significant difference between half-width in Nox (n = 125 spikes) and CHox (n = 429 spikes) cells (1.9 ± 0.1 and 1.8 ± 0.1 ms, respectively, P = 0.24 by t-test). Therefore, it appears that the initial efflux of oxidizable molecules from the fusion pore increases after CHox. However, the duration of vesicle docking appears unchanged, suggesting no differences in fusion pore open time in CHox vs. Nox type I cells.

Relative effects of exogenous Ado in augmenting catecholamine secretion from type I cells exposed to Nox vs. CHox. Ado A_{2a} and A_{2b} receptors are expressed in rat type I cells, and exogenous Ado is known to stimulate catecholamine secretion from the intact rat CB (6, 10, 17). Given that stimulus-evoked catecholamine secretion was augmented in type I cells exposed to 24 h of CHox (Fig. 1), we wondered whether this was accompanied by an enhancement in Ado signaling. To address this, we first compared the effects of exogenous Ado on catecholamine secretion from type I cells grown in sister cultures exposed to 24 h of Nox and CHox. As illustrated in Fig. 3, A and B, exogenous Ado caused a dose-dependent stimulation of catecholamine secretion in Nox and CHox type I cells, with a threshold of ~1 μM and a maximal response at 100–200 μM. The dose-response relationship was fitted with a sigmoidal curve for Nox cells (n = 9) in Fig. 3C (r^2 = 0.94) and for CHox cells (n = 13) in Fig. 3D (r^2 = 0.98). Significant differences were observed between the two curves. For example, at each Ado concentration, catecholamine secretion was much higher for CHox than Nox cells; the maximal secretion rate increased ~6.7-fold from 142.3 ± 29.1 fC/min for Nox cells to 964.6 ± 162.4 fC/min for CHox cells (P < 0.001 by 1-tailed t-test). Also, the EC_{50} value for Ado shifted from 20.9 ± 1.8 μM in Nox cells to 77.6 ± 1.2 μM in CHox cells (P < 0.05).

Ado A_{2b} receptors mediate stimulatory effects of Ado on catecholamine secretion in Nox and CHox type I cells. The EC_{50} values for Ado-induced catecholamine secretion in Nox and CHox cells (Fig. 3, C and D) suggest a prominent role for the low-affinity A_{2b} receptor. This is consistent with previous studies on the intact rat CB, demonstrating that exogenous Ado stimulated catecholamine secretion in Nox via presynaptic A_{2b} receptors (10). However, given that the high-affinity A_{2a} receptors are known to be upregulated by CHox in O2-sensitive PC-12 cells (26, 27) and immortalized adrenomedullary chro-
maffin cells (3), it was of interest to investigate the contribution of each receptor in Nox and CHox type I cells. First, we treated Nox (Fig. 4A) and CHox (Fig. 4C) cells to saturating concentrations of Ado (100 µM) and low concentrations of the selective A2b receptor blocker MRS-1754 (100 nM), which is ~250 times more potent at A2b than at A2a receptors (16). Blockade of A2b receptors with MRS-1754 almost completely abolished Ado-evoked catecholamine secretion in Nox and CHox type I cells (Fig. 4; A and C); secretion was reduced by ~92% in Nox cells (Fig. 4B; P < 0.01 by 2-tailed t-test, n = 6) and by ~97% in CHox cells (Fig. 4D; P < 0.01 by 2-tailed t-test, n = 6). By contrast, MRS-1754 had no significant effect on basal secretion in Nox cells (0 ± 0 fC/min in control vs. 8.3 ± 4.4 fC/min during MRS-1754, P = 0.1 by 2-tailed t-test) and CHox cells (0 ± 0 fC/min in control vs. 37.6 ± 17.2 fC/min during MRS-1754, P = 0.1 by 2-tailed t-test).

To test whether there was a significant contribution by A2a receptors to Ado-evoked catecholamine secretion, we used the specific A2a receptor blocker SCH-58261, which shows ~50 times greater selectivity for A2a than A2b receptors (16). Even high concentrations of SCH-58261 (5 µM), >2,000 times the K_i value (~2 nM) for A2a receptors (16), failed to inhibit Ado-evoked catecholamine secretion in Nox and CHox type I cells (Fig. 5, A and C). Thus there was no significant difference between Ado-evoked secretion rate in the presence and absence of SCH-58261 in Nox cells (n = 5) cells (Fig. 5B; P = 0.81 by 2-tailed t-test) and CHox (n = 6) cells (Fig. 5D; P = 0.51 by 2-tailed t-test). Similarly, SCH-58261 had no significant effect on basal secretion in Nox cells (27.2 ± 27.2 fC/min in control vs. 14.0 ± 14.0 fC/min during SCH-58261, P = 0.71 by 2-tailed t-test) and CHox cells (27.5 ± 21.9 fC/min in control vs. 51.7 ± 41.6 fC/min during SCH-58261, P = 0.61 by 2-tailed t-test). Taken together, these data strongly suggest that Ado-evoked catecholamine secretion from type I cells depends almost entirely on Ado A2b receptor activation in Nox and after CHox.

Paracrine actions of endogenous Ado contribute to catecholamine secretion from type I cells via A2b receptors. We next investigated whether endogenous Ado arising within CB type I cell clusters regulates catecholamine secretion via autocrine-paracrine signaling. Given that only subpopulations of type I cells typically respond directly to chemosensory stimuli such as acid hypercapnia, we used the depolarizing stimulus high K+ to maximize extracellular Ado effects near the recorded cell. When Nox and CHox cells were stimulated with 75 mM K+ during two successive applications spaced >5 min apart, there was no significant difference in stimulus-evoked catecholamine secretion (Table 2; Control). In a separate series of experiments, we exposed Nox and CHox cells to the first high-K+ stimulus, perfused the cultures with 100 nM MRS-

### Table 2. Effects of endogenous adenosine on high-K⁺-evoked catecholamine secretion in Nox and CHox type I cells

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>n</th>
<th>High (75 mM) K⁺</th>
<th>High K⁺ + Antagonist</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>327 ± 66.9</td>
<td>332.9 ± 68.0</td>
<td>0.47</td>
</tr>
<tr>
<td>SCH-58261</td>
<td>10</td>
<td>357.9 ± 71.7</td>
<td>297.4 ± 60.0</td>
<td>0.36</td>
</tr>
<tr>
<td>MRS-1754</td>
<td>6</td>
<td>588.3 ± 54.8</td>
<td>450.3 ± 54.9</td>
<td>0.03*</td>
</tr>
<tr>
<td>CHox</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>1005 ± 170.1</td>
<td>848.7 ± 193</td>
<td>0.24</td>
</tr>
<tr>
<td>SCH-58261</td>
<td>10</td>
<td>1271 ± 204.3</td>
<td>1562 ± 691.8</td>
<td>0.68</td>
</tr>
<tr>
<td>MRS-1754</td>
<td>6</td>
<td>1341 ± 136.8</td>
<td>727.3 ± 120.8</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

Values are means ± SE in fC/min. *Statistically significant difference (by Kruskal-Wallis test).
1754 for several minutes, and then applied a second high-K⁺ stimulus in the presence of MRS-1754 (Figs. 6, A and C). Consistent with paracrine activation of A₂b receptors during high K⁺, evoked catecholamine secretion was significantly inhibited by ~23% in Nox cells and by ~45% in CHox cells in the presence of MRS-1754 (Table 2).

To test for possible paracrine activation of A₂a receptors by endogenous Ado during the high-K⁺ stimulus, we repeated the above-described experiments in the presence of the specific A₂a receptor antagonist SCH-58261 (5 μM). As illustrated in Fig. 6, B and D, and Table 2, SCH-58261 had no significant effect on 75 mM K⁺-evoked catecholamine secretion.

Exposure of type I cells to CHox and DFX augments chemostimuli-evoked intracellular Ca²⁺ transients. Catecholamine secretion from type I cells in response to chemostimuli is normally dependent on a rise in [Ca²⁺]ᵢ (Fig. 7A) (19, 33). To test the hypothesis that the augmented stimulus-evoked secretion in CHox and DFX type I cells (Fig. 1) is associated with an increase in Δ[Ca²⁺]ᵢ transients, we used ratiometric Ca²⁺ imaging. Nox type I cells responded to brief challenges of acid hypercapnia, hypoxia, and high K⁺ with a rise in [Ca²⁺]ᵢ (Fig. 7A). Interestingly, these stimuli evoked larger Δ[Ca²⁺]ᵢ transients in CHox and DFX than Nox type I cells (Fig. 7, B and C). Pooled data show a significant increase in stimulus-evoked Δ[Ca²⁺]ᵢ transients in CHox and DFX cells compared with Nox controls (Table 3). Thus, even though basal [Ca²⁺]ᵢ was similar in all conditions, the hypoxia- and acid hypercapnia-evoked Δ[Ca²⁺]ᵢ transients were ~1.5-fold larger in CHox and DFX type I cells than Nox controls (P < 0.05, n = 6 dishes per treatment). The mean basal [Ca²⁺]ᵢ in Nox, CHox, and DFX cells was ~87, ~94, and ~92 nM, respectively. Although high-K⁺-evoked Δ[Ca²⁺]ᵢ transients were almost twofold greater in CHox than Nox cells, these data did not reach statistical significance (P > 0.05, n = 6 dishes per treatment).

Fig. 6. Ado A₂b receptor antagonism blunts secretion evoked by high (75 mM) K⁺ in Nox and CHox type I cells. A: exemplar trace of high-K⁺-evoked secretion is significantly reduced by the presence of the A₂b receptor blocker MRS-1754 (100 nM) in a Nox cell. B: exemplar traces of high-K⁺-evoked secretion showing no effect of the A₂a receptor blocker SCH-58261 on secretion in a Nox cell. C: significant blunting of high-K⁺-evoked secretion by MRS-1754 in a CHox type I cell. D: no major effect of SCH-58261 on catecholamine secretion in a CHox cell.
The proportion of type I cells responding to chemosensory stimuli was also enhanced after CHox and DFX. For example, whereas ~31% of Nox cells responded to hypoxia with a detectable (>20 nM) rise in [Ca^{2+}], as many as ~87% and ~74% did so after exposure to CHox and treatment with DFX, respectively. Furthermore, whereas ~26% of Nox cells were sensitive to acid hypercapnia, ~69% of CHox cells and ~50% of DFX cells were sensitive to acid hypercapnia; however, these [Ca^{2+}] measurements are likely to be underestimated because of the pH sensitivity of the dye fura 2 (43). Thus exposure of type I cells to CHox or DFX resulted in an increase in [Ca^{2+}] transients induced by CB chemostimuli, as well as an increase in the proportion of responsive cells.

**Role of A_{2b} receptors in Ado-evoked intracellular Ca^{2+} signaling in type I cells.** As discussed above, exposure of type I cells to CHox led to an increase in stimulus-evoked [Ca^{2+}]_i transients and catecholamine secretion. Because paracrine activation of Ado A_{2b}, but not A_{2a}, receptors was the major contributor to the increased catecholamine secretion after CHox (Figs. 4–6), we next determined receptor contribution to [Ca^{2+}]_i transients mediated by exogenous Ado (100 μM). Control Nox type I cells responded to Ado with a rise in [Ca^{2+}]_i that could be reversibly blocked by the A_{2b} receptor blocker MRS-1754 (Fig. 8A); by contrast, perfusion with the A_{2b} blocker SCH-58261 was ineffective (Fig. 8B). After CHox, Ado-evoked [Ca^{2+}]_i transients in type I cells were more robust; however, the pharmacological profile did not differ from that of Nox cells (Fig. 8, C and D). Pooled data from this experimental series indicate that the mean Ado-evoked Δ[Ca^{2+}]_i, increased from 29.8 ± 1.0 nM in Nox type I cells (n = 12 dishes, 20–30 cells tested per dish) to 42.0 ± 2.6 nM in CHox type I cells (P < 0.01, n = 12 dishes, 20–30 cells tested per dish). Furthermore, the A_{2b} receptor blocker MRS-1754 (100 nM) reduced Ado-evoked Δ[Ca^{2+}]_i by ~92% in Nox cells and by ~87% in CHox cells (P < 0.001, n = 6 dishes). On the other hand, SCH-58261 (50 nM) had no significant effect on Ado-evoked Δ[Ca^{2+}]_i in Nox and CHox cells (P = 0.22 by 1-tailed t-test, n = 6 dishes). Thus, after CHox, the potentiation in Ado-evoked [Ca^{2+}]_i transients in type I cells occurs principally via A_{2b} receptor activation.

**DISCUSSION**

In this study we demonstrate that modifications of the Ado signaling pathway contribute to the augmentation of CB type I cell secretory activity after CHox. We obtained evidence that chemostimuli-, Ado-, and high-K^+-evoked increases in [Ca^{2+}]_i, and catecholamine secretion were greater in CHox than Nox type I cells. Interestingly, these effects of CHox could be mimicked by the iron chelator and HIF activator DFX, suggesting a role for the transcription factor HIF. Furthermore, we showed that the above-mentioned effects of Ado were mediated principally via the A_{2b} receptor, with negligible contributions from the A_{2a} receptor, which is also expressed in type I cells (10, 17). Lastly, we provided evidence that paracrine release of endogenous Ado augmented catecholamine secretion and that this effect was enhanced in CHox type I cells. Taken together, these data suggest that CHox augments type I cell stimulus-secretion coupling in part by the potentiation of paracrine signaling via Ado A_{2b} receptors. It is well known that chronic exposure of animals and humans to low P_{O_2}, for example, during ascents to high altitude, produces a time-dependent increase in ventilation, a phenomenon known as

**Table 3. Stimulus-evoked changes in intracellular Ca^{2+} concentration in Nox and CHox type I cells**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Nox</th>
<th>CHox</th>
<th>DFX</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% CO_2/pH 7.1</td>
<td>34.8 ± 2.3 (n = 6 dishes, 103 cells)</td>
<td>51.5 ± 6.1* (n = 6 dishes, 274 cells)</td>
<td>57.4 ± 6.8* (n = 6 dishes, n = 309 cells)</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>30.6 ± 1.0 (n = 6 dishes, n = 88)</td>
<td>46.3 ± 2.9* (n = 6 dishes, n = 218 cells)</td>
<td>57.5 ± 6.7* (n = 6 dishes, n = 210 cells)</td>
</tr>
<tr>
<td>30 mM K^+</td>
<td>102.1 ± 31.9 (n = 6 dishes, n = 329 cells)</td>
<td>198.7 ± 41.3* (n = 6 dishes, n = 316 cells)</td>
<td>206.7 ± 35.6* (n = 6 dishes, n = 416 cells)</td>
</tr>
</tbody>
</table>

Values are means ± SE in nM. *P < 0.05, †P < 0.01, ‡not significant (by 1-way ANOVA).
VAH. Although the CBs are known to play an essential role in the development of VAH, the underlying mechanisms are not completely understood (44–46). The results of the present study raise the possibility that presynaptic mechanisms involving enhanced paracrine Ado A2b receptor signaling may contribute to VAH. Moreover, our results demonstrate for the first time that the effects of CHox on Ado A2b receptor signaling in the CB are most likely attributable to the direct effects of hypoxia on type I cells, rather than secondary systemic changes in the circulation.

Quantal catecholamine secretion in type I cells after CHox. During random sampling of single type I cells within cell clusters, we found that CHox greatly augmented catecholamine secretion evoked by acid hypercapnia and the depolarizing stimulus high K+. This augmentation occurred via an increase in frequency and size of quantal events. In Nox cells, the mean Q was ~23 fC, a value similar to that previously reported for neonatal rat CB type I cells (4). However, in CHox cells, the mean Q increased to ~44 fC, representing a shift from ~70,528 molecules per vesicle in Nox cells to ~137,312 molecules per vesicle in CHox cells. This novel observation is consistent with an increase in vesicular volume or catecholamine concentration or both. Although it has been reported that in vivo exposure of rats to prolonged CHox can lead to a nearly threefold increase in dense-core vesicle volume in type I cells (29), it is unclear whether this would occur during the 24-h exposure employed in the present study. We also found a greater Ipeak in individual amperometric spikes in CHox than Nox cells, although the half-width was unchanged. If we assume that half-width is a measure of the fusion time of a vesicle with the plasma membrane and Ipeak is a measure of the amine concentration, these observations would suggest a greater catecholamine concentration per vesicle after CHox. Dopamine content and turnover rate in the CB are increased by CHox (21), attributable in part to increased tyrosine hydroxylase activity (20, 24), and this could contribute to an increased vesicular catecholamine concentration. The present data on primary CB chemoreceptor cells parallel those reported for two continuous O2-sensitive chromaffin cell lines, i.e., PC-12 (54) and MAH (3) cells, where hypoxia-evoked catecholamine secretion was greater following CHox as a result of an increase in size and frequency of quantal events.

Potential role of HIFs in enhanced Ado signaling in type I cells after CHox. HIFs are known to play a key role in mediating the effects of CHox via modification of gene expression (46, 57). The transcription factors HIF-1α and HIF-2α are constitutively synthesized during Nox and targeted for degradation via the ubiquitin pathway. However, during CHox, HIF-1α and HIF-2α are stabilized, and dimerization with the β-subunit leads to the regulation of gene expression. Recent studies demonstrate that HIF-1α plays a critical role in the development of VAH. For instance, HIF-1α and HIF-2α are expressed in CB type I cells and are upregulated by CHox (1, 47). Significantly, although wild-type (HIF-1α/β+) mice exhibit VAH during hypobaric hypoxia, the response is deficient in mice heterozygous for HIF-1α (HIF-1α+/−) (25). Conversely, in humans with Chuvash polycythemia and constitutively elevated HIF levels, VAH is potentiated (49). In the present study, exposure to the HIF activator DFX (in Nox) mimicked the effects of CHox in enhancing catecholamine secretion evoked by acid hypercapnia and high K+. Indeed, the frequency of quantal events and mean Q in DFX cells were nearly identical to those in CHox cells. Moreover, chemostimuli- and high-K+ evoked [Ca2+]i transients were similar in CHox and DFX cells. Thus our data support the hypothesis that HIF activation plays a key role in the alterations in stimulus-induced secretory functions of CHox type I cells.

The above-described considerations raise the possibility that the enhanced Ado signaling, which contributed to the augmentation of stimulus-induced catecholamine secretion in CHox type I cells, was HIF-dependent. Interestingly, the promoter regions of the genes encoding Ado A2a and A2b receptors have functional HIF binding sites, and both receptors are upregulated by CHox in other cell types (3, 28). In addition, the accumulation of extracellular Ado may increase during CHox via upregulation of 5’-nucleotidase (53) and changes in activity of adenosine kinase and 5’-nucleotidase (11, 27, 30). Thus it is plausible that activation of the HIF pathway during CHox could lead...
to increases in extracellular Ado and presynaptic Ado A2 receptors in the CB. Indeed, this mechanism could account for the observation that rats exposed to CHox in vivo experience an augmented HVR that is blocked by the general Ado receptor blocker 8-[(p-sulfophenyl)theophylline hydrate (56). While Ado receptors on afferent nerve terminals may also contribute (see below), the present study suggests that any modulatory role of catecholamine in this phenomenon should take into account the enhanced autocrine Ado A2b receptor signaling in CHox type I cells.

Controversy over roles of presynaptic Ado A2a and A2b receptors. Although Ado is generally regarded as excitatory in the CB, the receptor subtypes involved remain controversial. Ado A2a and A2b receptor mRNA expression has been found in the CB, and immunocytochemical and in situ hybridization studies have revealed that type I cells express high-affinity A2a receptors and/or low-affinity A2b receptors, while postsynaptic petrosal afferent terminals express A2a receptors (10, 18, 26). Distinguishing the presynaptic and postsynaptic roles of Ado with use of in vivo or in vitro preparations has proved challenging. For instance, exogenous Ado increases cat CB sinus nerve discharge in vivo (36, 37) and in vitro (48). Moreover, Ado increases ventilation rate in rats, and this is abolished after CSN section (38). Although these effects have typically been attributed to postsynaptic A2a receptor activation, data interpretation has been confounded by the presence of A2a and A2b receptors in the CB. More recently, Conde and co-workers (10) demonstrated that whereas A2a receptor antagonists, including SCH-58621, had no effect on hypoxia-evoked catecholamine secretion from whole rat CB, they partially inhibited hypoxia-evoked CSN discharge, suggesting the presence of postsynaptic A2a receptors. On the other hand, A2b receptor antagonists significantly reduced hypoxia-evoked catecholamine secretion and inhibited up to 25% of hypoxia-evoked CSN discharge in the same study (10). These data suggest that autocrine-paracrine activation of Ado A2b receptors on type I cells during chemotransduction contributes to CB chemoreceptor output. Taken together, these observations are consistent with our present results on dissociated CB cultures demonstrating that Ado-evoked catecholamine secretion from type I cells occurs predominantly via A2b receptors and that paracrine A2b receptor signaling contributes to stimulus-secretion coupling. However, we are unaware of any studies dealing with the functional significance of Ado signaling in the CB after CHox. We show for the first time that enhanced Ado signaling via A2b receptors is a major contributor to the augmentation of stimulus-evoked catecholamine secretion from type I cells following CHox. This likely involves a HIF-dependent upregulation of A2b receptors in type I cells; however, this possibility remains to be tested.

It should be noted, however, that other authors have reported A2a receptor-dependent effects of exogenous Ado on isolated type I cells. For instance, Ado was shown to inhibit TASK-1 K+ channels, leading to membrane depolarization, and voltage-gated Ca2+ entry in rat type I cells (59). On the other hand, Ado has also been shown to inhibit voltage-dependent Ca2+ currents and partially inhibit the hypoxia-evoked increase in [Ca2+]i (26). In another study on isolated cat CB type I cells, Ado was shown to increase [Ca2+]i via A2a receptors and also to prevent the desensitization of nicotinic ACh receptors (15). In these studies, the effects of Ado were attributed to the A2a receptor, because they could be blocked with the mixed A2a/A2b receptor agonist ZM-241385, or the effects could be mimicked by the A2a receptor agonist CGS-21680. However, in the former study, Ado-evoked responses were blocked with ZM-241385 at 10 μM, a concentration that appears to block all Ado receptor subtypes (2, 23, 31, 42). Thus a paracrine role for presynaptic A2a receptors, perhaps linked to the release of other CB transmitters, cannot be completely ruled out. However, in Nox and CHox CBs, presynaptic A2b receptors appear to be the receptors more effectively coupled to stimulus-evoked release of catecholamines, whose function in the CB is inhibitory in most species.

In conclusion, this work demonstrates that CHox in vitro augments stimulus-evoked catecholamine secretion from CB type I cells, in part via enhanced paracrine Ado A2b receptor signaling. It is plausible that this pathway represents one of several that contribute to CB sensitization during VAH in animals and humans exposed to CHox in vivo. The potential involvement of HIF-1α in the transcriptional upregulation of A2b receptors during CHox is discussed as a potential mechanism. Future studies are required to validate this hypothesis and to elucidate which of the several intracellular pathways associated with A2b receptor signaling (13) facilitates catecholamine secretion in type I cells.

ACKNOWLEDGMENTS

We thank Cathy Vollmer for technical assistance.

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GRANTS

This work was supported by Canadian Institutes of Health Research Operating Grant MOP 12037 to C. A. Nurse.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.L. and C.A.N. are responsible for conception and design of the research; S.L. performed the experiments; S.L. analyzed the data; S.L. and C.A.N. drafted the manuscript; S.L. and C.A.N. edited and revised the manuscript; S.L. and C.A.N. approved the final version of the manuscript.

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A2B RECEPTOR ACTIVATION AUGMENTS Ca²⁺-DEPENDENT SECRETION C749


