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Detection of hypoxia-evoked ATP release from chemoreceptor cells of the rat carotid body

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Abstract

The carotid body (CB) is a chemosensory organ that detects changes in chemical composition of arterial blood and maintains homeostasis via reflex control of ventilation. Thus, in response to a fall in arterial PO_2 (hypoxia), CB chemoreceptors (type I cells) depolarize, and release neurotransmitters onto afferent sensory nerve endings. Recent studies implicate ATP as a key excitatory neurotransmitter released during CB chemoexcitation, but direct evidence is lacking. Here we use the luciferin–luciferase bioluminescence assay to detect ATP, released from rat chemoreceptors in CB cultures, fresh tissue slices, and whole CB. Hypoxia evoked an increase in extracellular ATP, that was inhibited by L-type Ca^{2+}channel blockers and reduced by the nucleoside hydrolase, apyrase. Additionally, iberiotoxin (IbTX; 100 nM), a blocker of O_2-sensitive Ca^{2+}-dependent K^+ (BK) channels, stimulated ATP release and largely occluded the effect of hypoxia. These data strongly support a neurotransmitter role for ATP in carotid body function.

Keywords: Carotid body slice; Hypoxia; ATP release; Type I cells; Bioluminescence; Iberiotoxin; Nifedipine; Neurotransmitter

In mammals exposure to acute hypoxia leads to rapid respiratory and cardiovascular adjustments that ensure sufficient oxygenated blood reaches the tissues. The main chemoreceptor organ that initiates acute respiratory reflex responses to low blood oxygen is the carotid body (CB), which receives afferent innervation via the carotid sinus nerve or CSN [1]. Although the sensory function of the CB has been known for some time, it is only recently that the cellular and molecular mechanisms underlying chemotransduction and sensory processing have begun to be elucidated [2]. The CB is composed of highly vascularized clusters of glomus or type I chemoreceptor cells that synapse with terminals of CSN fibers. In several species, glomus cells respond to hypoxia via inhibition of various K^+ conductances, which leads to or facilitates membrane depolarization, voltage-gated Ca^{2+} entry, and secretion of neurotransmitters [1–4].

A controversial issue is the identity of the excitatory neurotransmitter(s) that initiates the afferent chemosensory response and the once popular candidate, dopamine [1], has since fallen into disfavor at least in the rat CB [5,6]. Recent studies in this laboratory, based on a co-culture model consisting of chemoreceptor cell clusters and 'juxtaposed' afferent (petrosal) sensory neurons, have provided strong evidence for ATP, acting via postsynaptic P2X2–P2X3 purinoceptors, as an excitatory co-transmitter in the rat CB [7–9]. This idea was recently supported by the demonstration that CB-mediated, ventilatory responses to hypoxia were markedly impaired in knockout mice deficient in the P2X2 purinoceptor subunit [10]. According to this model, the ATP required for synaptic transmission was presumed to be co-stored with other classical transmitters (e.g., dopamine, ACh, 5-HT, and GABA) in the synaptic vesicles

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of chemoreceptor cells [1,7,11–14]. However, a direct demonstration of stimulus-evoked ATP release from these chemoreceptors, that is dependent on extracellular Ca^{2+}, is lacking. In the present study, we address this issue using rat CB cultures, tissue slices, and whole CB, together with the highly sensitive luciferin–luciferase bioluminescence assay for detection of extracellular ATP [15]. We show directly that hypoxia stimulates Ca^{2+}-dependent ATP release from rat CB chemoreceptor cells.

Materials and methods

Carotid body cell cultures. Primary cultures of rat chemoreceptor cells were obtained by combined enzymatic and mechanical dissociation of rat carotid bodies, dissected from 9- to 14-day-old rat pups (Wistar; Harlan, Madison, WI). The procedures were similar to those described previously [16]. Following inactivation of the enzyme with serum-containing growth medium (see below), a few drops of the dissociated cell suspension were applied to the center of modified 24-well dishes. In this modification, glass rings (1.8 cm diameter × 1.2 cm deep) were painted white on the exterior and glued with sylgard (Silgard; Paisley Products, Toronto) onto the inner surface of a 24-well culture dish lid so as to form a series of cylindrical wells. These modified dishes were designed: (i) to permit CB cell attachment and growth in the well whose bottom was pre-coated with a thin layer of matrigel; (ii) to allow easy access to the luminometer port; and (iii) to minimize light interference between neighboring wells. After an overnight incubation in a humidified atmosphere of 95% air:5% CO_2 at 37 °C to allow cell attachment, the cultures were flooded with 1.5–2 ml of growth medium. A litter of 12 pups provided dissociated carotid body cells for 3 wells. The normal growth medium consisted of F-12 nutrient medium (Gibco, Grand Island, NY, USA), supplemented with the following additives: 10% fetal bovine serum, 0.6% glucose, 80 $U l^{-1}$ insulin (Sigma), 2 mM L-glutamine, and 1% penicillin–streptomycin (Gibco).

Carotid body slices. Carotid bodies (CB) were dissected and placed in ice-cold F-12 medium that was equilibrated with a 95% O_2:5% CO_2 gas mixture at pH 7.4. After removal of excess tissue the CB was briefly stained with neutral red (20 μg/ml) for 10 min to aid viewing, before mounting in an agarose bed [17]. Tissue slices ~120 μm thick were obtained with the aid of a tissue vibroslicer (Leica; model VT 1000E) and kept in oxygenated F-12 medium for 30–60 min before use in release experiments. For each experiment, two slices from the central region of the CB were transferred to a single well of a modified 24-well dish (see above) before ATP release measurements were obtained. In some experiments a whole CB was transferred to a single well and used in release experiments.

Bioluminescence detection of ATP release. Prior to ATP release measurements the growth medium was removed and replaced with 900 μl of standard extracellular solution (ECS) containing in mM: NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 10; and Hepes, 10, at pH 7.4. Following the addition of 200 μl luciferin–luciferase solution (ATP determination kit; Molecular probes # A22066), the dish was placed in a Labsystem Luminoskan luminometer connected to a Pentium III computer. Readings of luminescence (relative light units or RLU) were collected, stored, and analyzed using Labsystems' Ascent Software. Hypoxic ECS was prepared by bubbling the solution with N_2 gas for 1 h prior to commencement of the experiments. For stimulus application, 900 μl of normoxic (control) ECS was replaced with an equal volume of hypoxic ECS, effectively reducing the PO_2 of the solution from ~150 mmHg to ca. 15–20 mmHg. The luminometer was set to monitor luminescence at intervals of 4 s for a period of 3 min. All release measurements were obtained at room temperature.

Statistical analysis. Data from multiple treatments were compared using the statistical software Origin 7.0 (OriginLab, Northampton, MA) and analyzed using the statistical analysis of variance (ANOVA) with the level of significance set at $p < 0.05$.

Drugs. Drugs were dissolved in the extracellular perfusate so as to obtain the desired concentration, and the pH was adjusted as necessary to 7.4. Cadmium chloride, apyrase, and tetraethyl ammonium chloride (TEA) were obtained from Sigma (Mississauga, ON, Canada); iberiotoxin and nifedipine were obtained from Alomone Laboratories (Jerusalem). The 24-well dish was removed between recordings to replace the ECF with either test or control solution containing fresh luciferin–luciferase.

Results

Hypoxia-induced ATP release from carotid body chemoreceptors

Extracellular ATP levels were measured using a bioluminescence assay and a Labsystem luminometer in rat CB cultures, tissue slices, and whole CB. In all 3 preparations, exposure to acute hypoxia (PO_2 = 15–20 mmHg) for 3 min caused a reversible increase in extracellular ATP levels as exemplified in Figs. 1A–C (upper traces). The histograms in Fig. 1 show normalized values of the bioluminescent signal (relative light units; RLU) for each preparation relative to its own initial (normoxic) control. In CB cultures and tissue slices there was a 2- to 3-fold increase in ATP levels during hypoxia, whereas a smaller increase (1.5- to 2-fold) was seen in whole CB. Among the different preparations the bioluminescent signal was greatest in CB slices compared to CB cultures and whole CB. Comparison with the standard curve (Fig. 1D) revealed that during hypoxia the ATP concentration reached ~500 nM in CB slices (Fig. 2). The hypoxia-induced luminescence signal was markedly inhibited by the general nucleoside triphosphate hydrolase, apyrase (2 U/ml), in both CB slices (Fig. 2) and cultures (Fig. 3), confirming that it was due to the generation of ATP. In fact, during hypoxia plus apyrase the luminescence signal actually fell below the control normoxic level (Fig. 2; upper middle trace), suggesting basal extracellular ATP levels were also reduced. During exposure to longer periods of hypoxia the luminescence signal tended to decay after 7–10 min (data not shown).

Hypoxia-induced ATP release is dependent on L-type Ca^{2+} channels

Studies based on measurements of catecholamine release and spectrofluorometric changes in intracellular Ca^{2+} indicate that the hypoxia-induced secretory response in CB chemoreceptor cells depends mainly on entry of extracellular Ca^{2+} via L-type Ca^{2+} channels [1,3,16]. To test whether or not the hypoxia-induced ATP release occurs via a similar mechanism we used cadmium, a general Ca^{2+} channel blocker, as well as ni-

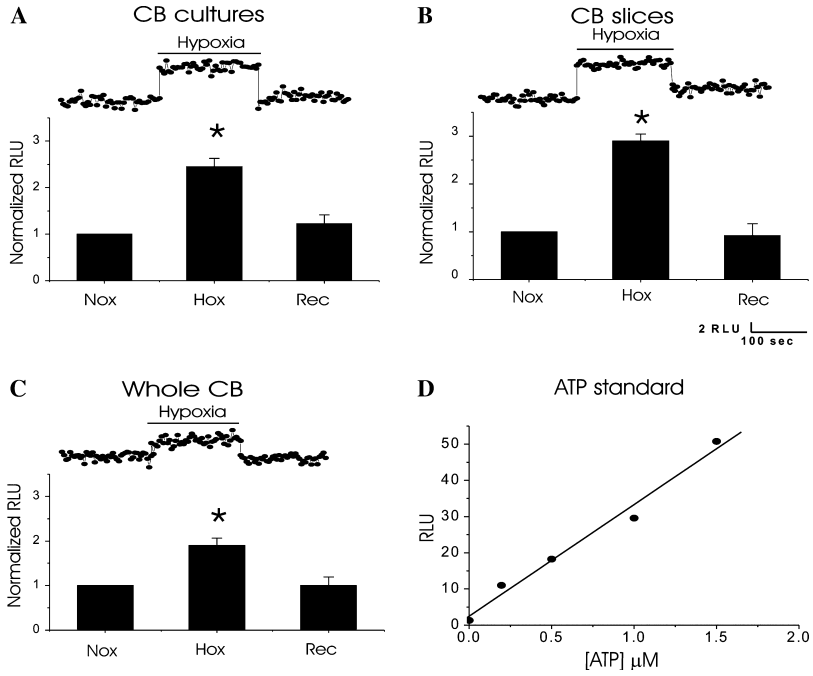

Fig. 1. Effect of hypoxia on ATP release in three different carotid body (CB) preparations. In all cases, the bioluminescence signal (relative light units or RLU) was normalized to control (normoxia). Hypoxia ($PO_2 \sim 15$–20 mmHg) caused a significant ($^*p < 0.01$) and reversible increase in extracellular ATP in CB cell cultures (A), CB slices (B), and whole CB (C). Upper trace in each (A–C) represents a sample recording of the luminescence signal in real time for each condition. Histograms show mean normalized values (±SEM) for a sample size $n = 6$. An ATP standard luminescence plot is shown in (D).

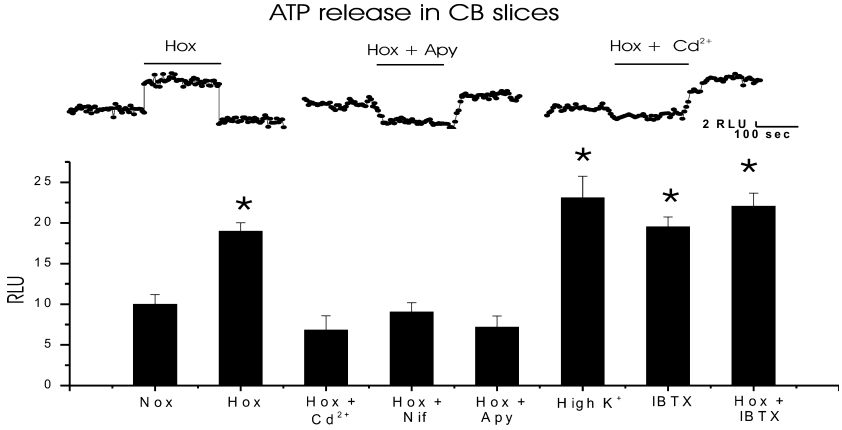

Fig. 2. Regulation of extracellular ATP in carotid body slices by various agents. For each treatment the bioluminescence signal (relative light units or RLU; mean ± SEM) indicative of extracellular ATP levels is shown. Compared to normoxic controls (Nox), ATP levels were significantly ($^*p < 0.01$; ANOVA) higher in slices treated with hypoxia (Hox), high K^+ (30 mM), iberiotoxin (IBTX, 100 nM), or hypoxia plus IBTX. The hypoxia-evoked increase in extracellular ATP was abolished in the presence of L-type Ca^{2+} channel blockers, cadmium (Cd^{2+}; 50 μM) and nifedipine (Nif; 50 μM), and the general nucleoside triphosphate hydrolase, apyrase (Apy; 2 U/ml). Notably, when hypoxia and IBTX (Hox + IBTX) were present together, their combined effects were non-additive, suggesting convergence onto a common pathway. Upper traces show typical luminometer records for the treatment indicated; note rebound stimulation of ATP release on removal of Cd^{2+}. Sample size was $n = 5$ for each treatment.

fedipine, a specific blocker of L-type Ca^{2+} channels. In CB slices (Fig. 2) and cell cultures (Fig. 3), both cadmium (Cd^{2+}; 50 μM) and nifedipine (Nif; 50 μM) abolished the increase in extracellular ATP seen during hypoxic exposure. There was a tendency for ATP levels to fall below basal (normoxic) control during exposure to hypoxia plus Cd^{2+}, and consistently, there was a rebound effect resulting in increased ATP levels for a brief period following Cd^{2+} removal (Fig. 2, upper right trace; $n = 6$). These effects, however, were not seen during application of hypoxia plus nifedipine (e.g. Fig. 3, upper right trace).

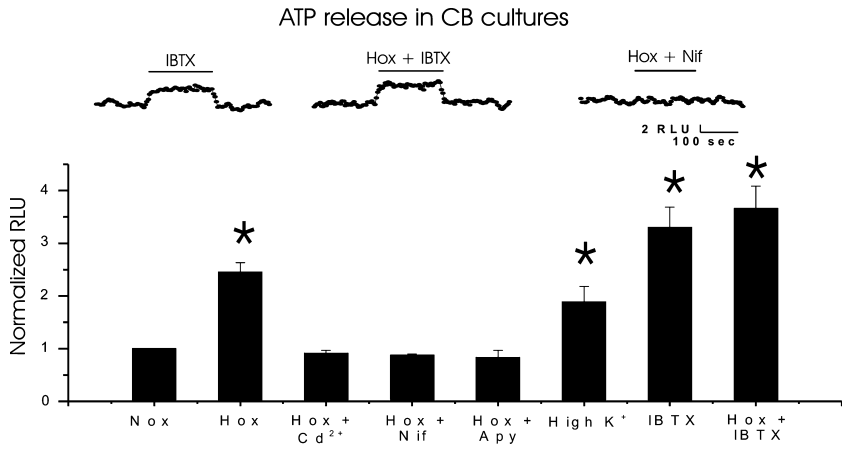

Fig. 3. Regulation of extracellular ATP in carotid body cultures by various agents. For each treatment the bioluminescence signal (relative light units or RLU), indicative of extracellular ATP levels, was normalized to that recorded in normoxia (Nox; control) for the same sample, to compensate for variations in cell number among the cultures. Compared to normoxic controls (Nox), ATP levels were significantly (*$p < 0.01$; ANOVA) elevated (2–3×) in cultures exposed to hypoxia (Hox), high K^+ (30 mM), iberiotoxin (IBTX, 100 nM), and hypoxia plus IBTX, as in CB slices (Fig. 2). Also, the hypoxia-evoked increase in extracellular ATP was abolished in the presence of L-type Ca^{2+} channels blockers, cadmium (Cd^{2+}; 50 μM) and nifedipine (Nif; 50 μM), and the general nucleoside triphosphate hydrolase, apyrase (Apy; 2 U/ml). When hypoxia and IBTX (Hox + IBTX) were present together, their combined effects were non-additive, suggesting convergence onto a common pathway as observed in CB slices (Fig. 2). Upper traces show typical luminometer records for the treatment indicated; sample size was $n = 5$ for each treatment.

ATP release in presence of K^+ channel blockers

In rat CB chemoreceptors hypoxia inhibits voltage- and Ca^{2+}-dependent maxi K^+ or BK [3,18], as well as voltage-independent, background or 'leak' TASK-1-like K^+ channels [19]. In cultures of rat CB chemoreceptor clusters [16] and CB tissue slices [17], blockade of O_2-sensitive BK channels with iberiotoxin (IBTX) or TEA leads to catecholamine secretion under normoxic conditions. In the present study, IBTX (100 nM) also stimulated ATP release (2- to 3-fold) in both CB tissue slices (Fig. 2) and cell cultures (Fig. 3). Notably, when IBTX was applied together with hypoxia, their combined effect on ATP release was largely occlusive (Figs. 2 and 3), suggesting that inhibition of BK channels is a major contributor to the secretory response evoked by hypoxia. Additionally, TEA (5 mM) stimulated ATP release (1.5- to 2-fold) in both preparations ($n = 5$; not shown), consistent with the idea that BK channels are normally open in rat chemoreceptor cell clusters under resting (normoxic) conditions [16,17]. Thus, by closing K^+ channels that are open at rest, both hypoxia and these K^+ channel blockers depolarize CB chemoreceptors, leading to voltage-gated Ca^{2+} entry and ATP release. Similarly, membrane depolarization induced by high extracellular K^+ also stimulates ATP release in CB tissue slices (Fig. 2) and cell cultures (Fig. 3).

Discussion

In this study we have developed a method for directly and continuously monitoring ATP secretion from carotid body (CB) slices, cell cultures, and whole CB in real time. The released ATP reacted with luciferin–luciferase in the extracellular solution, producing light that was detected by a luminometer. With this method we obtained direct evidence that hypoxia stimulates ATP release from CB chemoreceptors, thereby satisfying one important criterion in support of the proposed role of ATP as a key excitatory neurotransmitter in CB function. Confirmation that the luminescence signal arose from ATP was obtained in experiments where apyrase, a general nucleoside triphosphate hydrolase, markedly reduced the signal. Prior to these studies, the secretory status of CB chemoreceptors was routinely studied by monitoring catecholamine release by high performance liquid chromatography HPLC [1,16] or carbon fiber amperometry [17]. However, the role of catecholamine release in CB chemoexcitation has been questioned [1], and the available evidence in the rat indicates it is not causal to the hypoxia-induced increase in nerve activity [5,6]. On the other hand, ATP has recently been proposed as a key fast-acting co-transmitter that mediates the chemoexcitatory effects of hypoxia, and hypercapnia, by stimulating postsynaptic P2X2-containing purinergic receptors [7–10]. Moreover, in a recent study, hypoxia was found to stimulate adenosine release from whole rat CB, and ~40% of the extracellular adenosine was estimated to derive from the breakdown of extracellular ATP [20].

Mechanisms of ATP accumulation around CB chemoreceptors

In all three preparations tested, i.e., CB slices, monolayer cell cultures, and whole CB, hypoxia stimulated

ATP release and the effect was reversible upon re-introduction of normoxic solution. The amount of ATP released during hypoxia varied among the three preparations, with the greatest release detected in CB slices. Though whole CB contained the largest number of chemoreceptor cells, ATP detection in the surrounding fluid was limited by diffusion through the organ and enzymatic degradation by endogenous ATPases [20,21]. On the other hand, monolayer cell cultures contained the fewest chemoreceptor cells but the presence of minimal diffusion barriers meant that ATP released into the medium could directly interact with the luciferin–luciferase. Likewise, ATP released by cells at the center of the ~120 μm thick CB slices had relatively easy access to the surrounding fluid. These studies further emphasize the usefulness of tissue slices to study secretory function of CB chemoreceptors [17]. However, in the latter study slices were cultured for ~24 h in serum-containing medium before amine secretion was monitored by carbon fiber amperometry, whereas in the present study fresh slices were successfully used for monitoring ATP secretion within 30–60 min after sectioning. This, together with the observation that both hypoxia-induced ATP (this study) and adenosine [20] release were detectable in whole CB, suggests ATP released under these conditions is physiologically relevant and not a spurious artefact of cell or organ culture.

As expected for vesicular release, the hypoxia-induced increase in extracellular ATP around CB chemoreceptors was dependent on Ca^{2+} entry through voltage-gated Ca^{2+} channels. Thus, ATP release during hypoxia was inhibited by blockers (nifedipine and cadmium) of L-type Ca^{2+} channels, which are known to be present in these rat chemoreceptor cells [3,22,23]. This effect of hypoxia was likely mediated via membrane depolarization induced by closure of O_2-sensitive Ca^{2+}-dependent K^+ (BK) channels [3,18], and background TASK-like K^+ channels [19], present in rat chemoreceptor cells. Indeed, selective blockade of BK channels with iberiotoxin (IBTX) mimicked hypoxia in stimulating ATP release, and moreover, the presence of IBTX largely occluded the secretory response due to hypoxia. These results support a key role of Ca^{2+}-dependent BK channels in the hypoxic sensitivity of CB chemoreceptors in the rat [18]. Membrane depolarization induced by solutions containing high extracellular K^+ also stimulated ATP release.

Physiological significance of ATP release

The present study strongly supports a key role of ATP as a neurotransmitter during chemosensory transmission in the CB. Previous studies in this laboratory suggested that during both hypoxia and hypercapnia, ATP is co-released with ACh and mediates fast excitation by acting on postsynaptic ionotropic P2X2–P2X3 purinoceptors on afferent nerve terminals [7–9]. Furthermore, in mice deficient in the P2X2 subunit, the ventilatory response to hypoxia was markedly impaired, as was the hypoxia-induced afferent CB chemosensory discharge [10]. The direct demonstration that hypoxia stimulates ATP release from CB chemoreceptors satisfies one important criterion for an agent to act as a sensory neurotransmitter, i.e., that it be released during stimulus application. In the CB, this released ATP may also have additional presynaptic actions mediated by autocrine–paracrine stimulation of purinergic P2Y receptors present on neighboring receptor or glial cells [24,25].

Acknowledgments

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