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Inhibition of the hypercapnic ventilatory response by adenosine in the retrotrapezoid nucleus in awake rats

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Abstract

The brain regulates breathing in response to changes in tissue CO_2/H^+ via a process called central chemoreception. Neurons and astrocytes in the retrotrapezoid nucleus (RTN) function as respiratory chemoreceptors. The role of astrocytes in this process appears to involve CO_2/H^+ -dependent release of ATP to enhance activity of chemosensitive RTN neurons. Considering that in most brain regions extracellular ATP is rapidly broken down to adenosine by ectonucleotidase activity and since adenosine is a potent neuromodulator, we wondered whether adenosine signaling contributes to RTN chemoreceptor function. To explore this possibility, we pharmacologically manipulated activity of adenosine receptors in the RTN under control conditions and during inhalation of 7–10% CO_2 (hypercapnia). In urethane-anesthetized or unrestrained conscious rats, bilateral injections of adenosine into the RTN blunted the hypercapnia ventilatory response. The inhibitory effect of adenosine on breathing was blunted by prior RTN injection of a broad spectrum adenosine receptor blocker (8-PT) or a selective A_1 -receptor blocker (DPCPX). Although RTN injections of 8PT, DPCPX or the ectonucleotidase inhibitor ARL67156 did not affect baseline breathing in either anesthetized or awake rats. We did find that RTN application of DPCPX or ARL67156 potentiated the respiratory frequency response to CO_2 , suggesting a portion of ATP released in the RTN during high CO_2/H^+ is converted to adenosine

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Authors contribution

BF, LMO, DKM, ACT and TSM designed research; BF and LMO performed research; BF, LMO, ACT and TSM analyzed data; BF, ACT and TSM wrote the paper. BF, LMO, ACT, DKM and TSM performed critical review of the manuscript. All authors approved the final version.

and serves to limit chemoreceptor function. These results identify adenosine as a novel purinergic regulator of RTN chemoreceptor function during hypercapnia.

Keywords

RTN; purinergic signaling; breathing; adenosine; central chemoreflex

Introduction

The retrotrapezoid nucleus (RTN) contains a subset of neurons that are intrinsically sensitive to changes in CO₂ and pH (Mulkey et al., 2004; Nattie et al., 1993; Nottingham et al., 2001; Smith et al., 1989; Wang et al., 2013) and relay responses to respiratory control regions, such as the ventral respiratory complex to control breathing automaticity (Connelly et al., 1989, 1990; Guyenet and Bayliss, 2015; Li et al., 1999; Smith et al., 1989). Besides the intrinsic property, RTN neuronal mechanisms also involve an indirect activation by purinergic signaling, most likely from CO₂/H⁺-sensitive astrocytes (Barna et al., 2016; Gourine et al., 2010; Gourine et al., 2005a; Huckstepp et al., 2010b; Wenker et al., 2010; Wenker et al., 2012). Evidence also suggests that purinergic signaling is a unique feature of RTN chemoreception (Sobrinho et al., 2014). It was shown that CO₂/H⁺ facilitates ATP release at discrete locations near the RTN (Gourine, 2005; Gourine et al., 2005a; Sobrinho et al., 2014; Thomas and Spyer, 2000) and that RTN astrocytes, but not cortical astrocytes, respond to H⁺ with increased Ca²⁺-dependent exocytosis of ATP-containing vesicles (Kasymov et al., 2013). As described above, it is generally well accepted that CO₂-evoked ATP release from ventral medullary surface astrocytes contributes to respiratory drive by activating chemosensitive RTN neurons (Gourine et al., 2010; Wenker et al., 2010). We demonstrated that blocking P2 receptors at the RTN level decreased the hypercapnia ventilatory response (HCVR) (Barna et al., 2016; Sobrinho et al., 2014; Wenker et al., 2012). These results strongly suggest that purinergic signaling through P2 receptors enhances CO₂/H⁺-dependent output of the RTN. It is also well established in other brain regions that extracellular ATP can be rapidly metabolized to adenosine which can modulate neural activity by activation of adenosine receptors (A1-, A2A-B-, and A3-receptors) (Funk, 2013; Howell, 1993; Spyer et al., 1997; Thomas and Spyer, 1999, 2000). In the context of breathing, activation of A1 and A2 receptors generally results in suppression or enhancement of respiratory activity, respectively. Note that A3 receptors have relatively low expression in the central nervous system (Borea et al., 2015) and have not been implicated in control of breathing. Adenosine has been shown to contribute to the ventilatory response to hypoxia in mammals (Howell, 1993; Montandon et al., 2007). For example, exposure to hypoxia may trigger ATP release in respiratory centers including the Pre-Bötzinger complex and its subsequent conversion to adenosine has been shown to suppress respiratory output of this region and potentially contribute to central hypoxic respiratory depression (Angelova et al., 2015; Bissonnette, 2000; Bissonnette et al., 1991; Sheikhabaei et al., 2018). In terms of central chemoreception, previous work in rats and sheep showed that A1 receptors are highly expressed in the rostral ventrolateral medulla (Bissonnette and Reddington, 1991) in the vicinity of the RTN, and consistent with a role in central chemosensitivity, systemic application of a specific A1 blocker (DPCPX) potentiated the CO₂ ventilatory response in

juvenile awake rats (Montandon et al., 2007). However, despite evidence that CO_2/H^+ -evoked ATP release occurs in the RTN, the effects of adenosine signaling in the RTN on breathing and the HCVR have not been characterized.

The goals of this study are to determine whether and how ADO signaling in the RTN affects baseline breathing and the HCVR in anesthetized and awake rats. We find that RTN injections of ADO inhibited breathing and the HCVR by an A1-receptor dependent mechanism. We also found that blocking A1 receptors potentiated respiratory frequency responses to CO_2 , suggesting endogenous ADO produced in the RTN during hypercapnia limits CO_2/H^+ -dependent respiratory drive.

Methods

1) Animals

Animal use was in accordance with guidelines approved by University of São Paulo Institutional Animal Care and Use Committee (ICB/USP: 70/2012). *In vivo* experiments were performed on male Wistar rats weighing 250–330 g (3–5 months old; N = 52). All efforts were made to minimize animal discomfort and the number of animals used.

2) *In vivo* preparation and physiological recordings

Anesthetized rats—The surgical procedures and experimental protocols were done in bilaterally vagotomized, artificially ventilated urethane anesthetized rats prepared as described previously (Sobrinho et al., 2017; Sobrinho et al., 2014; Takakura et al., 2011; Takakura and Moreira, 2011). Briefly, general anesthesia was induced with 5% isoflurane in 100% O_2 . Artificial ventilation with 1.4–1.5% isoflurane in 100% O_2 was maintained throughout surgery. The surgical procedures (bilateral vagotomy, arterial cannulation, electromyography (EMG), and dorsal transverse access to the ventrolateral medulla) were standard. Bipolar electrodes were coupled to record the activity of diaphragm (Dia_{EMG}), and abdominal (Abd_{EMG}) muscles, and the amplification used for the signal was 5,000–10,000x. Rectal temperature (maintained at 37°C) and end-tidal CO_2 were monitored throughout the experiment with a capnometer (Columbus Instruments) that was calibrated twice per experiment.

On completion of surgical procedures, isoflurane was gradually replaced by urethane (1.2 g/kg iv. over 30 min). After injection of the intravenous anesthetic, the anesthesia level was monitored by testing for absence of withdrawal response and lack of arterial pressure (AP) changes due to firm paw pinch. The rats were ventilated with 100% oxygen throughout the experiment in order to silence peripheral chemoreceptors. Activation of central chemoreflex was done by hypercapnia (10% of etCO_2).

All analog data (end-expiratory CO_2 , EMG activities, and AP) were stored on a computer via a Micro1401 digitizer from Cambridge Electronics Design (CED, Cambridge, UK) and were processed with version 5 of Spike2 software (CED). Integrated electromyography activity ($^{\text{p}}$ EMG) was obtained after rectification and smoothing ($\tau = 0.003$ s) (Oliveira et al. 2016). Diaphragm, and abdominal muscle activities were determined by averaging $^{\text{p}}$ EMG

over 30 s and normalizing the result by assigning a value of 0 to the dependent variable recorded at 3–4% of end-expiratory CO₂ and a value of 9–10% of end-expiratory CO₂.

Conscious rats—Rats were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) combined with xylazine (7 mg/kg) and placed in a stereotaxic frame (model 900; David Kopf Instruments). Stainless steel cannulas were placed bilaterally into the RTN using the following coordinates: 2.3 mm caudal to lambda, 1.8 mm lateral to the midline, and 5.5 mm below dura mater. The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. Rats received a prophylactic dose of penicillin (30,000 IU) given intramuscularly and a subcutaneous injection of the analgesic Ketoflex (1%, 0.03 ml/rat) postsurgically. After the surgery, the rats were maintained in individual boxes with free access of tap water and food pellets.

Respiratory rate (f_R , breaths/min) and tidal volume (V_T , ml/kg) in conscious, freely moving rats were measured by whole-body plethysmography as described previously (Barna et al., 2016; Silva et al., 2016; Totola et al., 2017). All experiments were performed at room temperature (24–26°C). In brief, freely moving rats were kept in a Plexiglas recording chamber (5 L) that was flushed continuously with a mixture of 79 % N₂ and 21 % O₂ (unless otherwise required by the protocol) at a rate of 1 L/min. The pressure signal was amplified, filtered, recorded, and analyzed off-line using PowerLab software (ADInstruments). A volume calibration was performed during experiment by injecting a known air volume (1 ml) into the chamber. Animals were allowed ~30 min to acclimatize to the chamber environment at normoxia/normocapnia (21 % O₂, 79 % N₂, and < 0.5 % CO₂) before measurements of baseline AP and ventilation were taken. Hypercapnia was induced by titrating CO₂ into the respiratory mixture up to a level of 7% for 10 min. Measurements of f_R and V_T were taken during the last 2 min before exposure to the stimulus and during the 2 min period at the end of drug stimulus, when breathing stabilized. Changes in the f_R , V_T , and minute ventilation (V_E) ($f_R \times V_T$; ml/min/kg) were averaged and expressed as mean $\pm$ SE.

3) Drugs

All drugs were purchased from Sigma Aldrich (Sigma Chemicals Co.) unless otherwise stated. For experiments in anesthetized animals, ADO (1–50 mM in sterile saline, pH 7.4), 8-PT (100 μ M in sterile saline, pH 7.4) or DPCPX (5 μ M in sterile saline, pH 7.4) were injected into the RTN using single barrel glass pipettes (tip diameter of 25 μ m) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp.) All drugs contained a 2% dilution of fluorescent latex microbeads (Lumafluor, New York, NY) for later histological identification of the injection sites. For experiments in conscious animals, ADO (10 mM in sterile saline, pH 7.4), DPCPX (5 μ M in sterile saline, pH 7.4) or ARL 67156 (200 μ M in sterile saline, pH 7.4) were injected bilaterally using a 5 μ l Hamilton syringe connected to an injection needle positioned in the guide cannula (the tip of the needle extended 2.5 mm beyond the end of the cannula).

4) Chemoreflex analysis

In anesthetized rats, hypercapnia was produced by addition of pure CO₂ to the 100% O₂ supplied by artificial ventilation to increase the maximum end-expiratory CO₂ to 9–10%. End-expiratory CO₂ was maintained for 5 min, and CO₂ was removed. Each rat was submitted to three sessions of hypercapnia: one session 10 min after bilateral saline injections into the RTN, and two additional sessions, 10 and 60 min after bilateral injections of saline, ADO, 8-PT or DPCPX into the RTN.

Unanesthetized rats were allowed at least 30–40 min to acclimatize to the chamber environment at normoxia/normocapnia (21% O₂, 79% N₂ and <0.5% CO₂) before measurements of baseline breathing (V_T , f_R and V_E) were taken. Hypercapnia was induced by titrating CO₂ into the respiratory mixture up to a level of 7% (21% O₂, 69% N₂) for 10 min.

5) Histology

At the end of the experiments, all rats were deeply anesthetized with pentobarbital and a 2% solution of Evans blue was injected into the RTN (100 nl). Saline followed by 4% buffered formalin (pH 7.4) was perfused through the heart. The brains were removed and processed as described previously (Barna et al., 2016). Injection sites in the RTN were confirmed by inspection using an Axioskop 2 microscope (Carl Zeiss Microscopy). Sections from different brains were aligned with respect to a reference section, which was the most caudal section containing an identifiable cluster of facial motor neurons. To this reference section was assigned a value of 11.6 mm caudal to bregma (bregma –11.6 mm (Paxinos and Watson, 1998)). Levels rostral or caudal to this reference section were determined by adding or subtracting the number of intervening sections $\times$ 40 μ m.

6) Statistical analysis

Data normality was assessed using the Shapiro-Wilk test, and all the normally-distributed data were expressed as the mean $\pm$ SEM. Student t-test; One-way or two-way repeated measures (RM) ANOVA with Newman-Keuls's multiple comparisons as appropriate ($p < 0.05$). Statistical analysis was performed using Sigma Stat version 3.0 software (Jandel Corporation).

Results

1) Adenosine inhibits ventilation during hypercapnia in the RTN

The first series of experiments were designed to evaluate a dose-response curve to ADO (1, 5, 10, 25 and 50 mM - 30 nl) on the respiratory output. We recorded Dia_{EMG} and Abd_{EMG} in a total of 31 animals that received vehicle and increasing doses of ADO injected bilaterally into the RTN during continued exposure to hypercapnia (9–10% CO₂) (Figs. 1A–D). We found that bilateral injections of ADO (10–50 mM - 30 nl) into the RTN under anesthesia decreased respiratory activity in reversible and dose dependent manner. For example, ADO (10, 25 and 50 mM - 30 nl) ($N = 5\text{--}7/\text{group}$) into the RTN reduced the increase in Dia_{EMG} frequency (10 mM: $11 \pm 3\%$; 25 mM: $9 \pm 3\%$; 50 mM: $8 \pm 2\%$; vs. vehicle: $39 \pm 3\%$, ID₅₀ = 6.73 mM, $p < 0.001$), Dia_{EMG} amplitude (10 mM: $111 \pm 7\%$; 25 mM: $107 \pm 4\%$; 50 mM:

107 ± 3 % vs. vehicle: 190 ± 8 %, ID₅₀ = 6.29 mM, p = 0.001), and Abd_{EMG} frequency (25 mM: 33 ± 2 bpm; 50 mM: 34 ± 2 bpm vs. vehicle: 49 ± 2 bpm, ID₅₀ = 17.36 mM, p < 0.001) elicited by hypercapnia (Figs. 1A–C). RTN injections of lower concentrations of ADO (1 and 5 mM - 30 nl) (N = 5–7/group) did not change the increase in Dia_{EMG} frequency and amplitude, Abd_{EMG} frequency and amplitude (Figs. 1A–D).

The following experiment was performed in unrestrained awake rats using the threshold dose of adenosine (10 mM). Application of ADO into the RTN under hypercapnia in conscious rats (N = 5) reduced V_T (8.4 ± 0.5, vs. vehicle: 10 ± 0.6 ml/kg, p < 0.001), f_R (117 ± 7.7, vs. vehicle: 136 ± 5.4 bpm, p < 0.001) and V_E (978 ± 74 vs. vehicle: 1366 ± 104 ml/kg/min, p < 0.001) (Figs. 2B–E). ADO did not affect breathing under normocapnia condition (data not shown).

As shown in Figs. 1E, 2A, 3F, 4F, 5E and 6E all injections were centered 250–270 µm below the facial motor nucleus and 100–200 µm rostral regarding the caudal portion of the facial nucleus, lateral to pyramidal tract, and medial to the spinal trigeminal tract (Barna et al., 2016; Takakura et al., 2011; Takakura and Moreira, 2011). The microbeads used to identify injection sites showed a dispersion of around 250 µm in the rostro-caudal direction from the center of the injection. Misplaced injections that reached the facial motor nucleus or the parapyramidal region did not produce significant changes in breathing output (data not shown)

2) Respiratory effects elicited by blockade of the adenosine receptors in the RTN

Next, we used a non-specific ADO receptor blocker and a selective A1 receptor blocker to determine which receptors in the RTN mediate the inhibitory effects of ADO on breathing. Under urethane anesthesia, bilateral injection of a broad-spectrum adenosine antagonist (8-PT; 100 µM) (N = 6/group) into the RTN blocked the decrease in Dia_{EMG} frequency (30 ± 6 vs. ADO: 14 ± 2%, p = 0.032), Dia_{EMG} amplitude (180 ± 13 vs. ADO: 107 ± 7%, p < 0.001), and Abd_{EMG} frequency (57 ± 6 vs. ADO: 38 ± 5 bpm, p = 0.049) elicited by ADO (Figs. 3A–D). No significant changes were observed on Abd_{EMG} amplitude (Fig. 3E). Bilateral injections of a specific A1 receptor antagonist (DPCPX, 5 µM) (N = 6/group) into the RTN produced a further increase in Dia_{EMG} frequency (80 ± 7 vs. vehicle + CO₂: 56 ± 3%, p = 0.018) and Dia_{EMG} amplitude (177 ± 17 vs. vehicle + CO₂: 103 ± 24%, p < 0.011), without changing Abd_{EMG} frequency and amplitude (Figs. 4A–E). Interestingly, DPCPX alone potentiated respiratory frequency during sustained hypercapnia, suggesting a counter-regulatory role of endogenous ADO in RTN chemoreceptor function. All injections into the RTN were correctly placed under the facial motor nucleus (Figs. 3F and 4F). We also tested the effect of DPCPX on breathing and HCVR in conscious rats. Bilateral RTN injections of DPCPX (5 µM) (N = 4/group) did not affect baseline breathing (V_T: 4.9 ± 0.2 vs. baseline: 3.6 ± 0.5 ml/kg, p = 0.078; f_R: 79.9 ± 7.9 vs. baseline: 86.6 ± 3 bpm, p = 0.698 and V_E: 391.2 ± 45 vs. baseline: 314.1 ± 49 ml/kg/min, p = 0.439). However, A1 receptor blockade in the RTN did potentiate the ventilatory response to CO₂; DPCPX increased V_T (8.8 ± 0.4 vs. vehicle + CO₂: 7.5 ± 0.4 ml/kg, p < 0.001), f_R (131 ± 10.7 vs. vehicle + CO₂: 119 ± 7.9 bpm, p < 0.007) and V_E (1140 ± 43 vs. vehicle + CO₂: 891.6 ± 92 ml/kg/min, p <

0.001) in conscious rats (Figs. 5A-D). These results further suggest that ADO limits RTN chemoreceptor activity during hypercapnia.

3) Respiratory effects of inhibiting ectonucleotidase activity bilaterally in the RTN

Extracellular adenosine is thought to be primarily derived from the enzymatic breakdown of extracellular ATP (Cunha, 2001), which can be released from either neurons or glia via exocytosis (Burnstock, 2007). Given the established link between purinergic signaling and RTN astrocytes (Gourine et al., 2010), we hypothesized that CO₂/H⁺-evoked ATP release from astrocytes is metabolized to adenosine and subsequently modulates activity of chemosensitive RTN neurons by an A1 receptor dependent mechanism. Bilateral injections of ARL 67156 (200 μM) into the RTN of conscious rats (N = 6/group; Fig. 6E) did not affect baseline breathing (V_T: 5.2 ± 0.7 vs. baseline: 5.5 ± 0.8 ml/kg, p = 0.600; f_R: 85 ± 4 vs. baseline: 875 ± 5 bpm, p = 0.805 and V_E: 443 ± 67 vs. baseline: 479 ± 73 ml/kg/min, p = 0.694) (Figs. 6A-D). However, these same ARL 67156 treated animals showed an increase in the tachypneic response to CO₂ (170 ± 11 vs. vehicle + CO₂: 143 ± 8 bpm, p = 0.002) but without affecting the increase in V_T (8.7 ± 1.5 vs. vehicle + CO₂: 8.8 ± 1.3 ml/kg, p = 0.832), and V_E (1410 ± 175 vs. vehicle + CO₂: 1239 ± 158 ml/kg/min, p = 0.073) (Figs. 6B-D).

Discussion

Our data show for the first time that adenosine limits CO₂/H⁺-dependent output of the RTN in both anesthetized and awake rats by an A1 receptor dependent mechanism. At least one source of adenosine appears to be metabolism of extracellular ATP that is released in response to high CO₂. Consistent with this, blockade of A1 receptors or ectonucleotidase activity blunted the ventilatory response to CO₂/H⁺ but minimally affected baseline respiratory activity.

Purinergic signaling within the ventral medullary surface

Retrotrapezoid nucleus chemoreceptors neurons are well described to be directly activated by CO₂/H⁺ in several preparations (Holloway et al., 2015; Kumar et al., 2015; Mulkey et al., 2004; Onimaru et al., 2012; Shi et al., 2016; Takakura et al., 2014; Wang et al., 2013). The mechanism by which RTN chemoreceptors sense changes in pH involves TWIK-related acid-sensitive K-2 channels (TASK-2) and proton-activated receptor GPR4 (Kumar et al., 2015; Wang et al., 2013). The activity of chemosensitive RTN Ott et al., 2011) and distal sources including pulmonary receptors, hypothalamus, nucleus of the solitary tract (NTS), periaqueductal grey matter, spinal cord, dorsolateral pons and raphe nuclei (Barna et al., 2014; Moreira et al., 2007; Rosin et al., 2006; Takakura et al., 2006). If some of these inputs are chemosensitive themselves (e.g. NTS, raphe, hypothalamus), then part of the chemosensitivity of RTN neurons could be synaptically driven. Consistent with this, RTN neurons show a more vigorous firing response to CO₂/H⁺ in vivo as compared to more reduced preparations where many synaptic inputs have been eliminated (Guyenet et al., 2005).

More recently, a role for purinergic signaling in the ventral medullary surface has been found to contribute to RTN chemoreception (Gourine et al., 2010; Gourine et al., 2005b; Thomas et al., 1999; Thomas and Spyer, 2000). Results supporting this possibility include *i*) blocking P2 receptors in the RTN region decreased breathing activity elicited by hypercapnia *in vivo* and *in vitro* (Barna et al., 2016; Gourine et al., 2005; Sobrinho et al., 2014; Wenker et al., 2012); *ii*) disrupting ATP release with connexin hemichannel (Cx26) blockers also blunted CO₂/H⁺ sensitivity of RTN neurons (Huckstepp et al., 2010a; Huckstepp et al., 2010b; Meigh et al., 2014; Wenker et al., 2012); and *iii*) optogenetic or pharmacological manipulation of astrocytes within the RTN triggered ATP release and subsequent activation of RTN neurons and respiratory output (Gourine et al., 2010; Sobrinho et al., 2017; Sobrinho et al., 2014; Wenker et al., 2010; Wenker et al., 2012). Although it remains possible that ATP or other purinergic effectors are released by other cell types, astrocytes appear to be the most likely candidates as suggested by Dr. Gourine's laboratory (Kasymov et al., 2013; Turovsky et al., 2016).

Another open question is by what mechanism(s) does purinergic signaling alters the function of RTN chemosensitive neurons. As noted above, purinergic signaling directly activates RTN neurons; however, the identity of purinergic receptors expressed by RTN neurons has not been determined. Evidence also suggests that CO₂/H⁺-evoked ATP release prevents CO₂/H⁺-induced dilation of RTN arterioles, which presumably serves to maintain tissue CO₂/H⁺ and thus augment neural activity indirectly (Hawkins et al., 2017). The cellular and molecular basis for purinergic modulation of arteriole tone in the RTN remains unclear. The use of a new purinergic pharmacology and cell specific loss-of-function genetics will certainly improve our understanding of purinergic mechanisms in central chemoreception at the level of RTN region.

Adenosine signaling within the ventral medullary surface

Unlike classic neurotransmitters, intracellular ADO is not stored and release from vesicles but rather is generated by the highly regulated intracellular metabolic pathways like ATP production (King et al., 2006). The rapid extracellular conversion of locally released ATP to adenosine represents a potentially important source of this neurotransmitter in several tissues (Delaney and Geiger, 1998; Dunwiddie and Masino, 2001; Fredholm et al., 2007). It should be noted that the extracellular ADO might originate not only from neurons but also from glial cells (Halassa et al., 2007, 2009). Our evidence that blockade of A1 receptors minimally affects baseline breathing but increases the HCVR is consistent with the possibility that ATP is only released, presumably by RTN astrocytes, during exposure to high CO₂. Therefore, we suggest that the source of ADO in the RTN is CO₂/H⁺-evoked ATP release by astrocytes followed by conversion to ADO by local ectonucleotidase activity. Consistent with this, we show blocking ectonucleotidase activity with ARL67156 potentiated the effects of CO₂/H⁺ on respiratory frequency. These results are entirely consistent with the possibility that ADO derived from ATP metabolism limits chemoreceptor activity during high CO₂. Of course, we cannot exclude a possible direct release of ADO by astrocytes or other cells in the area.

The present data demonstrate that under hypercapnic conditions, the application of ADO within the RTN region was able to elicit an inhibitory effect on breathing output. This effect is mediated from the degradation of ATP to ADO and the activation of an A1 receptors-mediated inhibition that obscures the P2 excitation. The blockade of ADO receptors within the RTN eliminated the inhibitory effect of ADO under hypercapnia challenge. We are not suggesting that all ATP released under hypercapnia is instantaneously converted to ADO into the RTN. In fact, the blockade of ADO receptors produced a further increase (20–30% increase) in breathing under high levels of CO₂.

Although our results show that blockade of the ectonucleotidase activity produced an effect only on breathing frequency, without affecting V_T and V_E, we do not suggest ADO signaling in the RTN is specific to this respiratory parameter. There are important issues that must be taken into consideration to interpret these results. First, we performed experiments in conscious rats with bilateral injections of ectonucleotidase inhibitor in the RTN region, while previous studies manipulated ectonucleotidase activity in more reduced preparations (Funk et al., 2008; Witts et al., 2012). Second, the global activity distribution patterns of the multiple ectonucleotidases in brain nuclei remains poorly characterized. The levels of brain ectonucleotidase activity varies widely (from cortex to brainstem) (Kukulski et al., 2004). Moreover, differential distribution of ectonucleotidases could contribute to the ATP sensitivity of different regions of the respiratory column. Finally, ARL67156 is selective ectonucleotidase type 1 and is therefore of somewhat limited utility in relation to respiratory control because ectonucleotidase type 2 is considered the main enzyme expressed in the brainstem (Kukulski et al., 2004).

Adenosine signaling involves four well-characterized receptors subtypes (A₁, A_{2a}, A_{2b} and A₃). A₁ receptors are Gi-coupled receptors that are highly expressed in the brain where they are associated with activation of K⁺ channels and inhibition voltage-dependent Ca²⁺ channels leading to inhibition of neurotransmitter release (Burnstock et al., 2011; Stone et al., 2009). The A₂ receptors are Gs-coupled receptors that mediate both inhibitory and excitatory responses (Stone et al., 2009); however, due to their limited distribution in the brain adenosine signaling is generally considered inhibitory (Wieder et al., 2014). Consistent with this, we find that exogenous application of ADO into the RTN inhibited breathing, whereas application of A1 receptor blocker into the same region potentiated the ventilatory response to CO₂ and blocked subsequent ADO inhibition. These results indicate ADO signaling through A1 receptors inhibits RTN chemoreceptor activity. Since A1 receptors are express pre- and post-synaptically, it is not clear whether RTN neurons are intrinsically or synaptically inhibited by this mechanism. Furthermore, since ADO is also a potent vasodilator including in the RTN (Hawkins et al., 2017), and since vasodilation has been shown to suppress CO₂/H⁺-dependent output of the RTN presumably by facilitating removal of extracellular CO₂/H⁺ (Hawkins et al., 2017), it is also possible that the inhibitory effects of ADO on RTN chemoreception involve vasodilation and increased tissue pH.

ADO inhibits breathing in almost all mammals under anesthesia (Bissonnette et al., 1991; Burr and Sinclair, 1988; Eldridge et al., 1985; Koos and Matsuda, 1990; Lagercrantz et al., 1984; Schmidt et al., 1995; Wilson et al., 2004); however, the opposite has been shown to be the case in conscious humans (Fuller et al., 1987; Griffiths et al., 1997; Griffiths et al., 1990;

Reid et al., 1991). Contrary to these results, we found that application of ADO into the RTN had similar inhibitory effects on breathing in anesthetized and awake rats. Our results are also consistent with evidence from more reduced preparations that showed application of ADO agonists to inspiratory rhythm-generating brainstem spinal cord preparations inhibited respiratory rhythm (Herlenius and Lagercrantz, 1999; Herlenius et al., 1997). It is possible that the stimulatory action of adenosine in awake humans is mediated by its action on peripheral chemoreceptors (Fuller et al., 1987; Kowaluk et al., 1998; Reid et al., 1991). We believe that at least two mechanisms are involved in the inhibition of ventilation by ADO. An A2 receptor-mediated excitation of GABAergic neurons that in turn inhibits brainstem respiratory networks could be one contributor to the ADO-mediated inhibition *in vivo* (Koos and Chau, 1998; Koos et al., 2005; Mayer et al., 2006; Wilson et al., 2004). However this mechanism is not the first option because GABA neurons are very sparse in the RTN region, at least in rats (Weston et al., 2004). In addition, A2 receptors antagonists applied to the respiratory neurons do not prevent the inhibitory actions of ADO (Mironov et al., 1999).

Conclusion

The multiple purinergic mechanisms that determine the dynamics of the HCVR is key in the strategies to better understand the functional role of chemoreceptors neurons in the RTN on chemoreception. In the present study, we identify a role of the ADO receptors in the reduction of breathing under hypercapnia challenge. Degradation of ATP in the RTN will contribute to the reduction in the HCVR not only by removing the excitatory actions of ATP, but also by producing adenosine, which is inhibitory at the level of RTN.

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Highlights

- Neurons and astrocytes in the RTN function as respiratory chemoreceptors
- ATP is rapidly broken down to adenosine by ectonucleotidase activity
- Blocking A1- or NTPase attenuate the hypercapnia ventilatory response inhibition
- Adenosine act as a novel purinergic regulator of RTN chemoreceptor function

Figure 1. Adenosine in the RTN attenuates the hypercapnic ventilatory response in anesthetized vago-denervated rats

Changes in A and B) diaphragm (DiaEMG) and C and D) abdominal (AbdEMG) electromyography activities after bilateral injection of vehicle or increasing doses of ADO in the RTN under hypercapnic condition. E) Photomicrograph showing typical sites of bilateral injections in the RTN region. The latex microspheres are located ventral to the caudal portion of the facial nucleus in the RTN region. Computer-generated plot of bilateral injections of vehicle or ADO that were confined to the RTN region (bregma level -11.12 to -11.6 mm according to the Paxinos and Watson (1998)). *Different from vehicle (Two-way ANOVA; Newman-Keuls' test, $p < 0.05$, $N = 5-7$ /group of rats). Abbreviations: py, pyramidal tract; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Scale bar in E = 1 mm.

Figure 2. Adenosine in the RTN attenuates the hypercapnic ventilatory response in awake rats
 A) Photomicrograph of a coronal section showing the site of a bilateral injection in the RTN (arrows) and a computer-generated plot of bilateral injections of vehicle or ADO that were confined to the RTN region. B) Recordings showing the effect of bilateral injection of vehicle or ADO (10 mM - 100 nL) into the RTN region under normocapnia and hypercapnia (7% CO_2). Changes in C) tidal volume (V_T , ml/kg), D) respiratory frequency (f_R , bpm) and E) minute ventilation (V_E , ml/kg/min) elicited by vehicle or ADO bilaterally injected into the RTN region under hypercapnic condition. *different from vehicle (Student t-test, $p < 0.05$); $N = 5$ /group of rats. Abbreviations: py, pyramidal tract; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Scale in A = 1 mm.

Figure 3. Respiratory effects elicited by blockade of the adenosine receptors in the RTN
 A) Representative recording of (Dia_{EMG}) and abdominal (Abd_{EMG}) electromyography activities after vehicle, ADO (10 mM), 8-PT (100 mM) and 8-PT+ADO bilaterally injected into the RTN. Changes in B) Dia_{EMG} frequency, C) Dia_{EMG} amplitude, D) Abd_{EMG} frequency and E) Abd_{EMG} amplitude elicited by bilateral injection of vehicle, ADO, 8-PT or 8-PT+ADO into the RTN under hypercapnia (10% CO₂) challenge. F) Computer-generated plot of bilateral injections that were confined to the RTN region. *Different from vehicle, ⁺Different from ADO (One-Way ANOVA, Newman-Keuls' test, $p < 0.05$, $N = 6$ /group of rats). Abbreviations: py, pyramid; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Scale bar in F = 1 mm.

Figure 4. Blockade of the adenosine A1 receptors in RTN blocks the inhibitory effect on the hypercapnic ventilatory response in anesthetized vago-denervated rats

A) Representative recording of (Dia_{EMG}) and abdominal (Abd_{EMG}) electromyography activities after vehicle, ADO (10 mM), DPCPX (5 μ M) or DPCPX+ADO bilaterally injected into the RTN under hypercapnic condition. Changes in B) Dia_{EMG} frequency, C) Dia_{EMG} amplitude, D) Abd_{EMG} frequency and E) Abd_{EMG} amplitude elicited by bilateral injection of vehicle, ADO, DPCPX or DPCPX+ADO into the RTN under hypercapnia (10% CO₂) challenge. F) Computer-generated plot of bilateral injections that were confined to the RTN region. *Different from vehicle, +Different from ADO (One-Way ANOVA, Newman-Keuls' test, $p < 0.05$, $N = 6$ /group of rats). Abbreviations: py, pyramidal tract; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Scale bar in F = 1 mm.

Figure 5. Blockade of the adenosine A1 receptors in RTN blocks the inhibitory effect on the hypercapnic ventilatory response in awake rats

A) Recordings showing the effect of bilateral injection of vehicle or DPCPX (5 μ M - 100 nL) into the RTN region under normocapnia and hypercapnia (7% CO_2). Changes in B) tidal volume (V_T , ml/kg), C) respiratory frequency (f_R , bpm) and D) minute volume (V_E , ml/kg/min) elicited by bilateral injection of vehicle or DPCPX injection in the RTN region under normocapnia and hypercapnia. E) Computer-generated plot of bilateral injections that were confined to the RTN region. *Different from normocapnia, +Different from vehicle + CO_2 (One-way ANOVA, Newman-Keuls' test, $p < 0.05$, $N = 4$ /group of rats). Abbreviations: py, pyramidal tract; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Scale bar in E = 1 mm.

Figure 6. Inhibition of the ectonucleotidase in the RTN blocks the inhibitory effect on the hypercapnic ventilatory response in awake rats

A) Recordings showing the effect of bilateral injection of vehicle or ARL67156 (200 μ M -100 nL) into the RTN region under normocapnia and hypercapnia (7% CO₂). Changes in B) tidal volume (V_T, ml/kg), C) respiratory frequency (f_R, bpm) and D) minute ventilation (V_E, ml/kg/min) elicited by bilateral injection of vehicle or ARL67156 injection in the RTN region under normocapnia and hypercapnia. E) Computer-generated plot of bilateral injections that were confined to the RTN region. *Different from saline + vehicle, +Different from saline + CO₂ (One-way ANOVA, Newman-Keuls' test, $p < 0.05$, $N = 6$ /group of rats). Abbreviations: py, pyramidal tract; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Scale bar in E = 1 mm.