Respiratory Physiology & Neurobiology 200 (2014) 97–104

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Endogenous hydrogen sulfide in the rostral ventrolateral medulla/Bötzinger complex downregulates ventilatory responses to hypoxia

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ARTICLE INFO

Article history:
Accepted 11 June 2014
Available online 17 June 2014

Keywords:
Aminooxyacetate
Cystathionine beta-synthase
Hyperpnea
Central nervous system
Sodium sulfide
Ventilation

ABSTRACT

Hydrogen sulfide (H_2S) is now recognized as a new gaseous transmitter involved in several brain-mediated responses. The rostral ventrolateral medulla (RVLM)/Bötzinger complex is a region in the brainstem that is involved in cardiovascular and respiratory functions. Recently, it has been shown that exogenous H_2S in the RVLM modulates autonomic function and thus blood pressure. In the present study, we investigated whether H_2S, endogenously produced in the RVLM/Bötzinger complex, plays a role in the control of hypoxia-induced hyperventilation. Ventilation (V_E) was measured before and after bilateral microinjection of Na_2S (H_2S donor, 0.04, 1 and 2 pmol/100 nl) or aminooxyacetate (AOA, 0.2, 1 and 2 pmol/100 nl, a cystathionine β-synthase, CBS, inhibitor) into the RVLM/Bötzinger complex followed by a 60-min period of hypoxia (7% inspired O_2) or normoxia exposure. Control rats received microinjection of vehicle. Microinjection of vehicle, AOA or Na_2S did not change V_E in normoxic conditions. Exposure to hypoxia evoked a typical increase in V_E. Microinjection of Na_2S (2 pmol) followed by hypoxia exposure attenuated the hyperventilation. Conversely, microinjection of AOA (2 pmol) into the RVLM/Bötzinger complex caused an increase in the hypoxia-induced hyperventilation. Thus, endogenous H_2S in the RVLM/Bötzinger complex seems to play no role in the maintenance of basal pulmonary ventilation during normoxia whereas during hypoxia H_2S has a downmodulatory function. Homogenates of RVLM/Bötzinger complex of animals previously exposed to hypoxia for 60 min exhibited a decreased rate of H_2S production. Our data are consistent with the notion that the gaseous messenger H_2S synthesis is downregulated in the RVLM/Bötzinger complex during hypoxia favoring hyperventilation.

1. Introduction

Hydrogen sulfide (H_2S) has been recognized as an environmental contaminant and as a toxic molecule for many decades. More recently, this gas has been reported to be a cellular mediator that is endogenously produced, and influences physiological functions in mammalian tissues (Abe and Kimura, 1996; Hu et al., 2011; Yang et al., 2008). The H_2S raises from the catabolism of amino acids, such as cysteine and homocysteine predominantly by the pyridoxal-5′-phosphate-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Kabil and Banerjee, 2010; Qu et al., 2008). CBS predominates in the central nervous system (CNS) (Awata et al., 1995; Lee et al., 2009; Mustafa et al., 2009) whereas CSE is mainly expressed in peripheral tissues (Hosoki et al., 1997; Yang et al., 2008).

Inhalation of large quantities of H_2S results in cessation of ventilation, disturbance of oxygen homeostasis, and neurological dysfunctions (Almeida and Guidotti, 1999; Greer et al., 1995). Haouzi et al. (2008) have suggested that H_2S could be important for mediating the well-described effect of hypoxia on ventilatory and metabolic control in small mammals. Moreover, Hu et al. (2008) have documented the presence of CBS in the medullary respiratory center, and that exogenous H_2S affects respiratory activities in a biphasic pattern.

The structural–functional organization of pontine-medullary respiratory network seems to be based on a hierarchical order (Smith et al., 2007) and on functional compartments (Alheid and McCrimmon, 2008; Smith et al., 2009) that are arranged bilaterally in the rostro-caudal direction from the rostral pons to the caudal medulla (for review see Smith et al., 2013). Within this complex

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http://dx.doi.org/10.1016/j.resp.2014.06.007
1569-9048/

structure, the rostral ventrolateral medulla (RVLM)/Bötzinger complex is a group of neurons of the medullary reticular formation, caudal to the facial nucleus and ventral to the compact formation of the ambiguous nucleus. This region has been described to be involved in the compensatory responses to hypoxia (Hirooka et al., 1997; Nitsos and Walker, 1999) and to have important connections to two inspiration-related medullary areas, *i.e.*, the dorsal respiratory group (DRG) and the rostral ventral respiratory group (rVRG), suggesting that RVLM/Bötzinger complex neurons play a key role controlling breathing (Bongianni et al., 1991; Guyenet, 2000).

Considering that the RVLM/Bötzinger complex is an important brain structure that plays a role in the control of ventilation (de Paula and Branco, 2005) and that H_2S affects this structure (Guo et al., 2011), we tested the hypothesis that endogenously produced H_2S in the RVLM/Bötzinger complex plays a modulatory role in the ventilatory responses to hypoxia. To attain this goal, we measured pulmonary ventilation before and after pharmacological modulation of the H_2S-generating enzyme (cystathionine beta-synthase, CBS) in the RVLM/Bötzinger complex or administration of a H_2S donor to rats exposed to hypoxia. Moreover, to verify whether H_2S production is altered in tissue homogenates following hypoxia, we measured H_2S production rate in the RVLM/Bötzinger complex.

2. Materials and methods

2.1. Animals

Adult male Wistar rats were group-housed (four to five animals per cage) and acclimated (25 °C; 12:12-h light-dark cycle) for 1 week before experimental use. The rats had free access to water and food, and were housed in a temperature-controlled chamber at 25 °C (model: ALE 9902001; Alesco Ltda, Monte Mor, SP, Brazil). Experiments were performed on fully conscious, freely moving animals weighing 270–300 g. Animal care was carried out in compliance with the guidelines set by the Brazilian College of Animal Experimentation (COBEA), an affiliate of the international Council for Laboratory Animal Science (ICLAS), which included minimizing the number of animals used and their suffering, and had the approval of the Animal Care and use Committee of the University of São Paulo (n° 064/2012).

2.2. Drugs

Aminooxyacetate (AOA; 0.2, 1 and 2 pmol/100 nl, commonly used as a CBS inhibitor) and sodium sulfide (Na_2S; 0.04, 1 and 2 pmol/100 nl, a H_2S donor) (Kimura, 2010; Whiteman et al., 2011) were purchased from Sigma (S. Louis, MO, USA). These drugs were dissolved in phosphate-buffered saline (PBS; pH 7.0) just prior to microinjection procedure (Na_2S) or up to 1 week before experiment, and stored at −20 °C (AOA). The pH of the drugs was verified and adjusted to 7.4 when necessary.

2.3. Surgical procedures

Surgical procedures were performed under ketamine-xylazine anesthesia (100 and 10 mg/kg; respectively; 1 ml kg^{-1}, i.p.). Deep anesthesia was assessed by the absence of response to noxious stimulation (*e.g.*, paw pinch). Antibiotics (160,000 U/kg benzylpenicilin, 33.3 mg/kg streptomycin, and 33.3 mg/kg dihydrostreptomycin, i.m.; prophylactically) and analgesic medication (Flunexine; 2.5 mg/kg, s.c.) were provided immediately after the end of surgeries. The animals were fixed (prone) on a stereotaxic frame to be implanted with a stainless steel guide cannula (15 mm long, 22 gauge outer diameter) toward the RVLM/Bötzinger complex (for intra-RVLM/Bötzinger complex microinjection), according to the following stereotaxic coordinates (Paxinos and Watson, 2005): 3.2 mm caudal to the lambda; 1.8 mm lateral to the midline; 6.5 mm ventral to the skull surface). The guide cannulas were attached to the bone with stainless steel screws and acrylic cement. Tight-fitting stylets were kept inside the cannulas to prevent occlusion. Afterwards, a median laparotomy was performed so as to insert a temperature datalogger capsule (SubCue, Calgary, AB, Canada) into the peritoneal cavity. All animals were kept under deep anesthesia throughout the surgical procedures, receiving a supplementary dose of anesthetic whenever necessary. Before the experimental procedure, the animals were allowed to recover from surgical interventions for 5 days.

2.4. Microinjection

To perform microinjection within the RVLM/Bötzinger complex we used a microinjection device (model 310, Stoelting, Wood Dale, IL, USA) and a 10-μl syringe (Hamilton, Reno, NV, USA) connected to a microinjection needle (30-gauge outer diameter) with a polyethylene tube (PE-10). Microinjection was performed at a flow rate of 50 nl min^{-1}. The microinjection needle, 3.5 mm longer than the guide cannula, was inserted into the cannula solely at the moment of the microinjection. The animals in which the microinjection of AOA or Na_2S did not reach the RVLM/Bötzinger complex were grouped during the data analysis process, and then were used to compose the group peri-RVLM/Bötzinger complex to demonstrate that the significant effect of the drug is statistically significant if, and only if, the drug reaches RVLM/Bötzinger complex cells.

2.5. Ventilation

Measurements of ventilation (V_E) were performed by means of the body plethysmography method (Bartlett and Tenney, 1970). Each animal was individually placed in the Plexiglas chamber connected to a reference chamber of identical size and construction. The use of the reference chamber makes the system independent of minor pressure interferences. Pressure oscillations caused by animal's ventilation were detected by a differential transducer and amplified (MLT141 Spirometer, Power Lab; ADInstruments, Bella Vista, NSW, Australia). The recordings were analyzed using the software PowerLab (Chart 5; ADInstruments). The volume of calibration was performed during each V_E measurement throughout the course of the experiments by injecting a known air volume (1 ml) inside the chamber. Body temperature was recorded throughout the experiments at 5-min interval with the temperature datalogger capsule (SubCue, Calgary, AB, Canada) inserted into the peritoneal cavity to calculate tidal volume (V_T). V_T was calculated using the following formula (Malan, 1973):

$$V_T = V_K \times \frac{P_T}{P_K} \times \frac{T_A}{T_R} \times \frac{P_B - P_C}{(P_B - P_C) - (T_A/T_b) \times (P_B - P_R)}$$

where P_T is the pressure deflection associated with each V_T; P_K is the pressure deflection associated with injection of the calibration volume (V_K); T_b is deep body temperature; T_A is the air temperature in the animal chamber; P_B is the barometric pressure; P_R is the vapour pressure of water at T_b; P_C is the vapour pressure of water in the animal chamber; and T_R is the room temperature. V_E was calculated as the product of V_T and respiratory frequency (R_f). V_E and V_T are presented at the ambient barometric pressure, at body temperature, and saturated with water vapour at this temperature (BTPS).