Neuroscience 201 (2012) 146–156

HYDROGEN SULFIDE AS A CRYOGENIC MEDIATOR OF HYPOXIA-INDUCED ANAPYREXIA

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Abstract—Hypoxia causes a regulated decrease in body temperature (Tb), a response that has been aptly called anapyrexia, but the mechanisms involved are not completely understood. The roles played by nitric oxide (NO) and other neurotransmitters have been documented during hypoxia-induced anapyrexia, but no information exists with respect to hydrogen sulfide (H_2S), a gaseous molecule endogenously produced by cystathionine β-synthase (CBS). We tested the hypothesis that H_2S production is enhanced during hypoxia and that the gas acts in the anteroventral preoptic region (AVPO; the most important thermosensitive and thermointegrative region of the CNS) modulating hypoxia-induced anapyrexia. Thus, we assessed CBS and nitric oxide synthase (NOS) activities [by means of H_2S and nitrite/nitrate (NO_x) production, respectively] as well as cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) levels in the anteroventral third ventricle region (AV3V; where the AVPO is located) during normoxia and hypoxia. Furthermore, we evaluated the effects of pharmacological modifiers of the H_2S pathway given i.c.v. or intra-AVPO. I.c.v. or intra-AVPO microinjection of CBS inhibitor caused no change in Tb under normoxia but significantly attenuated hypoxia-induced anapyrexia. During hypoxia there were concurrent increases in H_2S production, which could be prevented by CBS inhibitor, indicating the endogenous source of the gas. cAMP concentration, but not cGMP and NO_x, correlated with CBS activity. CBS inhibition increased NOS activity, whereas H_2S donor decreased NO_x production. In conclusion, hypoxia activates H_2S endogenous production through the CBS-H_2S pathway in the AVPO, having a cryogenic effect. Moreover, the present data are consistent with the notion that the two gaseous molecules, H_2S and NO, play a key role in mediating the drop in Tb caused by hypoxia and that a fine-balanced interplay between NOS-NO and CBS-H_2S pathways takes place in the AVPO of rats exposed to hypoxia.

Key words: AVPO, hypothermia, H_2S, aminooxyacetate, CBS, hypothalamus.

Organisms ranging from protozoans to mammals respond to hypoxia by reducing their temperature (Wood, 1991; Malvin and Wood, 1992). Such a regulated drop in body temperature (Tb), that is, anapyrexia, often referred to as hypoxic hypothermia or regulated hypothermia, is thought to be beneficial because it increases the affinity of haemoglobin for oxygen and reduces the oxygen demand of tissues (Wood, 1991). Moreover, hypoxic anapyrexia is also known to be of medical interest during a number of conditions such as stroke and brain trauma avoiding injury during reoxygenation of tissues (Shankaran et al., 2005).

Similar to fever (Blatteis, 2006; Steiner et al., 2006), hypoxia-induced anapyrexia is also thought to be centrally mediated (Steiner and Branco, 2002). Several mediators have been documented to be involved in such a response, including adenosine (Barros and Branco, 2000, 2002; Barros et al., 2006), dopamine (Barros et al., 2004), opioids (Scarpellini Cda et al., 2009), serotonin (Gargaglioni et al., 2005), and the gaseous neuromodulators nitric oxide (NO) (Steiner et al., 2000) and carbon monoxide (CO) (Paro et al., 2002).

Recently, a third gaseous transmitter that regulates neuronal activity has been reported (Kimura et al., 2005). Hydrogen sulfide (H_2S) production is catalyzed by cystathionine β-synthase (CBS) or cystathionine γ-lyase (CSE), cf. (Abe and Kimura, 1996). CBS is the enzyme that predominates in the brain, whereas CSE is mainly found in peripheral tissues (cf. Yang et al., 2008). Moreover, an interaction between H_2S and nitric oxide synthase (NOS) has been reported, even though this interaction is far from fully elucidated and thought to be complex (cf. Wagner et al., 2009). Actually, the importance of NO, CO, and H_2S in the regulation of the stress axis has been recently revised and it has been suggested that these gases are not involved in the tonic regulation of the stress axis, but they may reduce its excessive activation by stressful stimuli (Mancuso et al., 2010).

H_2S acts on biological systems through a number of interconnected mechanisms (Wagner et al., 2009). In the brain, H_2S effects have been attributed to elevations of cyclic adenosine 3′,5′-monophosphate (cAMP) (Kimura, 2000; Dawe et al., 2008; Muzaffar et al., 2008); particularly in the hypothalamus, the gas has been shown to modulate autonomic function in freely moving rats (Dawe et al., 2008). Moreover, H_2S has been shown to inhibit mitochon-

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Abbreviations: AOAA, aminooxyacetate; AVPO, anteroventral preoptic region; AV3V, anteroventral third ventricle region; cAMP, cyclic adenosine 3′,5′-monophosphate; CBS, cystathionine β-synthase; cGMP, cyclic guanosine 3′,5′-monophosphate; CO, carbon monoxide; CSE, cystathionine γ-lyase; H_2S, hydrogen sulphide; i.c.v., intracerebroventricular; MPO, medial preoptic nucleus; NO, nitric oxide; NOS, nitric oxide synthase; NO_x, nitrite/nitrate; SD, standard deviation of the mean; Tb, core body temperature; Tbi, initial Tb; TCA, trichloroacetic acid; 3V, third ventricle.

0306-4522/12 $36.00
doi:10.1016/j.neuroscience.2011.11.030

drial respiration by blocking the cytochrome *c* oxydase, the terminal enzyme complex in the electron transport chain (Beauchamp et al., 1984).

Since hypoxia-induced anapyrexia is at least partially mediated by cAMP accumulation in the anteroventral preoptic region (AVPO)—hypothalamic region that has been reported to be involved in this thermoregulatory response (Steiner et al., 2002b; Barros et al., 2004, 2006; Gargaglioni et al., 2005; Scarpellini Cda et al., 2009) and appears to be the most sensitive preoptic site to thermoregulatory effects of cAMP (Steiner et al., 2002b), and anapyrexia is also observed after inhibition of oxidative phosphorylation in the central nervous system (Branco and Malvin, 1996; Nystul and Roth, 2004), we hypothesized that AVPO H_2S mediates hypoxia-induced anapyrexia in mammals and that this mechanism is at least partially dependent on cAMP. To test this hypothesis, we evaluated the effects of microinjections of pharmacological modifiers of the CBS-H_2S pathway given intracerebroventricular (i.c.v.) or into the AVPO on hypoxia-induced anapyrexia, and also measured the levels of H_2S, nitrite/nitrate (NO_x), cAMP, and cyclic guanosine 3′,5′-monophosphate (cGMP) in the anteroventral third ventricle region (AV3V; where the AVPO is located).

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats, obtained from institutional vivarium sources, 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), with a 12:12 h light:dark cycle (lights on at 6:00 AM). Experiments were performed on fully conscious, freely moving animals weighing 250–300 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 (Protocol # 020/2010).

Drugs

Aminooxyacetate (AOAA; inhibitor of the enzyme CBS (Kimura, 2010)), and sodium sulfide (Na_2S; H_2S donor (Wagner et al., 2009)) were purchased from Sigma (St. Louis, MO, USA). The drugs were freshly dissolved in pyrogen-free saline on the day of the experiment.

Surgeries

Surgical procedures were performed under ketamine-xylazine anesthesia (100 and 10 mg/kg, respectively; 1 ml/kg, i.p.). 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 (prostrate) on a stereotaxic frame to be implanted with a stainless steel guide cannula (15-mm long, 22-gauge outer diameter) in the third ventricle [3V; for i.c.v. microinjection] or (17 mm long, 22-gauge outer diameter) toward the AVPO (for intra-AVPO microinjection), according to the following stereotaxic coordinates (Paxinos and Watson, 2005). For the 3V: anteroposterior: −0.4 mm, lateral: 0.0 mm, and dorsoventral: −7.5 mm from bregma; for the AVPO: anteroposterior: +6.0 mm, lateral: −0.3 mm, dorsoventral: −7.5 mm from bregma. The guide cannula was fixed to the skull with stainless steel screws and acrylic cement. A tightly fitting stylet was inserted into the guide cannula to maintain patency and prevent infection. Afterward, 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 procedures, the animals were allowed to recover from the surgical interventions for 5 days.

Microinjection

To perform i.c.v. or intra-AVPO microinjection, 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. The microinjection needle, 0.1 (i.c.v.) or 2.0 (AVPO) mm longer than the guide cannula, was inserted into the guide cannula solely at the moment of the microinjection.

Histology

To evaluate the site specificity of effects of the drugs, at the end of the experiments aiming at performing microinjections into the AVPO, the animals were deeply anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively; i.p.) and transcardially perfused with phosphate-buffered saline followed by 10% buffered formalin solution. The brains were excised, sectioned (30 μm), and stained using the Cresyl-Nissl method.

Hypoxia

Rats were housed in acrylic, plethysmographic chambers (5 L) continuously ventilated with humidified room air. After the animals habituated to the experimental conditions (30 min), a humidified hypoxic gas mixture containing 7% O_2 (AGA, Brazil) was ventilated through the chamber for 60 min.

Tb measurements

Tb was recorded throughout the experiments at 5-min intervals with a temperature datalogger capsule (SubCue, Calgary, AB, Canada) inserted into the peritoneal cavity. Tb recorded during 40 min before any manipulation was used to calculate the initial Tb (Tbi).

AV3V dissection

To measure the AV3V levels of H_2S, NO_x, cAMP, and cGMP immediately after exposure to normoxia or hypoxia for 60 min, the animals were decapitated, the brains excised, and the AV3V region was dissected under a stereomicroscope based on the anatomical landmarks previously described (Knuepfer et al., 1984; Johnson, 1985), frozen under liquid nitrogen, and stored at −70 °C until assay. This procedure has been used in our previous studies (Steiner et al., 2002a,c; Soriano and Branco, 2010).

Assessment of H_2S concentration in the AV3V. H_2S levels were measured as previously described (Stipanuk and Beck, 1982; Singh et al., 2009; Francescato et al., 2011). AV3V samples were homogenized in potassium phosphate buffer (100 mM; pH 7.4) using a microprocessor (VirTis, Gardiner, NY, USA). Each sample (50% w/v; 100 μl) contained L-cysteine (10 mM; 20 μl), pyridoxal 5′-phosphate (2 mM; 20 μl), and PBS (30 μl). The reaction was performed in parafilmed eppendorf tubes and initiated by transferring the tubes from ice to bath at 37 °C. After incubation for 2 h, zinc acetate (1% w/v; 100 μl) was added to trap evolved H_2S followed by tricloroacetic acid (10% w/v; 100 μl) to