Endogenous preoptic hydrogen sulphide attenuates hypoxia-induced hyperventilation


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Abstract

Aim: We hypothesized that hydrogen sulphide (H₂S), acting specifically in the anteroventral preoptic region (AVPO – an important integrating site of thermal and cardiorespiratory responses to hypoxia in which H₂S synthesis has been shown to be increased under hypoxic conditions), modulates the hypoxic ventilatory response.

Methods: To test this hypothesis, we measured pulmonary ventilation (\( V_E \)) and deep body temperature of rats before and after intracerebroventricular (icv) or intra-AVPO microinjection of aminooxyacetate (AOA; CBS inhibitor) or Na₂S (H₂S donor) followed by 60 min of hypoxia exposure (7% O₂). Furthermore, we assessed the AVPO levels of H₂S of rats exposed to hypoxia. Control rats were kept under normoxia.

Results: Microinjection of vehicle, AOA or Na₂S did not change \( V_E \) under normoxic conditions. Hypoxia caused an increase in ventilation, which was potentiated by microinjection of AOA because of a further augmented tidal volume. Conversely, treatment with Na₂S significantly attenuated this response. The in vivo H₂S data indicated that during hypoxia the lower the deep body temperature the smaller the degree of hyperventilation. Under hypoxia, H₂S production was found to be increased in the AVPO, indicating that its production is responsive to hypoxia. The CBS inhibitor attenuated the hypoxia-induced increase in the H₂S synthesis, suggesting an endogenous synthesis of the gas.

Conclusion: These data provide solid evidence that AVPO H₂S production is stimulated by hypoxia, and this gaseous messenger exerts an inhibitory modulation of the hypoxic ventilatory response. It is probable that the H₂S modulation of hypoxia-induced hyperventilation is at least in part in proportion to metabolism.

Keywords aminooxyacetate, cystathionine beta-synthase, hyperpnea, hypothalamus, sodium sulphide, ventilation.

Hydrogen sulphide (H₂S) is a gaseous messenger present in many mammalian tissues and produced from cysteine by pyridoxal 5′-phosphate-dependent enzymes, namely, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). H₂S can also be synthesized from a two-step reaction sequentially catalysed by cysteine aminotransferase (CAT) and 3-mercaptopropionate sulfurtransferase (3-MST). CBS, CSE and CAT are considered cytosolic enzymes, whereas 3-MST has been found in both the cytosol
and mitochondrial matrix, intracellular sites where the enzyme has been shown to be involved in $H_2S$ production (Olson 2013).

Cystathionine $\beta$-synthase is the predominant isoform in the brain, including the preoptic hypotalamus (Kwiatkoski et al. 2012, 2013), whereas CSE is mainly found in peripheral tissues (Yang et al. 2008, Kimura 2013). $H_2S$ has been demonstrated to play an important role in physiological and pathophysiological mechanisms, acting as a smooth muscle relaxant (Dominy & Stipanuk 2004), and being protective in ischaemia (Geng et al. 2004) and hypoxic pulmonary hypertension models (Wei et al. 2008; for a review, see Kimura 2013). $H_2S$ can also act as a modulator of synaptic activity in the brain (Abe & Kimura 1996), and increase the survival rate of mice exposed to lethal hypoxia (Blackstone & Roth 2007).

$H_2S$ inhalation has been shown to modulate metabolic rate in small mammals (Haouzi et al. 2008), probably by depressing the activity of hypothalamic structures, which would be similar to the putative mechanism triggered by hypoxia. Hypoxia is classically known to cause an immediate increase in pulmonary ventilation ($V_E$). It is well established that carotid bodies are the main structures for detecting arterial PO$_2$ levels. However, the full manifestation of the ventilatory responses to hypoxia depends on both peripheral chemoreception (cf. Braga et al. 2007, Prabhakar 2013) and complex interactions at multiple levels among the brainstem and supraponsine structures.

The presence and participation of $H_2S$ in response to hypoxia have been described peripherally in the carotid body (Peng et al. 2010, Prabhakar 2013) but not yet in the central nervous system (CNS). Several studies have suggested that the hypothalamus, besides being involved in thermoregulation (cf. Almeida et al. 2012), modulates the ventilatory responses to hypoxia (Horn & Waldrop 1994, Horn et al. 1999, Dreshaj et al. 2003, Barros et al. 2006, Gargaglioni et al. 2006), thereby receiving the label for a key site of integration of hypoxic and thermal stimuli to breathing (Maskrey & Hinrichsen 1994, Barros et al. 2006, Gargaglioni et al. 2006). Studies have demonstrated that hypoxia induces transcription-regulating protein Fos in neurons of various hypothalamic nuclei (Horn & Waldrop 1994, Berquin et al. 2000), suggesting that these nuclei may be involved in the neuronal network that orchestrates regulatory and counter-regulatory mechanisms to properly respond to hypoxia challenges. In this context, it has been shown that the production of $H_2S$ is augmented in the preoptic hypothalamus of rats exposed to hypoxia; and besides, in this hypothalamic area $H_2S$ modulates the hypoxia-induced thermoregulatory changes (Kwiatkoski et al. 2012).

Considering that the hypothalamus is an important brain structure that plays a role in the control of ventilation (Maskrey & Hinrichsen 1994, Ni et al. 1996, Hinrichsen et al. 1998, Boden et al. 2000a,b) during hypoxia (Horn & Waldrop 1994, Horn et al. 1999, Dreshaj et al. 2003, Barros et al. 2006, Gargaglioni et al. 2006), and that hypoxia elicits an increase in the $H_2S$ synthesis in the preoptic hypothalamus (Kwiatkoski et al. 2012), it seems plausible to hypothesize that preoptic $H_2S$ modulates the ventilatory response to hypoxia, especially in a preoptic region involved in the respiratory response to hypoxia, the anteroventral preoptic region (AVPO; Barros et al. 2006, Gargaglioni et al. 2006). Motivated by the evidence that $H_2S$ production does exist in the AVPO (Kwiatkoski et al. 2013), we tested the hypothesis that pharmacological modulation of the enzymatic production of $H_2S$ in the AVPO affects the hypoxic ventilatory response. To attain this goal, we measured pulmonary ventilation before and after microinjecting aminooxyacetate (AOA; an inhibitor of the enzyme CBS) or Na$_2$S (a $H_2S$ donor) within the AVPO of rats exposed to hypoxia. Moreover, we measured the AVPO $H_2S$ production in rats exposed to hypoxia and microinjected with AOA.

**Materials and methods**

**Animals**

Experiments were performed on adult male Wistar rats weighing 260–300 g. Animals had free access to water and food and were housed in a temperature-controlled chamber at 24°C (model: ALE 9902001; Alesco Ltda., Monte Mor, SP, Brazil), with a 12 h : 12 h light : dark cycle (lights on at 06:00 AM). This study was conducted in compliance with the Institutional Ethics Committee on Animal Experimentation of the Medical School of Ribeirão Preto, University of São Paulo (protocol number: 020/2010), which complies with the current laws of our country, and conforms to several international conventions.

**Drugs**

Aminooxyacetate (inhibitor of the enzyme CBS) and sodium sulphide (Na$_2$S; $H_2S$ donor) were purchased from Sigma (St. Louis, MO, USA). Drugs were dissolved in pyrogen-free saline just prior to microinjection procedure (Na$_2$S) 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.
**Surgeries**

Surgical procedures were performed under ketamine-xylazine anaesthesia (100 and 10 mg kg\(^{-1}\), respectively; 1 mL kg\(^{-1}\), intraperitoneal, ip). Antibiotic solution (160 000 U kg\(^{-1}\) benzylpenicillin, 33.3 mg kg\(^{-1}\) streptomycin and 33.3 mg kg\(^{-1}\) dihydrostreptomycin; 1 mL kg\(^{-1}\), intramuscular) and analgesic (Flunixin; 2.5 mg kg\(^{-1}\), 1 mL kg\(^{-1}\), subcutaneous) were provided after surgery. Animals were fixed on a stereotaxic frame to be implanted with a stainless steel guide cannula (16-mm long, 22-gauge outer diameter) towards the third ventricle (3V; for the icv microinjection) or the AVPO (for the intra-AVPO microinjection), according to the following stereotaxic coordinates (Paxinos & Watson 2005): for the 3V: 0.5 mm posterior to the bregma, 0.0 mm to the midline and 6.0 mm ventral to the skull surface; for the AVPO: 0.0 mm to the bregma, 0.6 mm lateral to the midline and 6.0 mm ventral to the skull surface. Accuracy of the cannula placement was checked with a flow of saline into the 3V by hydrostatic pressure, and, after the end of experiments, with microinjection of methylene blue into the 3V post-mortem. The positioning of the guide cannula within the AVPO was verified by histological analysis. 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. To properly calculate \(V_T\), deep body temperature had to be recorded (see below Data processing and analysis); this was achieved by inserting temperature-measuring devices (data loggers; SubCue, Calgary, AB, Canada) into the peritoneal cavity of the rats. The rats were kept under deep anaesthesia throughout the surgical procedures, receiving a supplementary dose of anaesthetic if needed. Prior to the experimental procedures, the animals were allowed to recover from the surgical interventions (1 week).

**Microinjection**

To perform the icv or intra-AVPO microinjection, a microinjection pump (model 310; Stoelting, Wood Dale, IL, USA) was used, and a 10-µL syringe (Hamilton, Reno, NV, USA) was 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, 0.1 (icv) or 2.0 (AVPO) mm longer than the guide cannula, thereby protruding beyond the end of the guide cannula, was inserted into the guide cannula 1 h before the moment of the microinjection, so that the AVPO was reached only by the microinjection needle and 60 min before the microinjection procedure. The animals in which the microinjection of AOA or Na\(_2\)S did not reach the AVPO were grouped during the data analysis process, and then were used to compose the group peri-AVPO to demonstrate that the significant effect of the drug is statistically significant if, and only if, the drug reaches AVPO cells. The icv microinjection was performed into the 3V to potentiate drug delivery to hypothalamic structures, a procedure in line with previous observations (Salter et al. 1995, Soriano et al. 2012).

**Pulmonary ventilation**

Measurements of ventilation (\(V_E\)) were made by the whole body plethysmograph method (Bartlett & Tenney 1970). Unanesthetized rats were placed into a 3.9-L Plexiglas chamber at 25 °C and allowed to move freely, while the chamber was flushed with humidified air. During each measurement of \(V_E\), the airflow was interrupted for a short time (approx. 1 min), and the chamber remained closed. The pressure oscillations caused by respiration were detected by a differential transducer and amplified (MLT141 spirometer, Power Lab; AdInstruments, Bella Vista, NSW, Australia). The recordings were analysed using the PowerLab software (Chart 5; AdInstruments). The volume 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.

**Measurement of \(H_2S\) concentration in the AVPO**

\(H_2S\) levels were determined as previously described (Singh et al. 2009, Francescato et al. 2011, Kwiatkowski et al. 2012, 2013). The AVPO was sampled in a cryostat by a punch needle (0.9 mm inner diameter) from a 500-µm slice of the preoptic hypothalamus, based on the following landmarks: ventral, optic chiasm; dorsal, anterior commissure; median, the 3V. Bilateral punches of the AVPO were taken just above the dorsal boundary of the optic chiasm and at the left and right lateral wall of the 3V. AVPO samples were homogenized in potassium phosphate buffer (100 mM; pH 7.4) using a digital 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 plastic 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 trichloroacetic acid (10% w/v; 100 µL) to precipitate proteins and stop the reaction. After centrifugation, N,N-dimethyl-p-phenylenediamine sulphate (20 mM; 50 µL) in HCl 7.2 M followed by FeCl₃...
(30 mM; 50 µL) in HCl 1.2 M was then added to 50 µL of the supernatant, and optical density was measured at 670 nm. The calibration curve of absorbance was obtained using Na2S solutions (0.1–100 µg mL$^{-1}$).

**Experimental protocols**

Throughout the experiments, including the preceding overnight period, animals were placed in the experiment room at 25 °C. Doses of AOA and Na2S were tested in pilot experiments and based on the literature and our previous studies (Kwiatkoski et al. 2012, 2013). Na2S was given at a very low dose, far from the toxic range. Each animal was used once only and received only one microinjection of AOA, Na2S or vehicle (saline). In all experimental protocols, each animal was individually placed in a Plexiglass chamber at 25 °C and allowed to move freely while the chamber was flushed with humidified air. After acclimatization for 40 min, basal $V_E$ (control) measurements were taken over a 30-min period. Subsequently, a hypoxic gas mixture (7% O2, N2 balance) was flushed into the chamber for 60 min. Control groups were exposed to a normoxic gas mixture (21% O2, N2 balance). $V_E$ was measured over 60 min after the beginning of normoxia/hypoxia exposure. All gas conditions were administered by a flow meter gas-mixing pump (Cameron Instruments GF-3/MP; Guelph, ON, Canada). O2 gas analyser (Raytech quadralyser 224A; Middletown, CT, USA) was used to monitor gas composition inside the chamber in all experimental protocols.

**Experiment 1: Effect of icv microinjection of AOA on $V_E$ during normoxia or hypoxia.** Before investigating the involvement of H2S specifically in the AVPO, we first tested the putative effect of the central inhibition of CBS with AOA on $V_E$ under normoxia or hypoxia conditions. After the acclimatization (40 min), basal $V_E$ was measured over 30 min. Subsequently, rats received icv microinjection of AOA (200 pmol 2 µL$^{-1}$) or vehicle (saline, 2 µL) and were exposed to normoxia or hypoxia for 60 min. $V_E$ was measured at 5, 10, 20, 30, 40, 50 and 60 min after the icv microinjection and normoxia/hypoxia exposure.

**Experiment 2: Effect of icv microinjection of Na2S on $V_E$ during normoxia or hypoxia.** To investigate the putative effect of increased availability of H2S in the CNS, unanesthetized, freely moving rats were microinjected within the 3V with a H2S donor (Na2S) and exposed to normoxia or hypoxia. After the acclimatization (40 min), the baseline of $V_E$ was measured over 30 min. Subsequently, the rats received icv microinjection of Na2S (260 nmol 2 µL$^{-1}$) or vehicle (saline, 2 µL) and were exposed to 21% O2 for 60 min (normoxia) or to a hypoxic gas mixture (7% inspired O2) flushed into the chamber for 60 min (hypoxia). $V_E$ was measured at 5, 10, 20, 30, 40, 50 and 60 min after the microinjection and normoxia/hypoxia exposure.

**Experiment 3: Effect of intra-AVPO microinjection of AOA on $V_E$ during normoxia or hypoxia.** After having collected data on the effect of pharmacological modulation of H2S production in the CNS, we then asked whether the effect of AOA microinjected specifically into the AVPO on pulmonary ventilation is potentiated or even different from that of the icv treatment. Unanesthetized, freely moving rats were microinjected into the AVPO with AOA (20 fmol 100 nL$^{-1}$) or vehicle (saline, 100 nL) and exposed to normoxia or hypoxia. Baseline of $V_E$ was measured over 30 min, after the acclimatization (40 min), and subsequently the rats received intra-AVPO microinjection of AOA or saline. $V_E$ was measured at 5, 10, 20, 30, 40, 50 and 60 min after the microinjection and exposure to normoxia or hypoxia.

**Experiment 4: Effect of intra-AVPO microinjection of Na2S on $V_E$ during normoxia or hypoxia.** We then investigated the putative effect of the increased availability of H2S specifically in the AVPO. After acclimatization (40 min), the baseline of $V_E$ was measured over 30 min, and subsequently the rats received intra-AVPO microinjection of Na2S (13 fmol 100 nL$^{-1}$) or vehicle saline (100 nL). $V_E$ measurements were taken at 5, 10, 20, 30, 40, 50 and 60 min after the rats were microinjected and exposed to normoxia or hypoxia.

**Experiment 5: Effect of icv microinjection of AOA on H2S levels in the AVPO of rats exposed to normoxia or hypoxia.** Having performed the in vivo approaches, our next experiment was to measure the endogenous production of H2S in the AVPO of animals exposed to normoxia or hypoxia. We then assessed the levels of H2S in the AVPO of rats that were acclimatized (40 min), icv microinjected with AOA (200 pmol 2 µL$^{-1}$) or vehicle (saline, 2 µL), and exposed to normoxia or hypoxia for 60 min. Immediately after the microinjection and exposure, the animals were decapitated and the brains processed as described above.

**Data processing and analysis**

Values are expressed as means ± SEM. Tidal volume ($V_T$) was calculated using the following formula (Malan 1973):

Data processing and analysis
Effect \( V_T \) and \( \dot{V}_E \) of icv microinjection of AOA

First, we investigated the putative central effect of AOA on \( \dot{V}_E \) during normoxia. Intracerebroventricular microinjection of AOA (200 pmol) or vehicle (saline 2 \( \mu l \)) did not significantly affect \( (P > 0.05) \) either \( \dot{f}R \) or \( V_T \), and consequently \( \dot{V}_E \) of rats under normoxia was not altered (Fig. 1a–c). Exposure to 7% O2 evoked a typical hypoxia-induced hyperpnea in both groups (treated and control; Fig. 2a–c). The icv microinjection of AOA (200 pmol) caused a significant increase in the hypoxic ventilatory response compared to the control group (AOA + hypoxia: 2046.10 ± 144.8 vs. saline + hypoxia: 1515.27 ± 84.6 mL kg\(^{-1}\) min\(^{-1}\); \( P < 0.05 \); Fig. 2c). This stimulatory effect of AOA on \( \dot{V}_E \) started 10 min after the beginning of hypoxia exposure and lasted 50 min (\( P < 0.05 \)). Such an effect on the ventilatory response was because of an increased \( V_T \) (AOA + hypoxia: 15.05 ± 1.04 vs. saline + hypoxia: 11.56 ± 0.75 mL kg\(^{-1}\); \( P < 0.05 \); Fig. 2b) as no significant difference (\( P > 0.05 \)) was observed in \( \dot{f}R \) (Fig. 2a).

**Effect of icv microinjection of Na2S on \( V_T, \dot{f}R \) and \( \dot{V}_E \) during normoxia or hypoxia**

Intracerebroventricular microinjection of a very low dose of Na2S (260 nmol 2 \( \mu l \)) did not change either \( \dot{f}R \) or \( V_T \), and consequently \( \dot{V}_E \) of rats under normoxia was not altered either (Fig. 3a–c). If the conclusion that the icv effect of AOA on \( \dot{V}_E \) during hypoxia depends on the reduction of the central levels of H2S is correct, the icv administration of the H2S donor would need to evoke opposite effects to those of AOA. To test this hypothesis, we microinjected Na2S into the 3V and evaluated its putative effects on \( \dot{f}R \), \( V_T \) and \( \dot{V}_E \) over 60 min of hypoxia exposure. As expected, a typical hypoxia-induced hyperpnea was observed; confirming our hypothesis, the icv microinjection of Na2S (260 nmol) significantly attenuated (\( P < 0.05 \)) this increase in pulmonary ventilation (saline + hypoxia: 1680.74 ± 115.1 vs. Na2S + hypoxia: 1229.34 ± 116.5 mL kg\(^{-1}\) min\(^{-1}\); \( P < 0.05 \); Fig. 4c). Such an attenuating effect of the H2S donor started 10 min after exposure and was maintained throughout the experiment (Fig. 4c). The increases elicited by hypoxia in both \( \dot{f}R \) (saline + hypoxia: 143.42 ± 7.1 vs. Na2S + hypoxia: 121.71 ± 7.6 cycles min\(^{-1}\); \( P < 0.05 \); Fig. 4a) and \( V_T \) (saline + hypoxia: 12.05 ± 0.9 vs. Na2S + hypoxia: 9.91 ± 0.7 mL kg\(^{-1}\); \( P < 0.05 \); Fig. 4b) were significantly attenuated by Na2S, thus accounting for the attenuated \( \dot{V}_E \) (Fig. 4c).
The putative effect of the inhibition of the H$_2$S production on $\dot{V}_E$ with the objective of directing our findings towards a specific set of cells in the preoptic hypothalamus. Similar to the effect of the icv microinjection, the intra-AVPO microinjection of AOA (20 fmol 100 nL$^{-1}$) or vehicle (saline, 100 nL) in rats exposed to normoxia evoked no significant changes ($P > 0.05$) in any of the parameters measured, fR, $V_T$ and $\dot{V}_E$ (Fig. 1d–f). We then asked whether the effect of AOA is similar to (or even more pronounced than) that of the icv treatment when the drug is given specifically into the AVPO. Hypoxia exposure induced a significant augmentation of $\dot{V}_E$ in all groups (Fig. 2d–f). Importantly, intra-AVPO microinjection of AOA (20 fmol) caused a significant increase ($P < 0.05$) in the hypoxic ventilatory response when compared to the respective control groups (group saline + hypoxia: 1472.13 ± 120.8 and group AOA peri-AVPO + hypoxia: 1539.63 ± 118.1 vs. AOA intra-AVPO + hypoxia: 2087.54 ± 159.4 mL kg$^{-1}$ min$^{-1}$; $P < 0.05$.

**Figure 1** Effect of icv or intra-AVPO microinjection of aminooxyacetate (AOA) on respiratory frequency (fR), tidal volume ($V_T$) and pulmonary ventilation ($\dot{V}_E$) of rats under normoxia. Panels show the time courses of fR, $V_T$ and $\dot{V}_E$ before and after the icv (a–c) or intra-AVPO (d–f) microinjection of AOA or vehicle. Arrow indicates the moment of the microinjection. Values are expressed as mean ± SE. Numbers of animals in each group are shown in parenthesis. AVPO, anteroventral preoptic region.
Fig. 2f). The effect of AOA (20 fmol 100 nL⁻¹) on $V_E$ was observed after 5 min of hypoxia exposure and was maintained elevated until the end of the experiment. Such an effect on the ventilatory response was because of an increased $V_T$ (group saline + hypoxia: 11.84 ± 0.7 and group AOA peri-AVPO + hypoxia: 12.16 ± 0.6 vs. AOA intra-AVPO + hypoxia: 15.91 ± 0.8 mL kg⁻¹; $P < 0.05$; Fig. 2e), as there was no significant difference in fR ($P > 0.05$; Fig. 2d).
Effect of intra-AVPO microinjection of Na$_2$S on VT, fR and $V_E$ during normoxia or hypoxia

As for the icv treatment, the putative effect of the H$_2$S donor on $V_E$ was also examined locally, specifically in the AVPO. Intra-AVPO microinjection of Na$_2$S had no effect ($P > 0.05$) on either fR or VT, and consequently $V_E$ of rats under normoxia was not altered (Fig. 3d–f). Having observed until this point of this study that the icv effect of AOA is similar to that of intra-AVPO, and that Na$_2$S given icv evokes effects opposite to those of AOA, we then asked whether the effect of the increased availability of H$_2$S in the AVPO is similar to that of the icv treatment. Typically, hypoxia induced an augmentation of $V_E$ in all groups (Fig. 4d–f). The intra-AVPO microinjection of Na$_2$S (13 fmol) caused a significant attenuation of $V_E$ ($P < 0.05$; Fig. 4f). The attenuating effect of Na$_2$S was significant and only observed when
this donor was given specifically into the AVPO, but not into the boundaries of this preoptic region (saline + hypoxia: 1645.00 ± 100.4 and Na2S peri-AVPO + hypoxia: 1564.74 ± 95.4 vs. Na2S intra-AVPO + hypoxia: 1191.42 ± 110.7 mL kg⁻¹ min⁻¹; P < 0.05; Fig. 4f). Such an effect of Na2S on VE was observed at the beginning of the hypoxic exposure and maintained throughout the experiment, and could be attributable to both a decreased fR (saline + hypoxia: 143.27 ± 4.9 and Na2S peri-AVPO + hypoxia: 137.79 ± 5.5 vs. Na2S intra-AVPO + hypoxia: 121.20 ± 5.4 cycles min⁻¹; P < 0.05; Fig. 4d) and an attenuated VT (saline + hypoxia: 12.09 ± 0.7 and Na2S peri-AVPO + hypoxia: 11.87 ± 0.6 vs. Na2S intra-AVPO + hypoxia: 10.00 ± 0.7 mL kg⁻¹; P < 0.05; Fig. 4e). After drug injection (Na2S), it was observed

Figure 4 Effect of icv or intra-AVPO microinjection of Na2S on respiratory frequency (fR), tidal volume (VT) and pulmonary ventilation (VE) of rats exposed to hypoxia (7% O2). Panels show the time courses of fR, VT and VE before and after the icv (a–c) or intra-AVPO (d–f) microinjection of aminooxyacetate (AOA) or vehicle. Arrow indicates the moment of the microinjection. Values are expressed as mean ± SE. Numbers of animals in each group are shown in parenthesis. *P < 0.05, vs. the respective control group(s). AVPO, anteroventral preoptic region.
statistical difference between groups at 5 min in intra-AVPO microinjected rats and at 10 min in icv treated animals. This is a short-period difference which may be because of drug diffusion from the CSF to tissues. Long-lasting effects on respiration were obtained when we microinjected the CBS inhibitor icv or intra-AVPO in hypoxic rats. In spite of the fact that H\(_2\)S has a transient existence in tissue, in both routes of administration, that is, icv and intra-AVPO, the H\(_2\)S donor also evoked long-lasting effects on the respiratory parameters in hypoxic rats.

**Effect of icv microinjection of AOA on the H\(_2\)S production in the AVPO following exposure to normoxia or hypoxia**

To verify whether our *in vivo* approaches agree with the hypothesis that hypoxia exposure affects the AVPO levels of H\(_2\)S and, further, that the microinjection of AOA is really capable of inhibiting the H\(_2\)S production in this region of the preoptic hypothalamus, we assessed the activity of AVPO production of H\(_2\)S in this preoptic region of rats that had been icv microinjected with AOA (200 pmol) or vehicle (saline, 2 \(\mu\)L) and exposed to normoxia or hypoxia for 60 min (Fig. 5). The basal levels of H\(_2\)S in the AVPO were 0.61 ± 0.05 \(\mu\)g of H\(_2\)S mg of protein\(^{-1}\) h\(^{-1}\). Intracerebroventricular administration of AOA to rats exposed to normoxia did not significantly change the AVPO levels of H\(_2\)S (0.64 ± 0.05 \(\mu\)g of H\(_2\)S mg of protein\(^{-1}\) h\(^{-1}\); \(P > 0.05\)). In agreement with our hypothesis, exposure to hypoxia caused a 64\% increase in H\(_2\)S production in the AVPO (1.00 ± 0.07 \(\mu\)g of H\(_2\)S mg of protein\(^{-1}\) h\(^{-1}\); \(P < 0.05\), when compared to basal levels, that is, the control group saline + normoxia). When AOA was icv administered to rats exposed to hypoxia, this hypoxia-induced increased production of H\(_2\)S was significantly attenuated (0.85 ± 0.03 \(\mu\)g of H\(_2\)S mg of protein\(^{-1}\) h\(^{-1}\); \(P < 0.05\), when compared to the group saline + hypoxia: 1.00 ± 0.07 \(\mu\)g of H\(_2\)S mg of protein\(^{-1}\) h\(^{-1}\); Fig. 5), proving that the treatment with AOA is indeed capable of reducing the production of H\(_2\)S in the AVPO. Cysteine, a CBS substrate, was used in this assay to allow the ‘signal’ to be amplified. Since all samples were treated equally, that is, with 10 \(\text{mM}\) cysteine, the H\(_2\)S production during hypoxia relative to the control condition was relatively more pronounced. Independently of the absolute values, compared to normoxia, the hypoxia group produced more H\(_2\)S, suggesting that the enzyme activity during hypoxia exposure was increased when compared to the respective control (normoxia) group.

**Effect of AOA or Na\(_2\)S on deep body temperature of rats exposed to normoxia or hypoxia**

As previously described by our group (Kwiatkoski et al. 2012) regarding the effects of AOA and Na\(_2\)S on deep body temperature, we found these drugs caused no effect during normoxia (data not shown). Intra-AVPO microinjection of AOA (20 fmol) attenuated (\(P < 0.05\)) hypoxia-induced drop in deep body temperature, known as anapyrexia (Kwiatkoski et al. 2012), compared to the control group (at 60 min of hypoxia exposure, control: 33.9 ± 0.31 °C vs. AOA: 35.7 ± 0.45 °C; panel a, Fig. 6). As for Na\(_2\)S (panel b, Fig. 6), the opposite effect was observed, that is, Na\(_2\)S microinjection within the AVPO exacerbated (\(P < 0.05\)) anapyrexia induced by hypoxia exposure when compared to the control group (at 60 min of hypoxia exposure, control: 34.15 ± 0.50 °C vs. Na\(_2\)S: 33.2 ± 0.63 °C). Icv microinjection of these drugs into rats exposed to normoxia or hypoxia (not shown) elicited similar effects to those seen in the groups microinjected within the AVPO.

**Relationships between deep body temperature and pulmonary ventilation (\(V_E\)) during hypoxia exposure**

Figure 7 shows the relationships between deep body temperature and \(V_E\) during hypoxia exposure in rats microinjected with AOA (panel a) or Na\(_2\)S (panel b) within the AVPO. AOA-treated rats had higher (\(P < 0.05\)) ventilatory response to hypoxia and lower (\(P < 0.05\)) deep body temperature compared to control. On the other hand, Na\(_2\)S-treated rats exhibited...
lower ($P < 0.05$) ventilation and a more pronounced fall ($P < 0.05$) in deep body temperature compared to control. The in vivo H$_2$S data indicated that during hypoxia the lower the deep body temperature the smaller the degree of hyperventilation (Fig. 7, panels a,b).

**Discussion**

In this study, we provide solid evidence that endogenously produced H$_2$S plays in the AVPO an inhibitory role in the ventilatory response to hypoxia. This finding is supported by the fact that microinjection of AOA, an inhibitor of CBS, within the AVPO promotes a substantial increase in pulmonary ventilation during hypoxia exposure. The inhibitory role of the gas was confirmed by the administration of a H$_2$S donor (Na$_2$S), which caused an opposite effect to that of AOA, that is, attenuates the hypoxia-induced hyperventilation. Moreover, since the levels of H$_2$S under hypoxic conditions were found to be increased in the AVPO, and prevented by the CBS inhibitor, our data directly demonstrate both the effectiveness of the treatment with the inhibitor and the existence of an AVPO H$_2$S endogenous source highly responsive to hypoxia. Our major interest was to assess the role of
endogenously produced H₂S (hence its physiological role). Therefore, to attain this goal, we tested the effect of a CBS inhibitor and measured the levels of H₂S in the AVPO. Na₂S was applied to verify whether increased availability of H₂S would produce an opposite response when compared to that of the enzyme inhibitor.

A previous study by Greer et al. (1995) reported the effects of exogenous H₂S on the respiratory brainstem network using in vitro preparations. They described a reduced or increased respiratory burst frequency depending on the preparation used, that is, medullary slice or brainstem-spinal cord. It is difficult to reconcile their data with our findings because in the present study we have performed experiments in conscious rats to address the role of endogenous H₂S in the AVPO during hypoxia exposure. We found that endogenous H₂S attenuates the ventilatory response to hypoxia. In vivo vs. in vitro differences, such as developmental period (neonatal vs. adult rats), bath temperature vs. body temperature, respiratory network (reduced vs. intact), and K⁺ concentration (Funk & Greer 2013) may account for distinct outcomes.

Studies have suggested that hypothalamic neurons project directly to respiratory centres in the brainstem, such as the Pre-Bötzinger complex, hypoglossal and phrenic nuclei, as well as the nucleus of the solitary tract, medullary raphe and retrotrapezoid nucleus (Young et al. 2005, Kuwaki 2010), which may account for the impact of pharmacological modulation of messengers/neurotransmitters/modulators in the AVPO on the respiratory system under hypoxic conditions (Barros et al. 2006, Gargaglioni et al. 2006). As a matter of fact, it is well known that the hypothalamus plays an important role in the regulation of breathing (Horn & Waldrop 1998, Kuwaki 2010). Boden et al. (2000a) have shown that additional respiratory drive occurs as a result of raised deep body temperature in rats, and that this additional drive is mediated by hypothalamic structures, especially the preoptic area of the hypothalamus (POA), which has been reported to be an important source of such drives to breathing (Boden et al. 2000a, Barros et al. 2006, Gargaglioni et al. 2006). According to this notion, hypothalamic nuclei have been reported to modulate responses to hypoxia and hypercapnia (Berquin et al. 2000): around 21% of neurons in the hypothalamus are activated by hypoxia, and 31% by hypercapnia (Dillon & Waldrop 1993). In addition, other studies have demonstrated that the preoptic hypothalamus participates in compensatory responses to hypoxia, including the hyperventilation (McGuire et al. 2004, Barros et al. 2006, Gargaglioni et al. 2006).

Neurons originating in (or passing through) the preoptic hypothalamus play a role in the processing of signals from the thermoregulatory system and hypoxia sensing, leading to an augmentation of pulmonary ventilation. This fact indicates that hypothalamic neuronal networks integrate ventilatory and thermal responses to hypoxia. It is fairly well established that hypoxia causes an increase in heat loss (Tattersall & Milsom 2003) and a decrease in heat production (Barros et al. 2001), thus resulting in a regulated reduction of deep body temperature (Steiner & Branco 2002). In this context, the POA is a hypothalamic area that has

Figure 7 Relationship between deep body temperature and pulmonary ventilation (\(V_E\)) in aminooxyacetate (AOA)-treated and saline-treated rats exposed to hypoxia (a). Relationship between deep body temperature and \(V_E\) in Na₂S-treated and saline-treated rats exposed to hypoxia (b). The direction of time is shown with arrows, and the times corresponding to the earliest (5 min) and latest (60 min) hypoxia exposure data points are indicated. Values are expressed as mean ± SE. Numbers of animals in each group are shown in parenthesis.

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long been considered to be a thermoregulatory centre (Almeida et al. 2012, for reviews see, Steiner & Branco 2002, Boulant 2006). However, taking into consideration this study and previous data in the literature, it is worth highlighting that this integrative region of the preoptic hypothalamus plays not only a thermoregulatory role but also may modulate ventilatory responses to hypoxia (Hinrichsen et al. 1998, Mortola & Frappell 2000, Barros et al. 2006, Gargaglioni et al. 2006).

Only a few studies have investigated the role of modulators of the hypoxic ventilatory response specifically in the AVPO (Barros et al. 2006, Gargaglioni et al. 2006). Gargaglioni et al. (2006) demonstrated that serotonin (5-HT), acting on two types of 5-HT receptors (5-HT1A and 5-HT1D) plays an inhibitory role in the hyperventilation induced by hypoxia, as the antagonism of these two receptors in the AVPO enhanced this response. In the study by Barros et al. (2006) AVPO adenosine receptors (A1) were antagonized and, in consequence, a higher hypoxia-induced hyperventilation was observed when compared to control groups. This study adds a novel, robust component to the scenario of the involvement of a forebrain region in the control of breathing during hypoxia, demonstrating that H2S plays a crucial role in the respiratory adjustments to hypoxia in the preoptic hypothalamus, more specifically in the AVPO.

Since the effects of the H2S donor and the CBS inhibitor given specifically in the AVPO of rats exposed to hypoxia were quite similar to those of the icv administration, it seems plausible to propose that the AVPO is the site of the preoptic hypothalamus (or of the CNS) where the gas is substantially produced and exerts noticeable effects on pulmonary ventilation under hypoxic conditions. This conclusion is based on the following findings: the icv microinjection of AOAs reduced the hypoxia-induced increase in the H2S production in the AVPO, and caused an additional increase in VT, and consequently in pulmonary ventilation; similarly, when not only AOAs but also Na2S were microinjected directly into the AVPO the effects were qualitative and quantitatively similar to those of the icv treatments in rats exposed to hypoxia.

A recent study (Asimakopoulou et al. 2013) has demonstrated that AOAs do inhibit CBS. However, this inhibitor is not selective to this enzyme, but also acts on CSE inhibiting its activity. In light of this, it is possible that in our experiments part of the effects observed with AOAs microinjection may be through the CSE-H2S pathway rather than the CBS one. Nonetheless, the expression of CBS is higher in the brain compared to CSE, which would predominantly favour the H2S production via CBS. In either case, the net effect of the inhibition of CBS and/or CSE will result in a reduced H2S production as we observed (Fig. 5). Olson (2013) has suggested that the H2S-mediated O2 sensing (in the carotid body) is likely to be regulated through inactivation of H2S rather than through regulation of H2S production. In this case, the mitochondrial oxidation and diffusion out of the cell offer a way of regulating H2S concentration. During hypoxia, H2S is less oxidized and hence its availability is increased. In the present data, it is possible that part of the increased levels of H2S during hypoxia is resultant of this non-enzymatic pathway. However, our data showed that AOAs elicits an attenuation of the hypoxia-induced increase in H2S suggesting that the increased levels of H2S during hypoxia comes at least in part from an enzymatic pathway.

Taking advantage of the deep body temperature recordings used to calculate VT, we could compare the responses induced by AOAs and Na2S under hypoxic condition obtained in the present study with those obtained in a previous study, confirming that our data were quite consistent and highly reproducible in terms of the thermal responses to hypoxia exposure (Kwiatkoski et al. 2012). The results were similar, that is, H2S acting in the AVPO significantly exacerbated hypoxia-induced anapyrexia. Conversely, by inhibiting H2S production with AOAs in the AVPO the drop in body temperature induced by hypoxia was decreased. Therefore, it is likely that in this study the modulatory effect of H2S on hypoxia-induced hyperventilation was at least in part in proportion to metabolism (Fig. 7). Although it is recognized the influence exerted by hypothalamic structures on brainstem respiratory neural network, once drug microinjection was applied within the AVPO, it is most likely that the H2S-induced increased anapyrexia response to hypoxia has potentiated the already installed hypoxia-induced hypometabolism, which in turn may have attenuated the hyperventilation induced by this condition of low level of inspired oxygen.

In general, physiological processes need to be properly regulated to avoid exaggerated or insufficient responses. Hypoxia-induced hyperventilation is an energetically costly response, and as such must be tightly controlled (and not exaggerated). Therefore, it is crucial the existence of counter-regulatory mechanisms to avoid overexpression of the ventilatory response. In this context, our data show that during hypoxia H2S in the AVPO is part of the counter-regulatory responses to hyperventilation. Responses induced by hypoxia, such as respiratory (e.g. hyperpnea), cardiovascular and metabolic adjustments, have been reported to confer protection against low oxygen availability, mainly in oxygen-sensitive tissues, for example, heart and brain (Wood 1991, Gordon 2001, Braga et al. 2007). According to this concept, the
decrease in O2 consumption during hypoxia is accompanied by (i) a reduction of deep body temperature (Gautier 1996, Saiki & Mortola 1997, Barros et al. 2001, Hauzi et al. 2008), (ii) a leftward shift in the oxyhemoglobin dissociation curve, with a resultant improvement of O2 loading in the lungs and (iii) an attenuation of the hyperventilation (Wood 1991, Steiner & Branco 2002).

In conclusion, AVPO H2S has no role in the maintenance of breathing during normoxia, but exerts a robust inhibitory modulation of breathing during hypoxia. This H2S-induced inhibition of breathing during hypoxia is at least in part favoured by the effect of the gas on deep body temperature. Thus, our data suggest that endogenous H2S down-modulates exaggerated ventilatory responses by favouring the occurrence of hypoxia-induced anapyrexia, thus further preventing an excessive energetically costly response under hypoxic conditions.

Conflict of interest
The authors declare that there is no conflict of interest.

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