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Central administration of aminooxyacetate, an inhibitor of H2S production, affects thermoregulatory but not cardiovascular and ventilatory responses to hypercapnia in spontaneously hypertensive rats

João Paulo J. Sabino[a], Renato N. Soriano[b], Bruna M. Santos[c], Alberto F. Donatti[d], Rodrigo R. Fernandez[c], Glauber S.F. da Silva[e], Luiz G.S. Branco[c,*]

[a] Department of Biophysics and Physiology, Federal University of Piaui, Teresina, PI, Brazil
[b] Division of Physiology and Biophysics, Department of Basic Life Sciences, Federal University of Juiz de Fora, Governador Valadares, MG, Brazil
[c] Department of Morphology, Physiology and Basic Pathology, Dental School of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, SP, Brazil
[d] Faculdade Estacio de Sa de Campo Grande, Campo Grande, MS, Brazil
[e] Federal University of Minas Gerais, Institute of Biological Science, Department of Physiology and Biophysics, Belo Horizonte, MG, Brazil

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ABSTRACT

Hydrogen sulfide (H_2S) is classically known for its toxic effects. More recently H_2S has been documented as a neuromodulator. Here we investigated the central effects of aminooxyacetate (AOA; inhibitor of the H_2S-synthesizing enzyme cystathionine β-synthase, CBS) on cardiovascular, respiratory and thermoregulatory responses to hypercapnia in spontaneously hypertensive rats (SHR). To attain this goal we measured mean arterial pressure (MAP), heart rate (HR), ventilation (V_E), and deep body temperature (Tb) of SHR and (normotensive) Wistar Kyoto (WKY) rats before and after microinjection of AOA (9 nmol/μL) or saline into the fourth ventricle immediately followed by 30-min hypercapnia exposure (7% inspired CO_2). In saline-treated WKY rats, hypercapnia caused an increase in MAP accompanied by bradycardia, an increase in V_E, and a drop in Tb. In AOA-treated WKY rats exposed to hypercapnia, the drug did not affect the increased MAP, potentiated the bradycardic response, attenuated the increased V_E, and potentiated the drop in Tb. In saline-treated SHR, in comparison to the saline-treated WKY rats, hypercapnia elicited a minor, shorter-lasting increase in MAP with no changes in HR, evoked a greater increase in V_E, and did not induce a drop in Tb. In AOA-treated SHR exposed to hypercapnia, the drug did not change the hypercapnia-induced cardiovascular and ventilatory responses while permitted a drop in Tb. Our findings indicate that AOA, an inhibitor of H_2S production, modulates cardiorespiratory and thermoregulatory responses to hypercapnia in normotensive rats, whereas hypertension development in SHR is accompanied by suppression of the AOA effect on the cardiovascular and respiratory responses.

1. Introduction

Hydrogen sulfide (H_2S) is an important gaseous neuromodulator in the peripheral and central nervous systems (CNS). In the CNS H_2S is synthetized by the enzymes cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3MST) (Abe and Kimura, 1996; Shibuya et al., 2009), whereas cystathionine γ-lyase (CSE) is the isoform that predominates in peripheral tissues (Yang et al., 2008). CBS is ubiquitously expressed in adult brain, being mainly expressed in neuroepithelial cells in the ventricular zone at early developmental stages. During the late embryonic and neonatal periods, its expression changes to radial glial cells and then to astrocytes. The enzyme is expressed most intensely in the cerebellar molecular layer and hippocampal dentate gyrus, preferentially in cerebellar Bergmann glia and in astrocytes throughout the brain (Enokido et al., 2005).

Our laboratory has demonstrated the propyretic role of the peripheral H2S-CSE pathway in thermoregulation (Soriano et al., 2018) as well as the functions of the central H2S-CBS pathway on the cardiovascular, respiratory and thermoregulatory systems during hypoxia exposure in normotensive and spontaneously hypertensive rats (SHR) (da Silva et al., 2017; Donatti et al., 2014a, 2014b, Kwiatkoski et al., 2014, 2012, Sabino et al., 2017, 2016). Additionally, da Silva et al. (da Silva et al., 2014) have documented that central H_2S plays an excitatory role mediating the ventilatory response to hypercapnia in normotensive rats.

Hypercapnia, an increased fractional concentration of inspired CO_2,

* Corresponding author at: Dental School of Ribeirão Preto, University of São Paulo, Av. do Café s/n., 14049-900, Ribeirão Preto, SP, Brazil.
E-mail address: branco@forp.usp.br (L.G.S. Branco).

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ultimately induces physiological adjustments, such as an increase in arterial blood pressure, hyperventilation, and a decrease in deep body temperature (Tb) (Branco and Wood, 1994; Dias et al., 2007), which are integrated and driven by CBS-bearing regions of the brainstem, particularly the caudal part of the nucleus tractus solitarius (cNTS) (Kara et al., 2003; Nattie, 2011, 1999), respiratory centers (Guyenet et al., 2010), and the rostral ventrolateral medulla (RVLM) (Duan et al., 2015).

Arterial hypertension has been shown to be associated with autonomic changes, such as enhanced sympathetic activity and decreased parasympathetic activity. These autonomic changes appear to be influenced by an enhanced chemoreflex sensitivity and an a reduced baroreflex sensitivity, and, curiously, have been reported to be similar in both humans and SHR (Frohlich and Pfeffer, 1975; Geraldes et al., 2014). Consistent with the notion that autonomic alterations are present in SHR, it has been documented a higher baseline of Tb in these hypertensive rats (Campos et al., 2014; Collins et al., 1987).

Therefore, since hypercapnia stimulates chemoreflex responses (Guyenet et al., 2010), which have been reported to be altered in SHR (Li et al., 2016), investigating the central effects of AOA, an inhibitor of the H_2S-synthesizing enzyme CBS, on autonomic and ventilatory responses to hypercapnia is an attempt to shed light on the advances in comprehension of the central mechanisms responsible for and associated with arterial hypertension. To the best of our knowledge, there is no data reporting whether central endogenous H_2S acts on the cardiovascular, respiratory and thermoregulatory systems in SHR exposed to hypercapnia. Similarly, the role of central endogenous H_2S in cardiovascular and thermoregulatory responses to hypercapnia in normotensive rats has not yet been reported in the literature.

Based on previous studies and pilot experiments with a H_2S donor we hypothesized that AOA exerts central effects on physiological responses to hypercapnia and that these effects are likely to be mediated by H_2S, even though we do not neglect the fact that other AOA targets (mentioned below) might be involved. Thus, the aim of the present study was to investigate whether AOA modulates cardiovascular, ventilatory and thermoregulatory responses to hypercapnia in (normotensive) WKY rats and SHR.

2. Material and methods

2.1. Animals

The present study was performed with male SHR and Wistar Kyoto (WKY) rats (17–20 weeks old; weighing between 280–300 g). The animals had free access to water and food and were kept at 25 °C of ambient temperature and exposed to a light-dark cycle of 12:12 h. The surgeries techniques and the experimental protocols were approved by the Local Committee of Ethics in Animal Research – School of Medicine of Ribeirao Preto, University of Sao Paulo (protocol 064/2012).

2.2. Drugs

Aminooxyacetate (AOA, 9 nmol/μL), Sigma, St. Louis, MO, USA, an inhibitor of the H_2S-synthesizing enzyme CBS, was freshly dissolved in sterile saline on the experiment day, and pH was adjusted to 7.4. Since AOA is not highly specific, it important to mention that the drug may stimulate GABA synthesis as well as inhibit mitochondrial respiration (discussed below). Importantly, the dose of AOA had no effect on the parameters baseline, and was chosen based on pilot experiments and previous studies (da Silva et al., 2014; Sabino et al., 2017, 2016).

2.3. Surgical procedures

Under general anesthesia with ketamine-xylazine (100 and 10 mg/kg, respectively; 1 mL/kg, i.p.) each animal was fixed on a stereotaxic frame to be implanted with a stainless steel guide cannula (15-mm long, 22-gauge outer diameter) into the fourth ventricle (4 V), as reported elsewhere (Soriano et al., 2012). This surgical procedure was performed to allow an intra-4 V microinjection; stereotaxic coordinates: anteroposterior, 11.9 mm caudal to bregma; lateral, 0.0 mm; dorsoventral, 7.4 mm ventral from the surface of the skull (Paxinos and Watson, 2007). Thereafter, a median laparotomy was performed to insert a temperature datalogger capsule (SubCue, Calgary, AB, Canada) into the peritoneal cavity. The animals were prophylactically injected with antibiotics (160,000 U/kg benzylpenicilin, 33.3 mg/kg streptomycin, and 33.3 mg/kg dihydrostreptomycin, i.m.; prophylactically) and received analgesic medication (flunixin meglumine; 2.5 mg/kg, s.c.) immediately after the surgeries, and were allowed to recover over a period of six days before the experimental protocols.

One day before the experimental protocols, for pulsatile arterial pressure (PAP) recordings each rat was deeply anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively; 1 mL/kg, i.p.) to be implanted with a polyethylene catheter into the left femoral artery. The catheter was subcutaneously tunneled to the back of the neck.

2.4. Recordings of cardiovascular and respiratory parameters and Tb

PAP recordings were used to quantify mean arterial pressure (MAP) and heart rate (HR). Respiratory frequency (F), tidal volume (V_T) and minute ventilation (V_E) were obtained by whole-body plethysmography in unanesthetized rats (Bartlett and Tenney, 1970). Tb was measured by means of the temperature datalogger capsule inserted into the peritoneal cavity. Cardiovascular and respiratory parameters were recorded and analyzed as previously reported (da Silva et al., 2014; Donatti et al., 2014b; Sabino et al., 2016, 2014).

2.5. Experimental procedures

On the day of the experiment the rats were maintained in the experiment room at 25 °C. During the acclimatization period of the experimental procedures, the freely moving rat was put in a Plexiglass chamber which was ventilated with humidified room air for 60 min. Basal measurements of cardiovascular and respiratory parameters and Tb were acquired under normocapnia (0% CO_2, 21% O_2 and N_2 balance) for 30 min. Thereafter, each rat received only one microinjection of vehicle (sterile saline) or AOA into the 4 V by means of a Hamilton syringe (5 μL) and a dental needle (Missy, 200 μm OD). The location of the intra-4 V microinjection was histologically verified. Immediately after the intra-4 V microinjection, a hypercapnic gas mixture (7% CO_2, 21% O_2 and N_2 balance) was flushed into the chamber for 30 min. Cardiovascular and respiratory parameters and Tb were measured for 30 min under the hypercapnia exposure. Cardiovascular and thermoregulatory responses to hypercapnia were assessed every 3 and 5 min, respectively, whereas ventilatory responses were evaluated at 5, 10, 20 and 30 min. All gases conditions were flushed by a flow meter gas-mixing pump (Columbus Instruments Pegas 4000, OHIO, USA). A gas analyzer (Gas Analyzer, AdInstruments, Sydney, Australia) was used to monitor gas composition inside the chamber in all experimental protocols.

2.6. Actions of central endogenous H_2S on cardiovascular, ventilatory and thermoregulatory responses to hypercapnia

To investigate the role of central endogenous H_2S in the cardiovascular system, pulmonary ventilation and Tb during hypercapnia exposure, the rats were intra-4 V microinjected with AOA immediately before being exposed to 30-min hypercapnia. The control group received an intra-4 V microinjection of sterile saline (vehicle of AOA).

2.7. Statistical analyses

The results are expressed as means ± SEM. Statistical differences in

Table 1
Basal values of MAP, HR, F, V_T, V_E and Tb in WKY and SHR.

	MAP (mmHg)	HR (bpm)	F (cycles. min^{-1})	V_T (mL. kg^{-1})	V_E (mL.kg. min^{-1})	Tb (°C)
WKY	114 ± 1	307 ± 4	110 ± 3	6.66 ± 0.2	698.48 ± 40.8	35.9 ± 0.1
SHR	159 ± 2*	331 ± 6*	96 ± 3*	7.32 ± 0.4	739.30 ± 39.0	37.3 ± 0.1*

* $P < 0.05$ compared to WKY.

basal values (MAP, HR, F, V_T, V_E, and Tb) were evaluated by two-way ANOVA followed by Tukey's post-hoc test. Alterations induced by hypercapnia and AOA (ΔMAP, ΔHR, ΔF, ΔV_T, ΔV_E, and ΔTb) were statistically evaluated by multifactorial ANOVA for repeated measures followed by Tukey's post-hoc test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Basal values of cardiovascular and respiratory parameters and of Tb in WKY rats and SHR

Table 1 shows the basal values of MAP, HR, F, V_T, V_E, and Tb in WKY rats and SHR. MAP, HR, and Tb were statistically higher ($P < 0.05$) in SHR than in WKY rats. Respiratory frequency (F) of SHR was statistically lower ($P < 0.05$), whereas V_T and V_E were statistically similar between SHR and WKY rats ($P > 0.05$).

3.2. Comparison between saline-treated WKY rats versus saline-treated SHR

Tables 2 and 3 show the comparison between saline-treated WKY rats versus saline-treated SHR exposed to 30-min hypercapnia (7%CO_2 and 21%O_2). Regarding the cardiovascular responses (Table 2), changes in MAP were significantly lower ($P < 0.05$) in SHR than WKY rats at 27 and 30 min, and the bradycardic response (changes in HR), observed in WKY rats, was absent in SHR and statistically different ($P < 0.05$) from that of WKY rats (from 3 to 24 min). Regarding the respiratory responses (Table 3), changes in pulmonary ventilation (V_E) resulted in a statistically higher ($P < 0.05$) V_E in SHR than WKY rats at 5, 10 and 20 min, whereas the thermal responses (changes in Tb; Table 3), the hypothermic response, observed in WKY rats, was almost absent in SHR, being statistically different ($P < 0.05$) from that of WKY rats only at 30 min.

Table 2
Comparison between saline-treated WKY rats versus saline-treated SHR exposed to 30-min hypercapnia (7%CO_2 and 21%O_2).

	Δ MAP		Δ HR	
Time (min)	WKY	SHR	WKY	SHR
3	12 ± 2	13 ± 2	−38 ± 2	20 ± 7*
6	7 ± 3	7 ± 2	−43 ± 5	11 ± 7*
9	2 ± 2	5 ± 5	−43 ± 5	11 ± 9*
12	5 ± 2	3 ± 6	−38 ± 8	−2 ± 12*
15	7 ± 2	2 ± 5	−33 ± 10	−1 ± 12*
18	10 ± 3	3 ± 4	−29 ± 14	2 ± 13*
21	11 ± 3	3 ± 2	−26 ± 14	3 ± 13*
24	8 ± 3	1 ± 3	−28 ± 12	1 ± 14*
27	14 ± 2	1 ± 4*	1 ± 15	9 ± 13
30	15 ± 2	0 ± 4*	7 ± 12	0 ± 11

* $P < 0.05$. Changes in mean arterial pressure (ΔMAP; mmHg) and heart rate (ΔHR; bpm).

3.3. Cardiovascular responses to AOA during hypercapnia exposure

Fig. 1 depicts representative recordings of pulsatile arterial pressure, MAP, and HR of WKY rats (upper panel) and SHR (bottom panel) under basal condition and after an intra-4 V microinjection of saline or AOA followed by hypercapnia exposure. Fig. 2 shows changes in MAP (upper panel) and HR (bottom panel) of WKY rats (left panels) and SHR (right panels) after an intra-4 V microinjection of saline or AOA immediately followed by 30-min hypercapnia exposure. In saline-treated WKY rats, hypercapnia induced an increase in MAP and a reduction in HR compared to baseline. Microinjection of AOA in WKY rats did not change the hypercapnia-induced increase in MAP. Regarding HR of WKY rats, AOA potentiated the hypercapnia-induced reduction in HR when compared to the saline group. In saline-microinjected SHR, compared to saline-treated WKY rats, hypercapnia elicited a minor and shorter-lasting increase in MAP, and did not induce bradycardia. AOA administration did not significantly change ($P > 0.05$) either MAP or HR responses to hypercapnia in SHR (Fig. 2).

3.4. Respiratory responses to AOA during hypercapnia exposure

Fig. 3 exhibits representative recordings of pulmonary ventilation of WKY rats (upper panel) and SHR (bottom panel) under basal condition and after an intra-4 V microinjection of saline or AOA followed by hypercapnia exposure. Fig. 4 shows changes in F (upper panel), V_T (middle panel) and V_E (bottom panel) of WKY rats (left panels) and SHR (right panels) after an intra-4 V microinjection of saline or AOA immediately followed by 30-min hypercapnia exposure. In saline-microinjected WKY rats, hypercapnia induced an increase in F, V_T and V_E when compared to baseline. AOA microinjected in WKY rats attenuated the hypercapnia-induced increase in F, V_T and V_E. As shown in the Table 3, in saline-microinjected SHR hypercapnia elicited an increase in F statistically similar ($P > 0.05$) to that found in saline-treated WKY rats, but evoked a greater increase ($P < 0.05$) in V_T and V_E. In SHR, AOA did not statistically change ($P > 0.05$) the respiratory response to hypercapnia (Fig. 4).

3.5. Thermoregulatory response to AOA during hypercapnia exposure

Fig. 5 shows the changes in Tb of WKY rats (left panel) and SHR (right panel) after an intra-4 V microinjection of saline or AOA immediately followed by 30-min hypercapnia exposure. In saline-treated WKY rats, hypercapnia induced a drop in Tb (the hypothermic response). When AOA was microinjected into the 4 V of WKY rats the drug significantly ($P < 0.05$) potentiated the hypercapnia-induced decrease in Tb in comparison to saline-treated WKY rats. In saline-microinjected SHR, hypercapnia did not induce a statistically significant ($P > 0.05$) drop in Tb. Microinjection of AOA into the 4 V of SHR elicited a remarkable drop in Tb which was statistically significant at the 30th min of exposure to hypercapnia ($P < 0.05$; Fig. 5).

3.6. Relationship between V_E and Tb

The relationship between V_E and Tb in WKY rats as well as in SHR (Fig. 6) highlights the observation that the decreased hypercapnic ventilatory response seen in AOA-treated WKY rats took place due to a

Table 3
Comparison between saline-treated WKY rats versus saline-treated SHR exposed to 30-min hypercapnia (7%CO_2 and 21%O_2).

	ΔF		ΔVT		ΔVE		ΔTb	
Time (min)	WKY	SHR	WKY	SHR	WKY	SHR	WKY	SHR
5	37 ± 8	50 ± 5	1.7 ± 0.4	4.9 ± 0.6*	469.5 ± 75.6	1045.6 ± 108.8*	−0.01 ± 0.09	−0.07 ± 0.03
10	34 ± 6	44 ± 5	2.1 ± 0.3	4.6 ± 0.4*	506 ± 86.8	954.3 ± 116.6*	−0.12 ± 0.06	0.04 ± 0.05
15							−0.24 ± 0.05	0.01 ± 0.07
20	31 ± 8	42 ± 3	2.2 ± 0.3	4 ± 0.2*	490.1 ± 74.2	837.7 ± 55.7*	−0.53 ± 0.08	0.04 ± 0.08
25							−0.56 ± 0.09	−0.01 ± 0.08
30	38 ± 7	40 ± 3	2.6 ± 0.3	3.6 ± 0.2	604.5 ± 67.2	760.1 ± 46.8	−0.65 ± 0.06	−0.05 ± 0.1*

* $P < 0.05$. Changes in respiratory frequency (ΔF; cycles. min^{-1}), tidal volume (ΔVT; mL.Kg^{-1}), pulmonary ventilation (ΔVE; mL.Kg. min^{-1}), and deep body temperature (ΔTb; ºC).

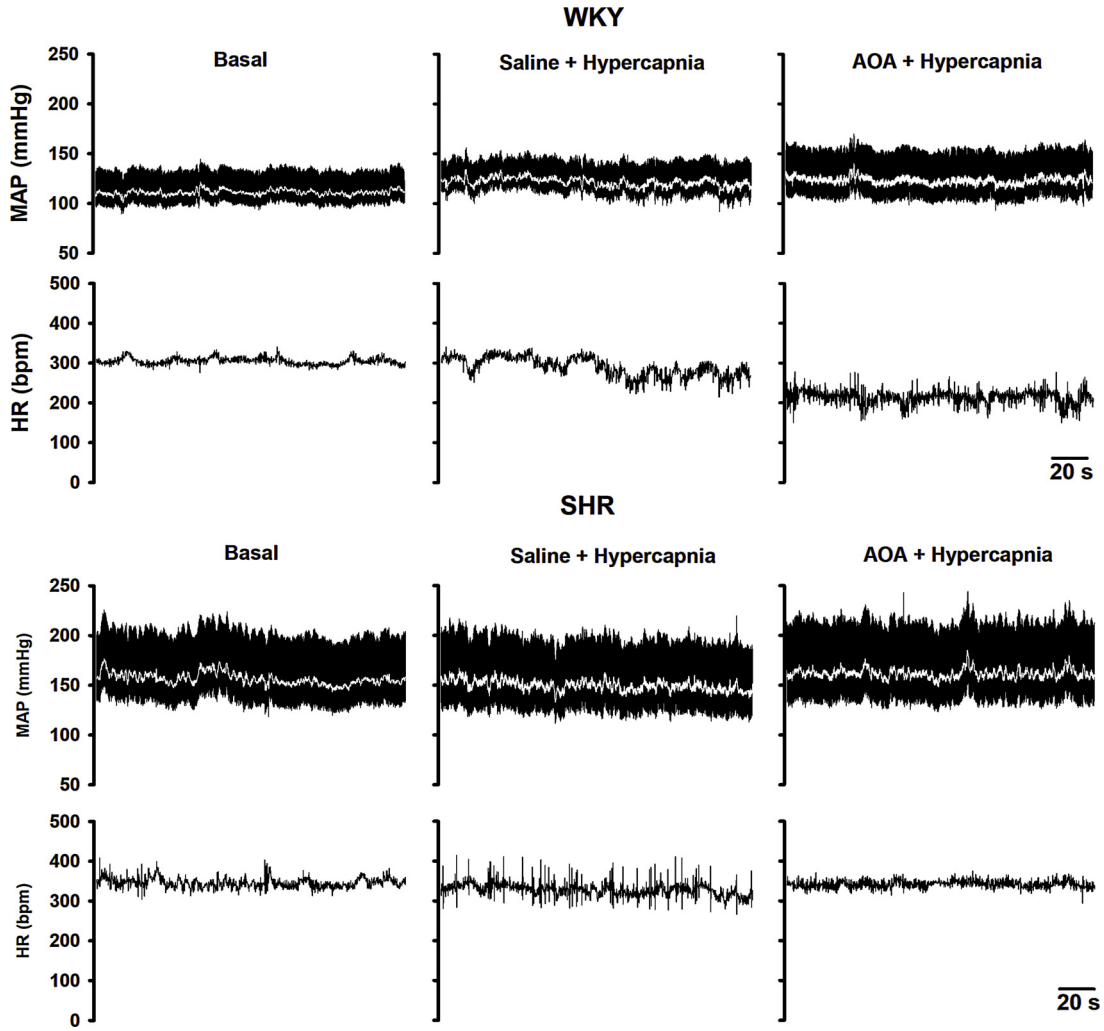

Fig. 1. Representative recordings of pulsatile arterial pressure, mean arterial pressure (MAP; white tracings) and heart rate (HR) of WKY rats (upper panels) and SHR (bottom panels) kept under normocapnia (basal), microinjected into the fourth ventricle with saline or AOA and exposed to hypercapnia.

lower Tb and a more pronounced hypothermic response in these animals. In SHR, which present a higher baseline of Tb (Table 1) and did not exhibit a hypothermic response, the ventilatory response to hypercapnia was higher when compared to that of WKY (Table 3; Fig. 6).

4. Discussion

The modulatory role of H_2S in autonomic and respiratory control has been documented in vitro and in vivo (da Silva et al., 2017). However, the fact that arterial hypertension is one of the two most important causes of mortality in the world indicates that more experimental approaches are required to unravel some of the neurochemical aspects of this clinical entity. Thus, since arterial hypertension is associated with enhanced chemoreflex sensitivity and hypercapnia stimulates chemoreflex responses, which has been shown to be modulated by H_2S and altered in SHR, the objective of the present study was to investigate whether central endogenous H_2S plays a modulatory role in autonomic and ventilatory responses to hypercapnia in hypertensive rats. During 30-min hypercapnia exposure we assessed cardiovascular, respiratory and thermoregulatory adjustments after an intra-4 V

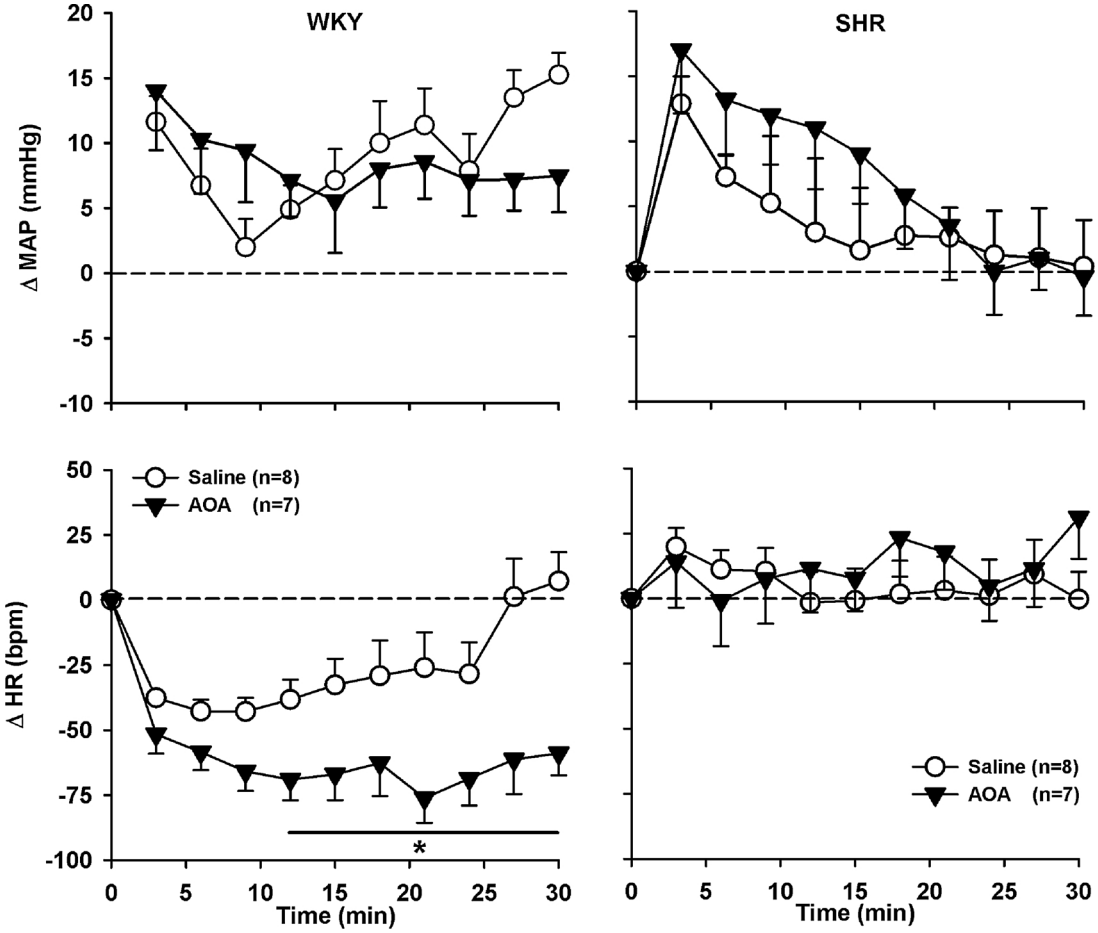

Fig. 2. Effect of an intra-fourth ventricle microinjection of saline (1 μL) or AOA (9 nmol/1 μL) immediately followed by 30-min hypercapnia exposure (7%CO_2 and 21%O_2) on mean arterial pressure (MAP, upper) and heart rate (HR, bottom) of WKY rats and SHR. MAP and HR are shown as changes from baseline. Number of animals is shown in parenthesis. *, $P < 0.05$ compared to saline-treated WKY rats. Differences between WKY vs SHR are shown in Table 2.

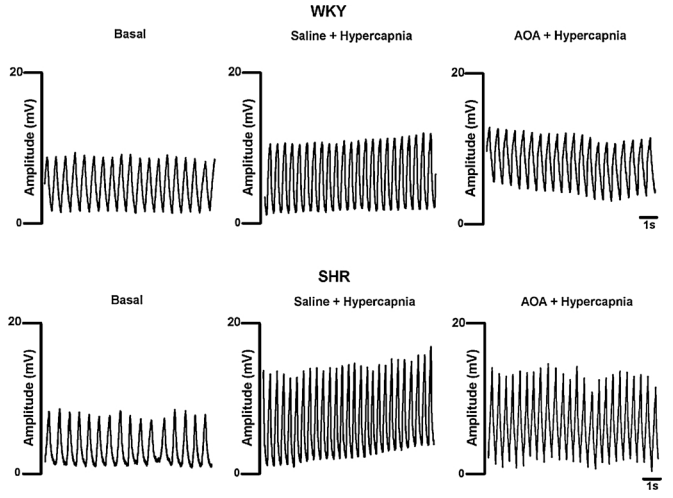

Fig. 3. Representative recordings of pulmonary ventilation (measured in voltage deflections) of WKY rats (upper panels) and SHR (bottom panels) kept under normocapnia (basal), microinjected into the fourth ventricle with saline or AOA and exposed to hypercapnia.

microinjection of AOA, a drug which reduces the endogenous synthesis of the gaseous neuromodulator by inhibiting the H_2S-synthesizing enzyme CBS.

At this point it is important to mention that in the literature AOA has not been considered to be a highly selective inhibitor of CBS; however, as far as we know, this is the most potent pharmacological tool available to inhibit the CBS-H_2S pathway (Asimakopoulou et al., 2013). Nevertheless, we cannot neglect that at least part of the effects reported herein may be attributed to AOA effects on other targets, since the drug has been reported to stimulate GABA synthesis in rat hippocampus and striatum (Ayala-Grosso and Urbina-Paez, 1999) as well as induce neurotoxicity (Du et al., 1998; Urbanska et al., 1991) and neuronal cell loss in the striatum (Beal et al., 1991) and hippocampus (McMaster et al., 1991) through inhibition of mitochondrial respiration (Brouillet et al., 1994). These processes are comparable to that of H_2S (Whiteman et al., 2011).

Besides mentioning the aspects related to other possible effects of AOA, we have to highlight that AOA-induced neuronal damages were found only at concentrations higher than that of the present study. Also, the time course of these effects were longer (ranging from 3 to 6 h post injection) than the experimental protocols of our present study. Furthermore, we previously addressed the potential side effects of AOA through the GABAergic mechanisms (da Silva et al., 2017). The experiments demonstrated an evident AOA dose-dependent inhibition of the respiratory activity both *in vitro* and *in vivo* approaches (the AOA concentrations were at the same range as in the present study). It was reported that the AOA-elicited effects remained unaffected when experiments were run in the presence of bicuculline (a GABA receptor antagonist). These findings remarkably suggest an absent effect of AOA-mediated potentiation of GABA actions. Additionally, pilot experiments performed with WKY rats strongly suggested that the effect of AOA observed in the present work is most likely to be mediated by inhibition of H_2S production, since intra-4 V administration of a H_2S donor (Na_2S)

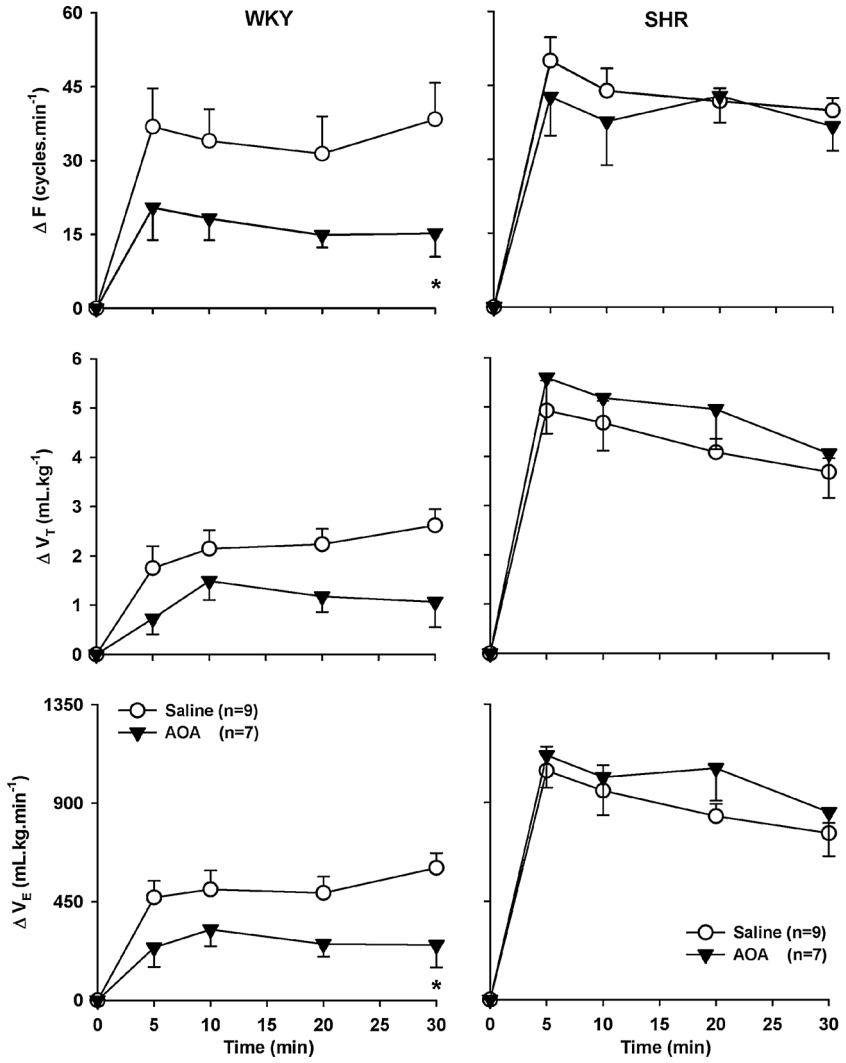

Fig. 4. Effect of an intra-fourth ventricle microinjection of saline (1 µL) or AOA (9 nmol/1 µL) immediately followed by 30-min hypercapnia exposure (7%CO_2 and 21%O_2) on respiratory frequency (F, upper), tidal volume (VT, middle) and pulmonary ventilation (VE, bottom) of WKY rats and SHR. F, VT and VE are shown as changes from baseline. Number of animals is shown in parenthesis. *, P < 0.05 compared to saline-treated WKY rats. Differences between WKY vs SHR are shown in Table 3.

evoked cardiovascular responses to hypercapnia which were quantitative and qualitatively opposite to that of AOA (not shown). Considering the abovementioned facts, we believe that major effects of AOA in the present study were not due to its side effects. Nonetheless, we cannot completely rule out the possibility that the effects observed herein can partly be attributed to some of the AOA off-targets.

Our findings showed that CBS inhibition with AOA did not alter the hypercapnia-induced cardiovascular and respiratory responses in SHR. Importantly, the dose of AOA used in the present study was at the very same intracerebroventricular dose that has been reported to be effective in modulating physiological responses to either hypoxia (which is a different stimulus evoking particular responses) or hypercapnia in normotensive rats (da Silva et al., 2014; Sabino et al., 2017, 2016), As reported in those studies (da Silva et al., 2014; Sabino et al., 2017,

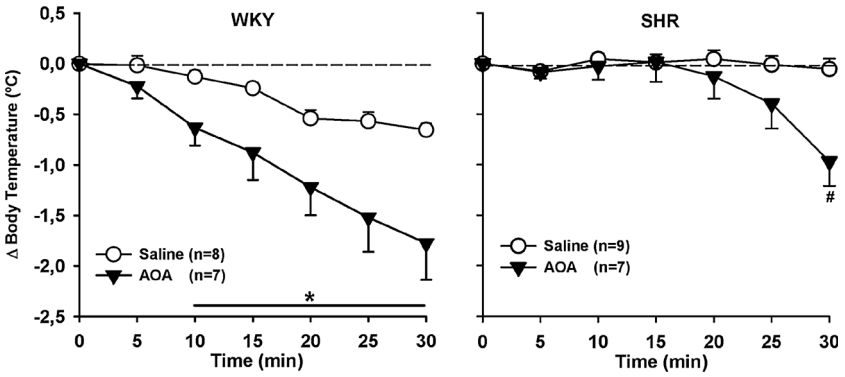

Fig. 5. Effect of an intra-fourth ventricle microinjection of saline (1 µL) or AOA (9 nmol/1 µL) immediately followed by 30-min hypercapnia exposure (7%CO_2 and 21%O_2) on deep body temperature (Tb) of WKY rats and SHR. Tb is shown as changes from baseline. Number of animals is shown in parenthesis. *, P < 0.05 compared to saline-treated WKY rats. #, P < 0.05 compared to saline-treated SHR. Differences between WKY vs SHR are shown in Table 3.

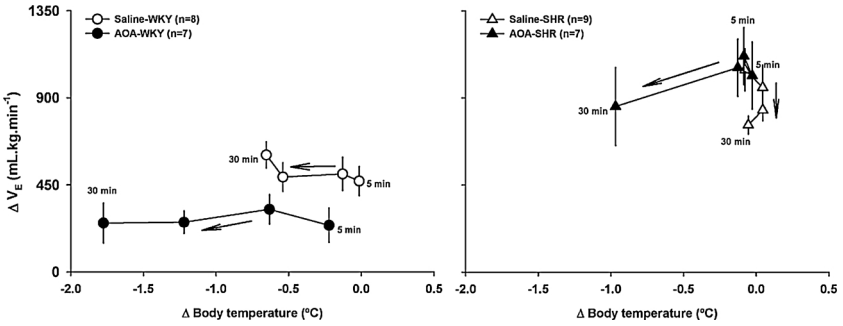

Fig. 6. Relationship between changes in pulmonary ventilation (ΔVE) and deep body temperature (ΔTb) in WKY rats and SHR at onset of the hypercapnic response (5–30 min). The direction of time is shown with arrows, and the time points corresponding to the earliest and latest data points are indicated. Number of animals is shown in parenthesis.

2016), as well as in the present one, the CBS inhibition took place mainly in the caudal brainstem cerebroventricular system, since AOA was microinjected into the caudal half of the 4 V (see ref. Soriano et al., 2012). Curiously, inhibition of the H_2S synthesis with AOA in the 4 V of SHR was permissive for the occurrence of a hypothermic response at the 30^{th} min of exposure to hypercapnia. On the other hand, the absence of alterations in cardiovascular and ventilatory parameters suggests that central (hindbrain) endogenous H_2S does not seem to be involved in the modulation of circulatory and respiratory responses to hypercapnia in SHR. It is important to highlight that these findings observed in SHR were substantially different from those seen in WKY rats.

In a previous work we investigated the actions of central H_2S on MAP and HR in SHR during activation of peripheral chemoreceptors by hypoxia exposure (Sabino et al., 2016). In that work, AOA has been shown to prevent the reduction in MAP, and induced tachycardia (Sabino et al., 2016). Conversely, here we observed that CBS inhibition with AOA during hypercapnia unexpectedly caused no change in the cardiovascular responses in SHR; and, interestingly, the bradycardic response to hypercapnia was not present in SHR. Taking that work and the present study together we suggest that cardiovascular response to hypoxia involves a modulatory control exerted by endogenous levels of H_2S, whereas the role of central endogenous H_2S in the cardiovascular responses to hypercapnia is negligible in SHR, at least in the hindbrain.

In the experiments with the control group it was investigated for the first time whether central endogenous H_2S plays a modulatory role in the MAP and HR responses to hypercapnia in normotensive WKY rats. AOA administration potentiated the bradycardic response to hypercapnia but did not cause any change in MAP. These data suggest that during chemoreceptors activation by hypercapnia exposure in normotensive WKY rats central H_2S modulates cardiac autonomic nervous system, probably by downmodulating cardiac parasympathetic activity, or upmodulating cardiac sympathetic activity. Additionally, we speculate that the effect of AOA seems to be associated with an increase in parasympathetic activity to the heart, which induced a greater bradycardic response and probably an attenuation of cardiac output. Nonetheless, the AOA-induced increased bradycardic response did not affect MAP, suggesting that the control of the vascular sympathetic activity was not altered or that AOA caused a sympathetic overactivity that prevented the reduction of MAP during hypercapnia.

It is worth mentioning some important data from the literature showing that during chemoreflex activation by hypoxia endogenous H_2S has been shown to play no role in the MAP and HR responses in normotensive Wistar rats (Sabino et al., 2016). Moreover, that work reported that AOA did not alter either MAP or HR during normoxia, regardless of being hypertensive or not (Sabino et al., 2016), indicating that AOA does not affect the cardiovascular control under normal conditions, i.e., inspiring room air.

Other laboratories have also investigated the central effects of H_2S on the cardiovascular system. Ufnal et al. (2008) reported that administration of a H_2S donor into the lateral cerebral ventricle (LCV) in WKY rats did not affect MAP and HR. Other study has documented that CBS expression in the rostral ventrolateral medulla (RVLM) was lower in SHR than in WKY rats, and that microinjection of adenovirus encoding enhanced CBS gene induced a significant increase in CBS protein in the RVLM of both groups. Consequently, an increase was triggered in systolic blood pressure, HR and urinary norepinephrine excretion in SHR (Duan et al., 2015). These authors suggested that the H_2S-CBS system has a key function in the maintenance of arterial hypertension and sympathetic activity, and that the reduced CBS expression in the RVLM can be a compensatory mechanism to hypertension in SHR (Duan et al., 2015).

We also investigated the effects of central H_2S on ventilatory parameters during chemoreceptors activation by hypercapnia exposure. In saline-treated SHR, hypercapnia evoked a greater increase in V_T and V_E compared to saline-treated WKY rats, suggesting that the established notion that hypertension is characterized by imbalances due in part to chemoreflex overactivity seems to be applicable to the enhanced ventilatory response to hypercapnia in SHR (Li et al., 2016). As observed in the cardiovascular parameters, endogenous H_2S did not affect the respiratory responses to hypercapnia in SHR. These findings indicate that central endogenous H_2S does not seem to play a role in pulmonary ventilation during hypercapnia in SHR. Compared to the effects observed during hypoxia, AOA has been shown to induce a reduction in breathing in response to hypoxia exposure in SHR (Sabino et al., 2016), indicating that, different from the hypercapnia exposure, during hypoxic stimulus endogenous H_2S plays a modulatory (excitatory) role in ventilatory control.

In our experiments with normotensive WKY rats exposed to hypercapnia, AOA caused a decrease in V_E, indicating that endogenous H_2S plays an excitatory role in breathing during hypercapnia exposure in normotensive rats (but not in SHR). Similarly, an excitatory effect of endogenous H_2S on pulmonary ventilation during chemoreflex activation by hypercapnia has been reported in normotensive Wistar rats (da Silva et al., 2014).

Regarding the effects of H_2S on the ventilatory responses to hypoxia, data available in the literature indicate an opposite function. It has been documented that H_2S into the preoptic area of the hypothalamus (Kwiatkoski et al., 2014) or into the RVLM (Donatti et al., 2014b) exerts an inhibitory function on breathing during hypoxic stimulus. On the other hand, other study demonstrated that peripheral H_2S may exert an excitatory action on respiratory parameters, since pharmacologic inhibition of CSE has been shown to induce an attenuation of sensory activity of the carotid bodies in response to hypoxia in rats, and that mice with genetic deletion of CSE exhibited a reduced activity of the carotid bodies and an attenuated ventilatory response to hypoxia (Peng et al., 2010).

Although the thermoregulatory control has been investigated in the hypothermic response to hypercapnia in several studies (Barros and Branco, 1998; Branco and Wood, 1994; Dias et al., 2007; Lai et al., 1981), for the first time it was investigated the role of central endogenous H_2S in the thermoregulatory response to hypercapnia in WKY rats and SHR. In saline-treated SHR, hypercapnia exposure did not cause a drop in Tb, i.e., hypercapnia did not induce a hypothermic response in hypertensive rats. This finding significantly differs from

that of the saline-treated WKY rats, suggesting that in SHR some factors seem to prevent the hypothermic response to hypercapnia. Consistent with the notion that the thermoregulatory control is altered in SHR not only in response to hypercapnia but also under normal conditions, we observed that Tb baseline was higher in SHR than in WKY rats (Table 1). AOA administered to the WKY rats potentiated the hypothermic response to hypercapnia, suggesting that central endogenous H_2S somehow limits the magnitude of the hypothermic response in these normotensive rats. Curiously, in SHR, which did not exhibit the hypothermic response to hypercapnia, CBS inhibition with AOA was permissive for the occurrence of a hypothermic response that was statistically significant at the 30th min of hypercapnia exposure. Given that the magnitude of the hypothermic response to hypercapnia in normotensive rats was limited by H_2S, probably through an anti-cryogenic action of the gas, and that this response was observed in SHR only after the inhibition of the H_2S synthesis with AOA, we speculate that SHR do not present this thermoregulatory response because of an enhanced anti-cryogenic action of H_2S. In these hypertensive rats the gaseous neuromodulator is likely to be more synthesized and/or more effective in brainstem sites involved in this thermoregulatory response.

However, due to the fact that Tb was altered by AOA, one might argue that the mentioned effect of the drug on the ventilatory response to hypercapnia is indirect. To evaluate this possibility we analyzed the relationship between V_E and Tb (Fig. 6). This figure showed that the decreased hypercapnic ventilatory response observed in AOA-treated WKY rats took place due to a lower Tb and a more pronounced hypothermic response in these animals (Fig. 6). Therefore, the effect of AOA on the ventilatory response was indirectly caused by a more pronounced drop in Tb in normotensive rats. Curiously, since SHR present a higher Tb (Table 1) and did not exhibit a hypothermic response, the ventilatory response to hypercapnia in these hypertensive animals was higher when compared to that of WKY (Table 3; Fig. 6). Therefore, given that hypercapnia did not induce a significant drop in Tb of SHR, saline-treated SHR (as well as AOA-treated SHR) presented a higher hypercapnic ventilatory response compared to that of WKY rats (Table 3). It is worth mentioning that in spite of the fact that AOA-treated SHR presented a significant fall in Tb only at 30 min their hypercapnic ventilatory response was not significantly different ($P > 0.05$) from that of saline-treated SHR (Fig. 6).

In summary, we present data suggesting that central AOA potentiates the cardiac (bradycardic) and thermal (hypothermic) responses to hypercapnia in normotensive WKY rats, whereas the attenuated respiratory response in these normotensive animals derives from the potentiated drop in Tb. On the other hand, in SHR the effect of the drug on the cardiovascular and respiratory responses to hypercapnia is somehow suppressed with the hypertension development. Nevertheless, AOA was responsible for permitting a late hypothermic response to hypercapnia in the hypertensive rats. It remains to be established the mechanisms underlying the AOA-evoked potentiation of the hypercapnia-induced bradycardic and hypothermic responses to hypercapnia in normotensive animals as well as the reported effects of the drug on the hypothermic but not on the cardiac and respiratory responses in the hypertensive rats.

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