Respiratory Physiology & Neurobiology 208 (2015) 15–20

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

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

Hydrogen sulfide activates the carotid body chemoreceptors in cat, rabbit and rat *ex vivo* preparations

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ARTICLE INFO

Article history:
Accepted 4 January 2015
Available online 10 January 2015

Keywords:
Carotid body
Hydrogen sulfide
Sinus nerve
Hypoxia

ABSTRACT

We and others previously reported experimental evidence suggesting an important role for hydrogen sulfide (H_2S) in oxygen sensing in murine carotid body chemoreceptors. More recent data implicated abnormal H_2S-mediated chemoreceptor signaling in pathological conditions such as chronic heart failure and hypertension. However, the idea of H_2S as a mediator of oxygen-sensing in chemoreceptors has been challenged. In particular, it was shown that exogenous H_2S inhibited the release of neurotransmitters (ACh and ATP) from the cat carotid body, raising the possibility that there exists significant species difference in H_2S-mediated signaling in chemoreceptors. This study was designed specifically to determine the effect of H_2S on chemoreceptors in different species. We conducted multiunit extracellular recordings of the sinus nerve in the *ex vivo* carotid body preparation taken from the rat, the cat and the rabbit. As observed in the mouse carotid body, H_2S donors (NaHS or Na_2S) evoked qualitatively similar excitatory responses of the afferent sinus nerves of the species studied here. The excitatory effects of the H_2S donors were concentration-dependent and reversible. The sinus nerve responses to H_2S donors were prevented by blockade of the transmission between type I cells and the afferent terminals, as was the response to hypoxia. These results demonstrate that exogenous H_2S exerts qualitatively similar excitatory effects on chemoreceptor afferents of different species. The role of endogenous H_2S-mediated signaling in carotid body function in different species awaits further investigation.

1. Introduction

The carotid body plays an important role in the maintenance of oxygen homeostasis in mammals. It has long been established that the type I glomus cells within the carotid body are able to sense acute hypoxia and in turn release neurotransmitters (*e.g.*, ATP and acetylcholine) to activate the afferent fibers running in the sinus nerve, which relay the information to the respiratory centers in the brain stem to cause adaptive changes in ventilation and the restoration of oxygen homeostasis (Prabhakar and Peers, 2014; Prabhakar, 2013).

The type I glomus cells can potentially produce and release many types of neurotransmitters, including dopamine, acetylcholine (ACh), adenosine and ATP (Nurse, 2014). Using a functional co-culture model of rat type I cell clusters and petrosal neurones, Nurse and colleagues demonstrated that ATP and ACh were the key excitatory neurotransmitters released by type I cells in the carotid body (Zhang et al., 2000; Nurse, 2010). Rong et al. (2003) further demonstrated that the hypoxic ventilatory responses and hypoxia-evoked afferent sinus nerve discharge were markedly attenuated in mice deficient in the P2X2 and P2X2/3 receptors, suggesting a central role for ATP in the transmission of chemoreceptor signals.

The exact mechanisms by which the type I cells respond to hypoxia, *i.e.*, the process of oxygen-sensing, are being debated (Peers et al., 2010; Prabhakar, 2013). Emerging evidence suggests that the gasotransmitters, including nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S), might play certain roles in mediating or regulating the cellular response to acute hypoxia in oxygen-sensitive tissues including the pulmonary artery and the carotid body (see Olson (2014) and Prabhakar and Peers (2014) for review). Hydrogen sulfide (H_2S) is an environmental hazard but is also endogenously synthesized in mammalian tissues from L-cysteine by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3MST), and cysteine aminotransferase (CAT) (Wang, 2012; Kimura, 2014). We reported earlier that CBS and CSE were present in the mouse carotid body and the chemoreceptors were activated by the H_2S donor, NaHS. Pharmacological blockade of endogenous H_2S production remarkably attenuated the chemoreceptor responses to hypoxia. Furthermore,

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http://dx.doi.org/10.1016/j.resp.2015.01.002
1569-9048/

exogenous H_2S resembled the strong inhibition by hypoxia of the BK_{Ca} channel currents in type I glomus cells, whereas lowering endogenous H_2S prevented the inhibition of BK_{Ca} channel currents by hypoxia (Li et al., 2010). Kemp and colleagues also reported that H_2S inhibited recombinant human BK_{ca} channels in HEK293 cells and native BK_{ca} channels in rat type I glomus cells (Telezhkin et al., 2010; Kemp and Telezhkin, 2014). Peng et al. (2010) further demonstrated that CSE knock-out mice had deficiency in carotid body function. These data together suggest that H_2S might mediate type I cell response to hypoxia, possibly *via* inhibition of BK_{ca} channel current.

However, the idea that H_2S might mediate oxygen-sensing in chemoreceptors has been contested (Buckler, 2012; Haouzi et al., 2011). Fitzgerald et al. (2011) reported that the H_2S donor Na_2S reduced the release of ACh and ATP from the cat carotid body *in vitro via* opening the ATP-dependent K^+ (K_{ATP}) channel. These later data in particular drastically contradicted with the previous reports of excitatory effects of exogenous H_2S on mouse and rat chemoreceptors and raised the possibility that a significant species difference might exist in H_2S-mediated effects on chemoreceptors.

The present study has been designed specifically to determine the effects of exogenous H_2S on the carotid body in different species. We found that H_2S donors, NaHS and Na_2S, both activated the sinus nerve in the *ex vivo* carotid body preparations taken from the rat, the cat and the rabbit. H_2S acted on type I cells since the afferent response to H_2S donors diminished after blockade of synaptic transmission in the carotid body, as were the response to hypoxia.

2. Materials and methods

All procedures involving animals were approved by the Ethical Committee of Shanghai Jiaotong University School of Medicine. A total of 5 adult Sprague-Dawley rats (200–250 g), 4 adult New Zealand rabbits (2.2–2.5 kg) and 4 cats (1.8–2.5 kg) of either sex were used in this study. Animals were killed by an overdose of pentobarbital *via* intraperitoneal injection. The bilateral bifurcation regions of the carotid artery were removed under the dissecting microscope and placed in ice-cold Krebs solution (composition in mM: NaCl, 113; KCl, 5.9; NaH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; $CaCl_2$ 1.25; glucose, 11.5). The bifurcation was then transferred to a recording chamber (5 ml) and was superfused continuously with oxygenated (21% O_2 + 5% CO_2) Krebs (10 ml/min) and kept at 37 °C. The central cut end of the sinus nerve emanating from the bifurcation was cleared of the connective tissue and was recorded using a suction electrode. The multiunit neuronal signal was amplified (20,000×), band-pass filtered (200–3000 Hz) and then sampled at 20 kHz into a computer using the Spike 2 data acquisition and analysis program (Cambridge Electronic Design, Cambridge, UK). The multiunit afferent signal was window-discriminated to generate the mean firing rate (time constant = 10 s) and was plotted as the rate histogram.

The preparation was allowed to stabilize for 1 h and was then challenged with hypoxia by switching the superfusate to Krebs bubbled with a hypoxic gas mixture (5% CO_2 + 5% O_2 + 90% N_2) for 3 min. To study the effects of H_2S on the chemoreceptors, the preparation was superfused with Krebs containing NaHS (10–300 μM) or Na_2S (10–100 μM) for 3 min. Calcium-free Krebs solution (composition in mM: NaCl, 113; KCl, 5.9; NaH_2PO_4 1.2; $MgSO_4$ 2.4; $NaHCO_3$ 25; glucose, 11.5; EGTA 1 mM) was utilized to investigate whether H_2S acted pre- (*i.e.*, on type I cells) or post-synaptically (*i.e.*, on afferent terminals). It is worth of mentioning that NaHS and Na_2S Krebs were prepared just prior to use by diluting freshly made stock NaHS or Na_2S solution (1 M) in Krebs that had been equilibrated with 21% O_2 + 5% CO_2.

Statistical analysis was performed using the Graphpad Prism 5. Values are expressed as mean ± SEM. To assess whether a treatment had an effect on the sinus nerve, the mean change in discharge rate at different time points was compared using one-way repeated measures ANOVA with the Dunnet's test. The mean peak response to different concentrations of NaHS or Na_2S was compared using one-way ANOVA with the Bonferroni test. A P value of less than 0.05 is considered as statistically significant.

3. Results

3.1. H_2S stimulates chemoreceptor afferents in different species

We have previously shown that the H_2S donor NaHS was able to stimulate the sinus nerve of mouse carotid body *ex vivo* (Li et al., 2010). Here, we first examined whether NaHS would have similar excitatory effects on the sinus nerve of other species, including the rat, the cat and the rabbit. Fig. 1A shows a representative recording of the sinus nerve activity during hypoxia and in the presence of NaHS in a rat preparation. As expected, hypoxia (5% O_2) elicited a significant rise in sinus nerve activity from the mean baseline rate of 21.9 ± 7.4 spikes/s to a mean peak of 165.8 ± 66.9 spikes/s ($P < 0.01$, $n = 5$). The nerve activity returned to the baseline level after cessation of the hypoxic stimulation. Application of NaHS also evoked reversible and concentration-dependent increases in sinus nerve discharge with a mean peak increase of 17.9 ± 4.4 ($n = 6$, $P < 0.01$), 36.1 ± 16.1 ($n = 4$, $P < 0.01$) and 77.6 ± 19.7 ($n = 5$, $P < 0.01$) following 30, 100 and 300 μM NaHS, respectively (Figure 1B). Similarly, the other H_2S donor, Na_2S (10, 30 and 100 μM) caused concentration-dependent activation of the sinus nerve with a mean peak increase of 15.9 ± 11.5 ($n = 2$), 49.1 ± 16.7 ($n = 4$, $P < 0.01$) and 102.3 ± 42.5 spikes/s ($n = 4$, $P < 0.01$), respectively (Fig. 1C). Lower concentration of the H_2S donors (10 μM NaHS, 3 μM Na_2S) were also tested but did not have significant effects on the afferent nerve activity.

The potential effects of H_2S donors on the cat chemoreceptors were examined in 6 *ex vivo* carotid body preparations taken from 4 cats. Fig. 2A shows a representative recording of the changes in the mean discharge rate of the sinus nerve during hypoxia and in the presence of 100 μM NaHS or 100 μM Na_2S. As with hypoxia, both NaHS and Na_2S evoked robust increases in sinus nerve activity. The mean peak responses were 123.3 ± 22.7 spikes/s to hypoxia ($n = 6$, $P < 0.01$), 32.4 ± 6.8 spikes/s to 100 μM NaHS ($n = 6$, $P < 0.01$) and 61.7 ± 11.5 spikes/s to 100 μM Na_2S ($n = 6$, $P < 0.01$), respectively. Lower concentration of the H_2S donors only caused minimal changes in the discharge rate of the sinus nerves with a mean peak increase of 2.7 ± 0.9 spikes/s to 30 μM NaHS ($n = 6$, $P > 0.05$) and 6.2 ± 2.7 spikes/s to 30 μM Na_2S ($n = 5$, $P > 0.05$), respectively.

We then tested the effects of hypoxia and H_2S donors on rabbit chemoreceptors in 7 *ex vivo* carotid body preparations taken from 4 rabbits. Again, like hypoxia, NaHS and Na_2S both caused marked increases in the mean discharge rate of the sinus nerve (Fig. 3A). The mean peak responses were 86.4 ± 21.2 spikes/s to hypoxia ($n = 5$, $P < 0.01$), 48.5 ± 17.9 spikes/s to 100 μM NaHS ($n = 4$, $P < 0.01$) and 78.7 ± 18.0 spikes/s to 100 μM Na_2S ($n = 6$, $P < 0.01$), respectively. Other concentration of the H_2S donors was also tested. 30 μM NaHS and 30 μM Na_2S caused a mean peak increase of afferent activity by 8.5 ± 1.3 spikes/s ($n = 3$, $P < 0.05$) and 13.3 ± 7.6 ($n = 6$, $P < 0.01$), respectively.

3.2. H_2S acts on type I cells

We have previously shown that H_2S donors activated the mouse sinus nerve by acting on the type I cells rather than the afferent