Protective effect of hydrogen sulfide on hypoxic respiratory suppression in medullary slice of neonatal rats

Ji-Gang Pan, Hai-Yan Hu [1], Jie Zhang, Hua Zhou, Li Chen, Yu-Hong Tang, Yu Zheng *

Department of Physiology, West China School of Preclinical and Forensic Medicine, Sichuan University, 3-17 Renmin South Road, Chengdu, 610041 Sichuan, PR China

ABSTRACT

Hydrogen sulfide (H_2S) has been shown to play a protective role in injury of cells induced by hypoxia. Little is known however about its effect on medullary hypoxia-induced rhythmic respiratory suppression. In the present study, a decrease in frequency of rhythmic discharge of hypoglossal rootlets was observed in medullary slices of neonatal rats perfused with 95% N_2–5% CO_2 to produce hypoxia. Perfusion with NaHS (H_2S donor) prevented the inhibitory effect of hypoxia on the burst activity of the rootlets, whereas such action of NaHS was suppressed by pretreatment with glibenclamide, a blocker of K_{ATP} channels. In addition, the increase in malondialdehyde content and the up-regulation of *c-fos* mRNA expression of the slices induced by hypoxia was significantly reduced by NaHS. These results indicate that exogenous H_2S may protect the medullary respiratory center against hypoxic injury via activation of K_{ATP} channels, reduction of lipid peroxidation and down-regulation of *c-fos*.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Recent studies indicate that hydrogen sulfide (H_2S) is a third gaseous messenger along with nitric oxide and carbon monoxide (Abe and Kimura, 1996; Wang, 2003; Qu et al., 2008). Physiological concentrations of H_2S can selectively enhance the induction of NMDA receptor-mediated long-term potentiation by increasing cAMP levels (Kimura, 2000). H_2S can also relax smooth muscle by activating the K_{ATP} channel (Wang, 2003). In addition, H_2S has been shown to protect the heart, kidney, liver, lung and brain in ischemia/reperfusion or hypoxic pulmonary hypertension models (Geng et al., 2004; Jha et al., 2008; Wei et al., 2008; Florian et al., 2008; Bos et al., 2009). H_2S can increase the viability of rat cardiac cells in hypoxia-induced cell injuries (Chen et al., 2009). Preinhalation of moderate H_2S also improved the survival rate of mice exposed to hypoxia (Blackstone and Roth, 2007). Our previous work showed that the endogenous H_2S synthase, cystathionine β-synthase (CBS), was present in the medullary respiratory center and exogenous H_2S could affect respiratory activities in a diphasic pattern manifested as an initial inhibition followed by excitation in medullary slices of neonatal rats (Hu et al., 2008), indicating that H_2S could be involved in the central control of rhythmic respira-

tion. Some respiratory disorders, such as apnea of newborn and central respiratory apnea, may be associated with hypoxic injuries of the respiratory centers. Whether H_2S could play a protective role in the respiratory disorders induced by medullary hypoxia remains unclear so far.

In our previous study, activation of K_{ATP} channels and the neuronal adenyl cyclase/cAMP pathway were found to be involved in the respiratory regulation by H_2S in medullary slices of neonatal rats (Hu et al., 2008). K_{ATP} channels play a pivotal role suppressing hypoxia/ischemia insults in the central neural system by limiting Ca^{2+} influx and release of glutamate and the apoptotic factor cytochrome *c* (Garcia et al., 1999; Liu et al., 2002). H_2S can also act as an antioxidant by increasing the level of glutathione and the activity of superoxide dismutase (Searcy et al., 1995; Kimura and Kimura, 2004). H_2S can protect rat cardiac cells against hypoxia/ischemic-induced oxidative stress injuries through scavenging reactive oxygen species (ROS) and lipid peroxidation production of malondialdehyde (MDA) (Geng et al., 2004; Chen et al., 2009).

It has been reported that maximal depression in burst frequency of medullary slices of mice was between 5 and 15 min during hypoxia (Ramirez et al., 1997). The immediate early gene, *c-fos*, as a marker of neuronal activity, underwent dramatic and transient expression in rapid response to a diversity of stimuli. Significant induction of *c-fos* mRNA was seen with hypoxic exposure as short as 15 min and the effects persisted for about 10 h in PC-12 cells (Prabhakar et al., 1995). In addition, *c-fos* and *bcl-2* genes were reported to participate in responses of neurons to

* Corresponding author. Tel.: +86 28 8550 3433; fax: +86 28 8550 3204.
  E-mail address: yzheng@scu.edu.cn (Y. Zheng).
  [1] Present address: Department of Physiology, Chengdu Medical College, Chengdu, Sichuan, PR China.

hypoxia/ischemia, the former promoted and the latter prevented neuronal injuries (Heurteaux et al., 1993; Chen et al., 1997).

In the present work, the *in vitro* medullary slices of neonatal rats were used to determine whether H_2S could protect the medullary respiratory centers subjected to hypoxia insults, and whether K_{ATP} channels, antioxidation and some immediate early genes are involved in the protective action of H_2S.

2. Methods

2.1. Animals

The experiments were performed on medullary slices of male or female neonatal (0–4 days) Sprague–Dawley rats. All procedures were reviewed and approved by the Sichuan University Committee on the Use of Live Animals in Research, and conformed to the Principles of Laboratory Animal Care (NIH publication No. 86-23 revised in 1985).

2.2. Electrophysiological studies

Medullary slices were prepared as described elsewhere (Ramirez et al., 1997; Hu et al., 2008). In brief, the brainstem was isolated in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 128 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgSO_4$, 24 $NaHCO_3$, 0.5 NaH_2PO_4, and 30 D-glucose equilibrated with carbogen (95% O_2–5% CO_2) at pH 7.4. The brainstem was secured in a vibratome with the caudal end up and sectioned. An 800–900 μm thick slice containing the pre-Bötzinger complex, a critical site for generation of the respiratory rhythm (Smith et al., 1991), was obtained, corresponding to the level of medulla from about 100 μm caudal to 700 μm rostral to the obex. The slice was then transferred immediately to a recording chamber and was continuously perfused with ACSF (28–29 °C) bubbled with 95% O_2–5% CO_2 at a rate of 9–10 ml/min. The preparation was allowed to stabilize for 30 min before starting experiments. Glass suction electrodes filled with ACSF were used to record the rhythmic respiratory activity from the cut ends of hypoglossal rootlets. Signals were amplified, filtered ($\tau = 0.001$ s, $F = 1$ kHz) and integrated with a time constant of 50 ms by BL-420 E^+ biological signal processing system (Taimeng Biotech. Co., China). The frequency and integrated amplitude of the burst discharge of the hypoglossal rootlets were analyzed. The slices were randomly divided into 4 groups ($n = 6$ for each): control (ACSF), N_2 (95% N_2–5% CO_2, hypoxia), N_2 + NaHS (200 μM) and N_2 + NaHS + glibenclamide (100 μM). NaHS (donor of H_2S) and glibenclamide (blocker of K_{ATP} channels, Gl) were purchased from Sigma (USA); other chemicals and reagents were of analytical grade. In the control group, slices were perfused with ACSF bubbled with 95% O_2–5% CO_2 throughout. In the N_2 group, slices were perfused with 95% N_2–5% CO_2 for 10 min after perfusion with 95% O_2–5% CO_2 ACSF for 8 min. In the N_2 + NaHS group, slices were perfused with 95% O_2–5% CO_2 and NaHS for 10 min after 8-min perfusion with 95% N_2–5% CO_2 and NaHS. In the N_2 + NaHS + Gl group, slices were perfused with Gl for 5 min, and then with Gl plus NaHS for 8 min before 10 min hypoxic insult plus Gl and NaHS. The slices subjected to hypoxia were exposed to 95% O_2–5% CO_2 for 10 min to restore normal O_2 supply at the end of N_2 perfusion. The total recording time of hypoglossal rootlet activity was 33 min.

2.3. Biochemical assay of MDA content

The slices were divided into 3 groups ($n = 6$ for each): control, N_2 and N_2 + NaHS. The discharge of hypoglossal rootlets was recorded as described above but without post-perfusion with 95% O_2–5% CO_2 ACSF. The slices were collected immediately and stored in a freezer (−76 °C). The slices were sagittally separated into two half parts.

One part was used to measure the MDA content. The other part was analyzed for *c-fos* and *bcl-2* mRNA level as described in the following section. The MDA content in the slices was measured with the thiobarbituric acid assay. The optical density value at 532 nm was measured with a spectrophotometer. The level of MDA was expressed as nanomoles per milligram protein.

2.4. Real-time RT-PCR analysis of c-fos and bcl-2 mRNA level

Total RNA extracted from the other halves of the slices with Trizol (Invitrogen, USA) was reversely transcribed into cDNA with Revert Aid™ First Strand cDNA Synthesis Kit (MBI, Lithuania). Aliquot of diluted first-strand cDNA was PCR amplified with a Real-Time PCR Detection System (FTC2000, Funglyn, Canada) as described (Jiang et al., 2007). The primers for *c-fos*, *bcl-2* and *β-actin* genes were as follows: (i) *c-fos*: forward 5′-ATCCGAAGGGAAAGGAATAA-3′; reverse 5′-TCTGGGAAGCCCAGGTCAT-3′; (ii) *bcl-2*: forward 5′-GATTGTGGCCTTCTTTGAGTT-3′; reverse 5′-AGTTCCACAAAGGCATCCCA-3′; (iii) *β-actin*: forward 5′-GCCAACACAGTGCTGTCT-3′; reverse 5′-AGGAGCAATGATCTTGATCTT-3′. The relative levels of each gene were calculated by the $2^{-\Delta\Delta CT}$ method (Jiang et al., 2007). *β-actin* was used as housekeeping gene. Each qRT-PCR assay was performed at least three times.

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. The significance of differences between groups was determined by repeated-measures ANOVA or one-way ANOVA followed by post hoc least significant difference (LSD) test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of H₂S on changes in burst discharge of the slices induced by hypoxia

There was no significant change in burst frequency of medullary slices in the control group ($P > 0.05$, Fig. 1Aa and Ba). When slices were made hypoxic by perfusion of ACSF bubbled with 95% N_2–5% CO_2, the burst frequency was increased after 2 min and then decreased by 7 min during hypoxia and 2 min post-hypoxia compared with the pre-control ($P < 0.05$, Fig. 1Ab and Bb). The percent change in frequency was decreased significantly at 7–8 min of hypoxia and at 2–3 min of post-hypoxia compared with the control group ($P < 0.05$, Fig. 1Ca). These results indicate that an inhibitory response of the burst discharge of the slices could be induced by hypoxia.

Perfusion of NaHS (200 μM) increased the burst frequency from the 6th min during NaHS perfusion alone to the 4th min during perfusion with N_2 plus NaHS compared to the pre-control ($P < 0.05$, Fig. 1Ac and Bc). The percent change in frequency was increased at the 2nd, 7th–8th min before hypoxia, at the 1st, 3rd, and 6th–10th min with hypoxia and at the first 2 min of post-hypoxia compared with the hypoxia group ($P < 0.05$, Fig. 1Ca). Thus H_2S could exert an excitatory effect on the respiratory activity and reverse the inhibitory effect of the burst discharge of the slices induced by hypoxia, suggesting that it could protect the medullary respiratory centers against hypoxic injury.

No significant changes were observed in the integrated burst amplitude of the slices in the control, N_2 and N_2 + NaHS groups versus pre-control ($P > 0.05$), as shown in Fig. 1Aa, b and c, respectively. Compared with the control group, the integrated burst amplitude decreased only at the 8th min with hypoxia in the N_2 group ($P < 0.05$,