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Hydrogen sulfide attenuates hypoxia-induced respiratory suppression in anesthetized adult rats

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ABSTRACT

Our previous study *in vitro* showed that hydrogen sulfide (H_2S) could protect the medullary respiratory centers from injury induced by acute hypoxia in brainstem slices of neonatal rats. The present study was carried out to determine if H_2S could exhibit similar protective effects in adult rats and to explore the underlying mechanisms of its protection. It was observed that hypoxia induced a diphasic respiratory response, an excitatory phase followed by an inhibitory one, as indicated by an increase followed by a decrease in frequency of rhythmic discharge of the diaphragm. Nissl staining revealed that some of the neurons in the medullary respiratory related nuclei were impaired in hypoxia rats. Hypoxia led to increases in the content of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), as well as a decrease in the level of Bcl-2 mRNA of the medulla oblongata. Intracerebroventricular injection of 2.5 mM NaHS (a donor of H_2S) or L-cysteine (L-Cys, a substrate for H_2S) could prevent inhibitory respiratory effect occurred in the rats with hypoxia. Exogenous application of NaHS and L-Cys could also reduce the content of MDA and the activities of SOD and GSH-Px, and increase the level of Bcl-2 mRNA expression of medulla oblongata caused by hypoxia. These results indicate that H_2S could protect the medullary respiratory centers against injury induced by acute hypoxia in adult rats partly due to its anti-oxidant and anti-apoptotic effects.

1. Introduction

In recent years, hydrogen sulfide (H_2S) has been recognized as the third endogenous gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO) (Abe and Kimura, 1996; Szabo, 2007; Wang, 2012). In mammalian tissues, H_2S can be endogenously produced from L-cysteine (L-Cys) catalyzed by three enzymes: two pyridoxal-5′-phosphate (PLP)-dependent enzymes, including cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS), and a PLP-independent enzyme, namely 3-mercaptopyruvate sulfur-transferase (3MST) (Stipanuk and Beck, 1982; Kimura, 2002; Chiku et al., 2009; Shibuya et al., 2009). CSE is mainly expressed in the cardiovascular system, liver, and kidney (Kimura, 2014). CBS has been found in the central nervous system (CNS), especially in the hippocampus and the cerebellum (Abe and Kimura, 1996). 3MST is localized to neurons (Shibuya et al., 2009). H_2S is usually stored as bound sulfane sulfur in neurons and astrocytes (Ishigami et al., 2009). Under the conditions of neuronal excitation or other stimulations, free H_2S is released from the bound sulfane sulfur (Tan et al., 2010). Free H_2S is mainly oxidized to thiosulfate, sulfite and sulfate; it can also be methylated to methanethiol and dimethylsulfide or be bound to methemoglobin (Lowicka and Beltowski, 2007; Tan et al., 2010).

H_2S has been known to play an important role in both physiological and pathological processes in various organs. A number of biological effects have been ascribed to H_2S such as neuromodulator, smooth muscle regulator, anti- or pro-inflammatory mediator, and antioxidant (Wang, 2012). Protective effects of H_2S on heart, kidney, liver, and brain in animal models of ischemia have been confirmed (Geng et al., 2004; Jha et al., 2008; Florian et al., 2008; Bos et al., 2009). It has also been shown to protect lung in hypoxic pulmonary hypertension models and to increase the viability of cardiac cells in hypoxic rats (Wei et al., 2008; Chen et al., 2009). H_2S is a powerful antioxidant which 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 is suggested that the respiratory activity could be affected by H_2S (Beauchamp et al., 1984; Reiffenstein et al., 1992). Our

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previous work showed that H_2S was involved in the central control of rhythmic respiration by opening ATP-sensitive potassium (K_{ATP}) channels and activating adenylyl cyclase-cyclic adenosine monophosphate (AC-cAMP) pathway of medullary neurons (Hu et al., 2008; Chen et al., 2013a,b), and exogenous and endogenous H_2S could prevent respiratory suppression induced by hypoxia via reduction of MDA content and down-regulation of c-fos mRNA expression (Pan et al., 2010, 2011), in *in vitro* medullary slices of neonatal rats. We also found that exogenous and endogenous H_2S was involved in the control of rhythmic respiration in adult rats *in vivo* (Li et al., 2014). However, little is known about the effects and underlying mechanisms of H_2S on the respiratory response induced by hypoxia in adult rats. The present study was undertaken to investigate if exogenous and endogenous H_2S could attenuate the respiratory suppression response to hypoxia and the underlying mechanisms in anesthetized adult rats.

2. Materials and methods

2.1. Animals and agents

Adult male or female Sprague-Dawley (SD) rats with body weight of 180–250 g were provided by the Experimental Animal Center of Sichuan University. All experimental procedures were compliant with the Sichuan University Committee Guidelines on the Use of Live Animals in Research, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 2010. NaHS, L-Cys, NH_2OH (an inhibitor for CBS), and pontamine sky blue were purchased from Sigma–Aldrich (USA).

2.2. Electrophysiological experimental procedures

Rats were anesthetized by intraperitoneal injection of 10% urethane (13 ml/kg body weight), then the trachea was cannulated and pairs of electrodes (made of 0.5 mm enamel-insulated stainless wires with tips exposed for 1–2 mm) were inserted into the diaphragm through an abdominal incision that was subsequently closed (Takeda and Matsumoto, 1998). The rats were then mounted on a stereotaxic apparatus with blunt ear bars. The skull was exposed and a hole was drilled for reaching the right lateral cerebral ventricle (coordinates: 1 mm posterior to bregma, 1.5 mm lateral to midline, and 4.0 mm vertical from the skull surface; Paxinos and Watson, 2005) to place a microsyringe for injection of chemicals. The average duration of surgery was approximately 40 min. At the end of the experiment, the rats were intracerebroventricularly injected with 5 μl of pontamine sky blue to visually confirm the injection site.

After approximately 30 min recovery from surgery, the rhythmic discharge of the diaphragm of each rat was measured and recorded via BL-420S Biological Signal Processing System (Taimeng Biotech Co., Chengdu, China). To study the effects of exogenous and endogenous H_2S on respiration of the rats with hypoxia, the animals were divided into four groups: hypoxia ($n = 7$), hypoxia + 2.5 mM NaHS ($n = 7$), hypoxia + 2.5 mM L-Cys ($n = 8$), and hypoxia + 2.5 mM L-Cys + 10 mM NH_2OH ($n = 6$). The rhythmic discharge of the diaphragm was recorded for 10 min as the baseline activities (pre-control) in each group before hypoxia. In the group of hypoxia, the rats were exposed to 8% O_2–92% N_2 for 60 min and then inhaled air for 40 min for recovery. In the other 3 groups, in addition to hypoxia, the rats were intracerebroventricularly injected with 5 μl of 2.5 mM NaHS, 2.5 mM L-Cys, and 2.5 mM L-Cys + 10 mM NH_2OH, respectively, at the 5th min from start of hypoxia; the control rats were injected with equal volume of solvent (saline) of the chemicals. The burst duration (BD), burst interval (BI), burst frequency (BF) and integrated amplitude (IA) of the diaphragmatic discharge of the animals were analyzed every 5 min during the whole 100 min experimental process.

2.3. Nissl staining

To analyze the damage of hypoxia on Nissl bodies of neurons in the medulla oblongata, the rats were divided into five groups ($n = 6$ for each group): control (without hypoxia and with intracerebroventricular solvent injection), hypoxia (with hypoxia and solvent injection), hypoxia + 2.5 mM NaHS, hypoxia + 2.5 mM L-Cys, and hypoxia + 2.5 mM L-Cys + 10 mM NH_2OH. The rhythmic discharge of the diaphragm was recorded as described above and then the rats were quickly perfused with normal saline, followed by 4% paraformaldehyde. The medulla oblongata were obtained, post-fixed in 4% paraformaldehyde, and stored in 30% sucrose solution at 4 °C until the specimens sank to the bottom of the container. The tissues were frozen and sectioned at 25 μm thickness in a freezing microtome. The sections were stained with 1% thionine. The neurons of pre-Bötzinger complex (pre-BötC), nucleus ambiguus (Amb), nucleus of solitary tract (NTS), facial nucleus (FN), and hypoglossal nucleus (12N) were examined under light microscope.

2.4. Biochemical analysis

For biochemical analysis, the rats were divided into five groups ($n = 6$ for each group): control, hypoxia, hypoxia + 2.5 mM NaHS, hypoxia + 2.5 mM L-Cys, and hypoxia + 2.5 mM L-Cys + 10 mM NH_2OH. When the hypoxia was finished, the medulla oblongata was immediately removed and washed in saline solution, and then stored at −80 °C. The content of MDA and the activities of SOD and GSH-Px were measured using test kits (Nanjing Jiancheng Bioengineering Institute, China).

2.5. Reverse transcriptase–polymerase chain reaction

For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, the rats were divided into five groups ($n = 8$ for each group): control, hypoxia, hypoxia + 2.5 mM NaHS, hypoxia + 2.5 mM L-Cys, and hypoxia + 2.5 mM L-Cys + 10 mM NH_2OH. The discharge of diaphragm was recorded as described above and then the medulla oblongata tissues were quickly harvested. Levels of Bcl-2, Bax and Caspase-3 mRNA were analyzed by RT-PCR. Total mRNA from medulla oblongata tissues was extracted using TRIzol reagent (Invitrogen, USA). Approximately 3 μg of each RNA sample was reversely transcribed into cDNA. The sequences of primers, annealing temperatures, and annealing times were as follows: β-actin (432 bp; 58 °C; 30 s), sense: 5′-TCA GGT CAT CAC TAT CGG CAA T-3′, antisense: 5′-AAA GAA AGG GTG TAA AAC GCA-3′; Bcl-2 (440 bp; 55 °C; 40 s), sense: 5-′GGC ATC TTC TCC TTC CAG C-3′, antisense: 5′-TCC CAG CCT CCG TTA TCC-3′; Bax (258 bp; 52.5 °C; 30 s), sense: 5′-CAT CCA GGA TCG AGC AGA-3′, antisense: 5′-AAG TAG AAG AGG GCA ACC AC-3′; Caspase-3 (217 bp; 49 °C; 30 s), sense: 5′-AAC GAA CGG ACC TGT GG-3′, antisense: 5′-GGG TGC GGT AGA GTA AGC-3′. The PCR mixture was amplified for 35 cycles. The RT-PCR products were electrophoresed on an agarose gel and the statistical analyses were performed.

2.6. Statistical analysis

All data were expressed as mean ± S.E.M. The significance of differences in the same group was analyzed with repeated measures analysis of variance (ANOVA) followed by the LSD test. The significance of differences between groups was determined by one-way ANOVA. $P < 0.05$ was considered statistically significant.