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Chronic intermittent hypoxia promotes expression of 3-mercaptopyruvate sulfurtransferase in adult rat medulla oblongata

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ABSTRACT

The present experiments were carried out to investigate the expression of 3-mercaptopyruvate sulfurtransferase (3MST) in medulla oblongata of rats and effects of chronic intermittent hypoxia (CIH) on its expression. Sprague Dawley adult rats were randomly divided into two groups, including control (Con) group and CIH group. The endogenous production of hydrogen sulfide (H_2S) in medulla oblongata tissue homogenates was measured using the methylene blue assay method, 3MST mRNA and protein expression were analyzed by RT-PCR and Western blotting, respectively, and the expression of 3MST in the neurons of respiratory-related nuclei in medulla oblongata of rats was investigated with immunohistochemical technique. CIH elevated the endogenous H_2S production in rat medulla oblongata ($P < 0.01$). The RT-PCR and Western blotting analyses showed that 3MST mRNA and protein were expressed in the medulla oblongata of rats and CIH promoted their expression ($P < 0.01$). Immunohistochemical staining indicated that 3MST existed in the neurons of pre-Bötzinger complex (pre-BötC), hypoglossal nucleus (12N), ambiguous nucleus (Amb), facial nucleus (FN) and nucleus tractus solitarius (NTS) in the animals and the mean optical densities of 3MST-positive neurons in the pre-BötC, 12N and Amb, but not in FN and NTS, were significantly increased in CIH group ($P < 0.05$). In conclusion, 3MST exists in the neurons of medullary respiratory nuclei and its expression can be up-regulated by CIH in adult rat, suggesting that 3MST–H_2S pathway may be involved in regulation of respiration and protection on medullary respiratory centers from injury induced by CIH.

1. Introduction

Chronic intermittent hypoxia (CIH) is a very common physiological and pathological process, which may contribute to several chronic diseases and clinical conditions, such as heart failure (Giordano, 2005), inflammatory liver injury (Savransky et al., 2007), chronic obstructive pulmonary diseases (Chen et al., 2005), obstructive sleep apnea (OSA) (Xu et al., 2004), etc. In mammals, hypoxia often results in deleterious effects on central nervous system (CNS) including the respiratory centers. Neuronal structure and function of the brain were damaged or even dead after hypoxic/ischemic challenges (Kaur et al., 2011; Ding et al., 2013; Ogunshola and Antoniou, 2009), which may further affect the function of the CNS.

Hydrogen sulfide (H_2S) was known to be a toxic gas and an environmental hazard for many decades. However, it is now recognized that H_2S may serve as a gaseous mediator that is endogenously produced to influence biological functions in mammalian. Thus, H_2S is a physiologically important molecule and it has been referred to as the third gaseous mediator alongside nitric oxide (NO) and carbon monoxide (CO). The past decade has seen an exponential growth of scientific interest in the physiological and pathological significance of H_2S especially with respect to its role in the CNS. Endogenous H_2S facilitates long-term potentiation (Abe and Kimura, 1996), and regulates intracellular calcium concentration and pH level in brain cells. In addition, H_2S can relax smooth muscle (Hosoki et al., 1997; Zhao et al., 2001; Zhao and Wang, 2002), and functions as a physiologic vasodilator and regulator of blood pressure (Yang et al., 2008). More importantly, H_2S produces antioxidant, anti-inflammatory, and anti-apoptotic effects that may have relevance to neurodegenerative disorders (Hu et al., 2009; Hu et al., 2010). Therefore, H_2S is considered to be an important neuromodulator and neuroprotectant in the CNS.

H_2S is found to be produced endogenously in various parts of the body such as the heart (Geng et al., 2004), the blood (Zhao et al., 2001) and the CNS (Warenycia et al., 1989). The endogenous production of H_2S is controlled by three enzymes, namely cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and a newly identified enzyme, 3-mercaptopyruvate sulfurtransferase (3MST) (Shibuya et al., 2009). The expression of H_2S-producing enzymes is tissue specific. CBS is primarily found in the central nervous system, while CSE is

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mainly expressed in peripheral tissues (Zhao et al., 2003), and 3MST is localized to neurons in the CNS (Shibuya et al., 2009). CBS was traditionally believed to be the sole H_2S-producing enzyme in the brain. However, new evidence has demonstrated that the amount of H_2S produced by 3MST in the brain homogenates of CBS knockout mice is similar to that of wild-type mice, and 3MST produces H_2S more efficiently than does CBS (Shibuya et al., 2009). Shibuya et al. thought that 3MST produces greater quantities of neuronal H_2S compared to CBS and contributes to ~90% of total H_2S production in the brain (Shibuya et al., 2009). Thus, 3MST is maybe a more important H_2S-producing enzyme in the brain. Our previous work had showed that CBS was present in the medullary respiratory centers and H_2S could be involved in the central control of rhythmic respiration (Hu et al., 2008). In addition, both endogenous and exogenous H_2S may protect the medullary respiratory centers against hypoxic injury in medullary slices of neonatal rats (Pan et al., 2010, 2011). However, whether 3MST was also present in the medullary respiratory centers and H_2S produced under action of 3MST in medulla oblongata was involved in the respiratory response and protective effects of respiratory centers against injury induced by chronic hypoxia was still unknown.

The aim of the present work was to study the expression of 3MST in the medulla oblongata of rats and effects of CIH on its expression.

2. Materials and methods

2.1. Experimental design

Adult male Sprague Dawley (SD) rats weighing 200–250 g were obtained from the Experimental Animal Center of Sichuan University. All experimental procedures were compliant with the Sichuan University Committee on the Use of Live Animals in Research, which is in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 2010.

Sixty animals were randomly divided into two groups. (1) Con group ($n = 30$): the rats were maintained in normal air (21% O_2) in a plexiglass chamber for 6 h daily for 14 days. (2) CIH group ($n = 30$): the CIH model was achieved according to the method described by Xue et al. (1988). The rats were exposed to hypoxia (10% O_2 -90% nitrogen) in a plexiglass chamber 6 h daily for 14 days. Immediately after the last hypoxia treatment, arterial blood gas analysis of 5 randomly chosen rats from each of the two groups was performed according to the method of Gueye et al. (2001) to examine whether the model of hypoxia was established successfully. Each group of animals was divided into four subgroups: group for Nissl staining and immunohistochemical staining ($n = 6$); group for measurement of endogenous production of H_2S ($n = 8$); group for RT-PCR assay for expression of 3MST mRNA ($n = 8$); and group for Western blot analysis for the expression of 3MST protein ($n = 8$).

2.2. Nissl staining

The rats were deeply anesthetized with 10% chloral hydrate (3 ml/kg body weight, i.p.) and perfused transcardially with 200 ml of normal saline and then with 300 ml of 4% paraformaldehyde (Sigma, USA) in phosphate buffered saline (PBS) for 1 h for fixation. The medulla oblongata was removed and post-fixed in 4% paraformaldehyde for 4 h. Then, the specimens were processed and embedded in paraffin. Transverse serial sections were made at the level of the medulla from about 800 μm caudal to 3000 μm rostral to the obex and the blocks were cut at intervals of 35 μm with 5 μm in thickness of each section. For each level, two sections were obtained, one of which was stained with 0.5% thionine (T7029, Sigma, USA) and the other one was stained for the immunohistochemical study. The nuclei of the medulla oblongata, including the pre-Bötzinger complex (pre-BötC), hypoglossal nucleus (12N), ambiguous nucleus (Amb), facial nucleus (FN) and nucleus tractus solitarius (NTS), were identified according to the rat brain stereotaxic atlas (Watson and Paxinos, 2005), and the neurons of the nuclei were examined under a light microscope. Images were captured using a Mshot color video camera (MD50, China) mounted on an Olympus microscope (CX41, Japan).

2.3. Measurement of endogenous production of H_2S

The rats were anesthetized and then decapitated. Fresh medulla oblongata was dissected and immediately frozen in liquid nitrogen. The endogenous production of H_2S by medulla oblongata tissue homogenates was measured using the methylene blue assay method (Zhao et al., 2001; Stipanuk and Beck, 1982; Cheng et al., 2004) with slight modifications. In brief, medulla oblongata tissues were homogenized in a 10-fold volume of ice-cold potassium phosphate buffer (pH 7.4). The tissue homogenates (0.1 ml) were incubated with L-cysteine (10 mM), α-ketoglutarate (2.5 mM) and pyridoxal-5-phosphate (2 mM). The final volume was 1 ml. A 20 ml tube containing two pieces of filter paper (1 × 2 cm) soaked with zinc acetate (1%; 0.5 ml) was put inside the vial. The vial was then flushed with a slow stream of nitrogen gas for 20 s and then capped with an airtight serum cap. The vials were then transferred to a 37 °C shaking water bath. After 90 min, trichloroacetic acid (TCA; 50%; 0.5 ml) was injected into the reaction mixture through the serum cap. Another 60 min was allowed for the trapping of evolved H_2S by the Zn acetate solution as Zn sulfide. Then the serum cap was removed into a tube that contained 3 ml water and N,N-dimethyl-p-phenylenediamine sulphate (20 mmol/l; 0.5 ml) in 7.2 mol/l HCl and $FeCl_3$ (30 mmol/l; 0.4 ml) in 1.2 mol/l HCl was added to the inner tube. After 20 min, absorbance at 670 nm was measured with spectrophotometry to represent the levels of H_2S.

The calibration curve of absorbance versus H_2S concentration was obtained by using NaHS (Sigma, USA) solution of varying concentrations. The calibration curve was linear from 0 to 1 mmol/l NaHS.

2.4. RT-PCR analysis

Fresh medulla oblongata tissues were obtained from anesthetized rats and immediately frozen in liquid nitrogen. The expression of 3MST mRNA was quantified by reverse transcription-polymerase chain reaction (RT-PCR) using GAPDH mRNA as an internal standard. Total RNA was extracted from medulla oblongata tissue, and samples of total RNA were quantified by reading the optical density at 260 nm. Three micrograms of total RNA from each sample was processed directly to cDNA with the First Strand cDNA Synthesis Kit (Fermentas, USA), according to the manufacturer's instructions. The primer sequences used in this study were as follows: 3MST forward, 5′-ACATCC CGGCTCAGTAAACA-3′ and reverse, 5′-TGTGTCCTTCACAGGGTCTTC-3′; and GAPDH forward, 5′-ACAGCAACAGGGTGGTGGAC-3′ and reverse, 5′-TTTGAGGGTGCAGCGAACTT-3′. The amplification reactions were carried out for 35 cycles as follows: for 3MST, denaturation at 94 °C for 30 s; annealing at 60 °C for 30 s; and extension at 72 °C for 35 s; and for GAPDH, denaturation at 95 °C for 30 s; annealing at 60 °C for 30 s; and extension at 72 °C for 20 s. These reactions for 3MST and GAPDH yielded products of 292 bp and 252 bp, respectively. The PCR products were separated on 1.5% agarose gel stained with Gelview.

2.5. Western blot analysis

Fresh medulla oblongata obtained from anesthetized rats were lysed in ice-cold Radio Immunoprecipitation Assay (RIPA) lysis buffer containing: 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 50 mM Tris–Cl, pH 7.4, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin. The lysates were centrifuged at 12,000 *g* for 15 min at 4 °C, and the resulting supernatants were determined by Bio-Rad assay kit. Supernatant samples (50 μg/μl) were prepared for Western blot with loading buffer. Samples were separated on a 12.5% SDS-PAGE gel for