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Hydrogen sulfide protects neonatal rat medulla oblongata against prenatal cigarette smoke exposure via anti-oxidative and anti-inflammatory effects

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ABSTRACT

We previously demonstrated that hydrogen sulfide (H_2S) protected neonatal rat medulla oblongata from prenatal cigarette smoke exposure (CSE) via anti-apoptotic effect. The present work further investigated the involvement of anti-oxidative and anti-inflammatory effects of H_2S in the protection. Pregnant Sprague-Dawley rats were randomly divided into NaCl, CSE, CSE + NaHS (a donor of H_2S) and NaHS groups. All the tests were performed with corresponding neonatal rats. Nissl staining revealed that NaHS treatment ameliorated neuronal chromatolysis in the hypoglossal nucleus and nucleus ambiguus resulted from prenatal CSE. Moreover, NaHS eliminated decrease of glutathione level, increase of malondialdehyde content and inhibition of superoxide dismutase activity within neonatal rat medulla oblongata caused by prenatal CSE. NaHS also relieved the up-regulation of tumor necrosis factor-α, interleukin-1β and interleukin-6 in the medulla oblongata of the neonatal CSE rats. These results suggest that H_2S can alleviate prenatal CSE-induced injuries of neonatal rat medulla oblongata through anti-oxidative and anti-inflammatory effects.

1. Introduction

Maternal smoking continues to be a public health concern for its adverse impacts. Prenatal cigarette smoke exposure (CSE) has been reported to cause low birth weight, induce malformation and increase the risk of sudden infant death syndrome (SIDS). Cigarette smoke contains more than 4000 harmful gradients, among which nicotine and carbon monoxide (CO) are shown as the major ones in the context of prenatal cigarette smoke exposure, as they could rapidly cross the placental barrier. Nicotine was reported to promote contraction of uterine arteries (Xiao et al., 2007), which may decrease uterine blood flow and thus lead to insufficient blood supply to fetus. CO can bind to myoglobin and hemoglobin, which respectively impairs cardiac output and reduces blood oxygen saturation, resulting in ischemia and hypoxia. The resulting ischemia and hypoxia further disturb the development of brain, including medulla oblongata (Wixey et al., 2011), which accommodates the centers for life maintenance. Clinic investigation discovered close link of prenatal CSE to medullary developmental abnormalities, such as hypoplasia and neuronal immaturity of the hypoglossal nucleus (Ottaviani et al., 2006). Besides, animal experiments showed that prenatal exposure to nicotine altered nicotine receptor subtypes in nucleus ambiguus (Kamendi et al., 2006). Furthermore, prenatal CSE induces oxidative stress and inflammation within different tissues, which play vital roles in the corresponding injuries and may also explain the prenatal CSE-induced medullary impairment.

Hydrogen sulfide (H_2S), a traditional toxic gaseous molecule and environment hazard, has been regarded as an endogenous signal molecule since it was found to be produced in rat hippocampus modulating long-term potentiation (Abe and Kimura, 1996). Accumulating evidences have shown that H_2S plays considerable roles in numerous physiological and pathophysiological processes. This may benefit from its widespread production by enzymes in tissues and its ability to sulfhydrate the cysteine residues within a large number of proteins, which modulates their conformation, and thus their functions (Paul and Snyder, 2015).

H_2S has now been regarded as an important neuroprotective factor, which is largely ascribed to its anti-oxidative and anti-inflammatory

Abbreviation: CSE, cigarette smoke exposure; GSH, glutathione; IL-1β, interleukin-1β; IL-6, interleukin-6; MDA, malondialdehyde; OD, optical density; RT-PCR, reverse transcription-polymerase chain reaction; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α

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effects. For example, NaHS (a donor of H_2S) administration reversed neuron loss and movement dysfunction in a rat model of Parkinson's disease (Hu et al., 2010). NaHS also improved neurological symptoms and brain infarct of rats subjected to global cerebral ischemia-reperfusion (Yin et al., 2013). These protective actions were dependent on anti-oxidative and anti-inflammatory effects of H_2S (Hu et al., 2010; Yin et al., 2013). Besides, our investigations showed that H_2S relieved hypoxia-induced damage of rat respiratory centers by anti-oxidative effect (Li et al., 2015). Since we have indicated that H_2S defended neonatal rat medulla against prenatal CSE (Nie et al., 2013), the aim of the present work was to further explore the involvement of its anti-oxidative and anti-inflammatory effects in this protection. We found that NaHS administration ameliorated neuronal chromatolysis within hypoglossal nucleus and nucleus ambiguus, alleviated oxidative stress and inflammatory response in CSE rat medulla oblongata. These results suggest that H_2S, via anti-oxidative and anti-inflammatory effects, protects neonatal rat medulla oblongata against prenatal CSE.

2. Materials and methods

2.1. Animal grouping and prenatal cigarette smoke exposure

Adult female Sprague-Dawley rats weighing 220–270 g and male ones weighing 280–320 g were obtained from 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. The rats were housed in an animal room with an alternating 12 h light/dark cycle at 22 °C and were placed in cages with a size of 420 × 240 × 170 mm with free access to food and water. The food was regular for rat breeding supplied by Sichuan University. The bedding material is wood shavings with SPF level also provided by Sichuan University. The relative humidity was kept at the level of 50%-65%. Timed pregnancies were established by overnight mating of a single mature male with two nulliparous females. The presence of spermatozoa in vagina was considered as the evidence of mating and the day was indicated as day 0 of gestation (gd 0).

Pregnant rats were randomly divided into NaCl, CSE, CSE + NaHS and NaHS groups (n = 6 in each group). The pregnant rats in the CSE group, according to the method adopted by Nie et al. (Nie et al., 2013), were exposed to cigarette smoke produced by 10 filtered cigarettes (Tianxiaxiu, 11 mg tar and 1 mg nicotine, China Tobacco Chuanyu Industrial Co., China) for 60 min each time (2 cigarettes for 10 min, with an interval of 2 min, repeated 5 times) and twice a day (starting at 09:00 a.m. and 16:00 p.m., respectively) in a self-designed static iron exposure chamber (dimension 80 × 60 × 50 cm, equipped with a small electric fan to mix the air in the chamber) during gds 7–20. During the CSE, two rat cages were put in the exposure chamber, with three pregnant rats in each cage. The rats in the CSE + NaHS group received the same exposure treatment as those in the CSE group. The rats in the CSE and CSE + NaHS groups were respectively administered 2.5 ml/kg physiological saline and NaHS (56 μmol/kg) by intraperitoneal injection at about 8:30 a.m. every day. Pregnant rats of the NaCl and NaHS groups received the same treatment of intraperitoneal injection as those of the CSE and CSE + NaHS groups, respectively, and were exposed to fresh air under the same condition. Physiological saline and NaHS solution were freshly prepared before intraperitoneal injection every day with NaHS (Sigma, USA) dissolved in physiological saline. The dosage and administration route were adopted according to our previous study (Nie et al., 2013), in which we tested several dosages of NaHS, and 56 μmol/kg NaHS showed the best protective effect. We have shown that maternal serum cotinine concentration from the CSE group achieves 92.3 ± 15.7 ng/ml (compared to 4.2 ± 1.0 ng/ml from the NaCl group) (Nie et al., 2013), achieving levels for judging active smoking (Klebanoff et al., 1998; Nafstad et al., 1996). Therefore, active maternal smoking during pregnancy was simulated in this study.

On gd 21, pregnant rats were allowed to undergo natural parturition, and the corresponding neonatal rats of 2 days old were sacrificed for examination. Neonatal rats from each pregnant rat were divided into 4 subgroups: group A for detection of neuronal lesion (n = 6); group B for measurement of glutathione (GSH) levels, malondialdehyde (MDA) contents and superoxide dismutase (SOD) activity (n = 6); group C for detection of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) mRNA levels (n = 6); group D for assessment of TNF-α, IL-1β and IL-6 protein levels (n = 5).

2.2. Morphological observation

Neonatal rats were anaesthetized by ether inhalation and decapitated in a fume hood. The brainstem was dissected in ice-cold physiological saline and immersed in 4% paraformaldehyde overnight at 4 °C. Then the specimens were embedded with paraffin, followed by preparation of transverse sections with 5 μm thick from 100 μm caudal to 600 μm rostral to the obex. The blocks were cut at intervals of 20 μm. For every level, one section was obtained for Nissl staining with 0.5% thionine (Sigma, USA) to evaluate the damage of neurons in hypoglossal nucleus and nucleus ambiguus, respectively. The neurons were examined under a light microscope and images were captured with an Mshot color video camera (MD50, China) mounted on an Olympus microscope (Olympus, Japan).

Neuronal chromatolysis, defined as dispersion or loss of Nissl bodies, was evaluated based on Nissl staining. The chromatolytic and total neurons within the hypoglossal nucleus and nucleus ambiguus were manually counted, and the proportions of chromatolytic neurons versus total neurons were compared among groups. The area for optical density (OD) analysis encompassed the entire hypoglossal nucleus and nucleus ambiguus, respectively. Integrated OD of Nissl bodies in every section were measured from all motoneurons within the nuclei. The somas were manually outlined, and the integrated OD was then automatically measured by using Image J software (NIH, USA). Mean OD was defined as integrated OD divided by corresponding area. The OD measurement and neuron counting were performed on the same sections at a 200 × magnification by an observer blind to the experimental grouping.

2.3. Assessment of oxidative stress

Neonatal rats were anaesthetized by ether and decapitated. The medulla oblongata was rapidly isolated in ice-cold physiological saline and then immediately frozen in liquid nitrogen for preservation. For oxidative stress assay, newborn rat medulla oblongata were weighted and homogenized in proper volume of 4 °C physiological saline. The homogenate was then centrifuged at 4000 × *g* for 10 min at 4 °C. The supernatants were harvested to determine GSH levels, MDA contents and SOD activity with GSH assay kit, MDA assay kit and SOD assay kit (Nanjing Jiancheng Bioengineering Institute, China), respectively, in accordance with the manufacture's instruction.

Briefly, the GSH levels were assessed by its reaction with dithio-bis-nitrobenzoic acid which produces a yellow compound. The absorbance of this compound at 420 nm was measured, based on which the GSH levels were calculated and presented as mg/g protein. The MDA contents in medulla oblongata were determined with the thiobarbituric acid (TBA) method. This method was based on the spectrophotometric measurement of the color produced by the reaction of TBA and MDA. The absorbance of TBA reactive substances (TBARS) at 532 nm was measured for calculation of MDA contents, which were presented as nmol/mg protein. The activity of SOD in medulla oblongata was detected with xanthine oxidase method. This assay used the xanthine-xanthine oxidase system to produce superoxide ions, which reacted with 2-(4-iodophenyl)-3-(4-nitrophenol-5-phenlyltetrazolium chloride) to generate a red formazan dye, and the absorbance at 550 nm was