Hydrogen-rich saline ameliorates hippocampal neuron apoptosis through up-regulating the expression of cystathionine β-synthase (CBS) after cerebral ischemia-reperfusion in rats

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Abstract

Objective(s): This study aimed to evaluate the potential role of hydrogen in rats after cerebral ischemic/reperfusion (I/R) injury.

Materials and Methods: The experimental samples were composed of sham group, model group of rats that received middle cerebral artery occlusion (MCAO) for 2 hr followed by reperfusion for 24 hr, and the hydrogen saline group treated by hydrogen-rich saline (1 ml/kg) after MCAO. Hydrogen sulfide (H2S), S100-β-protein (S100-β), and neuron-specific enolase (NSE) levels were measured; the levels of malondialdehyde (MDA), reactive oxygen species (ROS), and superoxide dismutase (SOD) were detected; the histologic structure and apoptotic cells of hippocampus were observed; the expressions of cystathionine β-synthase (CBS), nuclear factor erythroid 2-related factor 2 (Nrf2), and hemeoxygenase-1 (HO-1) were measured. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference (LSD) test.

Results: Our results showed that hydrogen up-regulated H2S levels via promoting the expression of CBS in the hippocampus, and its treatment alleviated oxidative stress via activating the expression of Nrf2 and HO-1, and then cell apoptosis reduced, furthermore, brain function improved by down-regulating the levels of S100-βand NSE.

Conclusion: This study showed that hydrogen-rich saline ameliorates cell injury through up-regulating the expression of CBS in the hippocampus after cerebral ischemia-reperfusion (I/R) in rats, this provides new experimental evidence for the treatment of stroke with hydrogen saline.

Introduction

It is known that ischemic stroke characterized by the sudden loss of blood circulation is a major type of stroke (1) and has become the second leading cause of death globally (2, 3). Moreover, its high recurrence rate has economic burdens for the society. Studies have confirmed that cerebral ischemia has a complicated pathology closely related to oxidative stress (4-7). Accumulating evidence demonstrated that the main cause of neuron damage is not ischemia itself but the overproduction of reactive oxygen species (ROS) which attacks cells, resulting in ischemia/reperfusion (I/R) injury and neuronal cell death (8-10). Thus, a better understanding of the molecular and cellular mechanisms underlying oxidative stress injury after I/R may provide novel treatment for ischemic stroke. It has generated considerable interest in developing antioxidant therapies to combat ischemia-induced damage due to the close relationship between cerebral ischemia and oxidative stress (11).

Hydrogen sulfide (H2S), known as a toxic gas in nature (12) with an odorous smell, is synthesized from L-cysteine by enzymes such as cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and mercaptopyruvate sulfurtransferase (3MST) (13). CBS is predominantly responsible for the production of H2S in the central nervous system (14, 15). Recent studies have shown that small amounts of H2S are produced in the brain (14), and it has been proven to be an endogenous factor that regulates cellular function (16). Previous research has investigated that H2S has a protective effect against cerebral injury in rodent models (17). So, up-regulating the expression of CBS to promote H2S synthesis may be a therapeutic strategy for stroke.

Hydrogen is a gas that can be used for diving (18). However, studies have confirmed its anti-oxidative capabilities in animal models since Ohsawa et al. reported that hydrogen inhalation could protect the brain against cerebral I/R injury (19). In our previous researches, we also found that hydrogen has a...
Measurement of H$_2$S levels

The biosynthesis of H$_2$S in the brain was measured as described previously (24). The optical absorbance was measured at 655 nm with a microplate reader (iMark; Bio-Rad). The H$_2$S concentration of each sample was calculated.

Measurement of ROS, malondialdehyde (MDA) and superoxide dismutase (SOD)

ROS and SOD are the indicators of oxidative stress. The homogenates of brain tissue were centrifuged at 3,000 rpm for 20 min at 4 °C. The supernatant was separated and the activity of ROS and SOD were determined using a detection kit (Jiancheng, China) as manual protocol. Optical density was determined using a spectrometer, both at 550 nm.

The concentration of MDA as a marker of lipid peroxidation, was measured using a detection kit (Jiancheng, China) following the manufacturer's protocol. Optical density was determined by a spectrometer at 532 nm.

Histopathological examination

Isolated ischemic cerebral tissues were fixed with 10% methanol, embedded in paraffin, tissues were sectioned at a thickness of 5 μm, stained with hematoxylin and eosin (HE), and observed under a light microscope (Olympus X71-F22PH, Japan) at 400 magnification. The total and injured neurons were counted in 12 different fields of microscope per sample, six samples in each group were counted.

Immunohistochemical staining

Immunohistochemical staining method refers to our previous studies (21). The ischemic cerebral tissues were fixed in 10% formalin, embedded in paraffin, and cut into 5-μm thick sections, stained with a CBS antibody (diluted 1:500, Cell Signaling Technology (CST), USA), followed by a secondary IgG antibody. Immunostaining was performed with diaminobenzidine (DAB). The DAB staining density was assessed with a microscopic image analysis system (GX51, Olympus, Japan).

TdT-mediated dUTP nick end labeling (TUNEL)

The ischemic cerebral tissue was fixed in 10% formalin, embedded in paraffin, and sectioned at a thickness of 5 μm; TUNEL staining was performed with an in situ cell death detection kit (Jiancheng, China) according to the manufacturer's protocol.

First, the sections were rinsed with PBS and were treated with 1% Triton X-100 for 3 min. Terminal deoxynucleotidyl transferase (TdT) was used to catalyze the addition of biotinconjugated dUTP to the 3′-OH ends of the DNA fragments subsequently. Streptavidin-HRP solution was added and reacted at 37 °C for 30 min. Finally, the slides were placed in DAB for 3 min and stained with Hematoxylin Harris. These analyses were performed at 100× magnification under a light microscope in 12 different fields using computer-aided software (Olympus X71-F22PH, Japan). The apoptosis cells were quantified using computer assisted image analysis system (Leica LAS Image Analysis V4.0).

Western blotting

The protein was extracted using an extraction kit (Beyotime Biotechnology, china). Equal amounts of lysate proteins (20 μg) were loaded onto SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% nonfat milk in TBS and Tween 20 (TBST) for 1 hr, the PVDF membrane...
was incubated with β-actin (1:2000, CST, USA), Nrf-2 (1:800, CST, USA), HO-1 (1:800, CST, USA) overnight at 4 °C. Then, membranes were washed three times with TBST and incubated with IgG secondary antibody (1:5000, Beyotime Biotechnology, China) for 1 hr at room temperature. After washing with TBST, the antibody-bound proteins were detected with the ECL chemiluminescence reagent (Beyotime Biotechnology, China). Protein levels were calculated relative to that of β-actin. The images were analyzed using the software package (Bio-Rad Laboratories Inc, Hercules, CA, USA).

Statistical analysis
When data were normally distributed, they were analyzed using SPSS 21.0 and are expressed as mean ± standard error (SEM). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference (LSD) test, and a value of *P*<0.05 was considered statistically significant.

Results

Effect of hydrogen on H$_2$S levels
The levels of H$_2$S in the brain tissue decreased in the I/R group compared with the sham group (*P*<0.05), but hydrogen increased the endogenous H$_2$S levels in the brain compared to the I/R group (*P*<0.05) (Figure 1).

Effect of hydrogen on S100-β and NSE levels
The levels of S100-β and NSE have similar trends, these increased in the I/R group compared with those of the sham group (*P*<0.05), but hydrogen treatment decreased their levels in the brain compared to the I/R group (*P*<0.05) (Figure 2). It indicated that hydrogen played a protective role against ischemic injury.

Changes of histopathological structure
The neurons in the CA1 area of the hippocampus were observed. Normal neurons had round nuclei, but the nuclei of necrotic neurons were irregular, shrinkage, and with deep staining. Figure 3A shows obvious morphological changes in the I/R group in which the body and nuclei of neurons were reduced with shrinkage, and percentage of injured cells detected in the I/R group was significantly higher than that in the sham group (*P*<0.05), but it decreased after hydrogen treatment (*P*<0.05) (Figure 3B).

Effect of hydrogen on ROS, MDA, and SOD levels
As shown in Figures 4B and C, ROS and MDA levels were much higher in the I/R group than those in the sham group (*P*<0.05). Treatments with hydrogen decreased ROS and MDA levels compared with the I/R group (*P*<0.05). On the contrary, the levels of SOD in the brain tissue decreased in the I/R group compared with the sham group (*P*<0.05), but hydrogen increased the endogenous H$_2$S levels in the brain compared with the I/R group (*P*<0.05) (Figure 4A).
Changes of apoptosis

The TUNEL staining is shown in Figure 5A, the gray value of positive TUNEL cells found in the I/R group was significantly higher than that of the sham group (P<0.05), but it decreased after hydrogen treatment (P<0.05) (Figure 5B).

Changes of CBS expression

The expression of CBS showed brown staining in cells (Figure 6 A). Its mean density in I/R group increased compared with the sham group (P<0.05). Moreover, it increased significantly higher in the hydrogen treatment group compared with I/R group (P<0.05) (Figure 6 B). It indicated that hydrogen up-regulated the CBS expression in ischemic hippocampus.

Changes of Nrf-2 and HO-1 expressions

The expression levels of Nrf-2 and HO-1 increased in the I/R group compared with the sham group (P<0.05), and the hydrogen treatment further up-regulated the protein levels of those compared to the I/R group (P<0.05) (Figure 7). It indicated that endogenous expression of Nrf-2 and HO-1 were activated after I/R, and hydrogen could further up-regulate the expressions of those.

Discussion

In the present study, we investigated the protective role of hydrogen in rats after I/R injury through up-regulation of H₂S levels, it was confirmed by H₂S assessment and CBS expression in the hippocampus. Meanwhile, the treatment of hydrogen reduced oxidative stress and down-regulated the percentage of apoptotic neurons in ischemic hippocampus, and then injuries of neurons were improved. These results indicated the protective effect of hydrogen on injured neurons. These are consistent with our previous research showing that hydrogen protected the neurons against I/R injury (20, 21). Furthermore, the novelty of this study is that hydrogen may achieve its protective effect by up-regulating the expression of CBS and H₂S levels in the hippocampus.

H₂S is the third gas signal molecule (24), has been recognized to play crucial physiological functions in the central nervous system (25, 26). The results were in accordance with previous reports that the expression of CBS and concentration of H₂S increased in the hippocampus of rats after brain ischemia (27), it indicated that hydrogen up-regulated the H₂S levels in ischemic brain, and H₂S could reduce cerebral I/R injury in the animal model (28-31). To further elucidate the mechanism of hydrogen in alleviating MCAO-induced cerebral ischemic injury, the present study investigated the effects on antioxidants. Oxidative stress is a core pathological component closely related to reperfusion injury accompanied with excessive ROS production (32, 33). Our results demonstrated that induction of I/R leads to elevated levels of ROS and MDA and a decrease of SOD. Increasing studies have shown that treatment with H₂S could inhibit apoptosis via blocking an ROS activated Ca²⁺ signaling pathway in hypoxia-induced hippocampal neurons (30) and improved ischemic damage and
apoptosis in cerebral ischemia through its antioxidant effects (34-36). As expected, we found that treatment with hydrogen reduced ROS and MDA levels in the ischemic brain of I/R rats, and increased the SOD activity as well as the expression of CBS, nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase-1 (HO-1). All these implied that treatment with hydrogen saline significantly suppressed oxidative stress in ischemic brain. Nrf2 is an endogenous cytoprotective factor that activates the transcription of antioxidant stress genes, including HO-1 against oxidative stress (37, 38). The activation of Nrf2/HO-1 antioxidant pathway has been shown to play an important neuroprotective role after ischemia reperfusion-induced brain injury (39-41). The results showed that hydrogen significantly regulated Nrf2/HO-1 levels in the ischemic model.

Conclusion
The present study demonstrated that hydrogen could protect neurons in the hippocampus against ischemic injury through up-regulation of the CBS expression and activating the Nrf2/HO-1 antioxidant pathway. This provides a new experimental basis for clinical application of hydrogen in the treatment of cerebral ischemic injury. But the specific mechanism of how hydrogen up-regulated CBS expression remains to be further explored, this is the subsequent target for us and other researchers.

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Conflicts of Interest
There are no conflicts of interest in this article.

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