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Original Article

Hydrogen sulfide protects focal cerebral ischemia-reperfusion injury in rats through the PI3K/Akt signaling pathway

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Abstract: A rat model of left middle cerebral artery occlusion was established. Rats were randomly divided into four groups: sham operation, ischemia-reperfusion (I/R), NaHS+I/R and inhibitor groups. HE staining showed that the neuronal structures were normal in the sham group. The extracellular space broadened and the number of neurons significantly reduced in the I/R group. Neuronal injury improved and the number of neurons increased after NaHS administration. Neuronal injury showed no significant change and the number of neurons decreased after the application of the Akt inhibitor wortmannin. The apoptotic rate of hippocampal neurons was detected using the TUNEL fluorescence staining assay. Results showed that the number of apoptotic cells significantly increased in the I/R group, but this number decreased after the application of NaHS. The number of apoptotic cells increased in the inhibitor group. The protective effect of NaHS was blocked by I/R. The change in pAkt expression was not obvious after I/R. The expression levels of caspase-9 and caspase-3 increased. NaHS increased the expression of I/R injury neurons but decreased the expression of caspase-9 and caspase-3. The protein expression of pAkt decreased in the inhibitor group. The expression levels of caspase-9 and caspase-3 increased. These results suggest that hydrogen sulfide can inhibit apoptosis and protect focal cerebral ischemia-reperfusion injury by activating phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway.

Keywords: Hydrogen sulfide, focal cerebral ischemia-reperfusion injury, PI3K/Akt, apoptosis, caspase-9, caspase-3

Introduction

Cerebral ischemic injury caused by cerebral blood flow decrease can damage the structures and functions in different brain regions, which is the main cause of death and disability in adults [1]. The mechanisms underlying cerebral ischemic injury include glutamate neurotoxicity, apoptosis-promoting and anti-apoptosis gene expression, oxidative stress and inflammatory reactions [2, 3]. Mattson et al. [4] showed that apoptosis plays an important role in neuronal death induced by cerebral ischemia. PI3K/Akt is a key link in the signal transmission of cell growth, metabolism and survival. When PI3K conformation is changed, Ser473 and Thr308 sites in Akt are phosphorylated into pAkt. pAkt further regulates a large number of downstream proteins, which include the caspase family. Caspases are major apoptosis regulatory genes which play key roles in neuronal apoptosis. Among the caspase family members, caspase-3 is one of the most important cell apoptotic performers [5].

Hydrogen sulfide is the third most endogenous gas signaling molecule which displays bioactivity, followed by NO and CO. H_2S has a complex bioactivity and is widely involved in the function regulation of various systems. Hu et al. [6] reported that the activation of the PI3K/Akt signaling pathway is involved in the protective effect of H_2S in the pre-processed myocardial ischemia-reperfusion rat model. Previous studies in our laboratory have found that the H_2S donor compound NaHS exerts a protective effect on neuronal injury induced by cerebral I/R [7]. In the present study, a rat model of left

middle cerebral artery occlusion was established. Whether or not hydrogen sulfide exerts its cytoprotective effect by inhibiting cell apoptosis via the PI3K/Akt pathway needs further investigation.

Materials and methods

Animal sources and grouping

A total of 48 SD male rats weighing 230±21 g were randomly divided into four groups: (1) sham operation group, (2) cerebral I/R group, (3) NaHS+I/R group, and (4) inhibitor group (NaHS+Wortmannin) + I/R. The animals in the I/R group were treated according to the animal model. After embolism for 10 min, the animals in the NaHS+I/R group were intraperitoneally injected with a 25 μmol/kg physiological saline NaHS solution. The rats in the sham and I/R groups were intraperitoneally injected with the isometric physiological saline solution at the same time points; the left hippocampi of the rats in the inhibitor group were injected with 16 μg/kg of the Akt inhibitor wortmannin by using a stereotaxic apparatus at 30 min before the I/R model was established [8]. NaHS and wortmannin were purchased from Sigma-Aldrich (St. Louis, Mo, USA). The solution was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Eighth edition, 2011). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University (Xinxiang, China).

Establishment of the animal model

The middle cerebral artery occlusion model was established in rats via the Longa suture-occluded method [9]. The rats were fasted for 12 h before the operation. To anaesthetise the rats, 3.5% chloral hydrate was intraperitoneally injected according to 1 ml/100 g. The rats were placed in a fixed position. The skin was cut open along the cervical median incision. The muscle groups were bluntly separated. The left common carotid artery, external carotid artery and internal carotid artery were successively separated. The external carotid artery and common carotid artery were ligated at the bifurcation of the internal and external carotid arteries. The distal end of the internal carotid artery was occluded with the artery clamp. The thread was passed above the common carotid artery ligature, and a loose knot was tied for reserve. An incision was cut open above the common carotid artery ligation. The nylon thread was slowly inserted into the internal carotid artery before the artery clamp was removed. The insertion was not stopped until the thread was hampered when the ligature was approximately 18-22 mm from the internal and external carotid artery bifurcation. The reversed thread on the common carotid artery was used to fix the ligature, and then the skin was sutured. The animals were returned to their cages. The environment temperature was 26°C. After ischemia for 2 h and anaesthesia by ether inhalation, the ligature was pulled gently until its movement was hampered, this suggested that the distal end of the thread reached the common carotid artery incision. The arteries were only separated in the sham group.

Neurological scores of rats

After reperfusion for 24 h, the rat neurological score was evaluated according to the Longa scoring system [9]: '0' when no obvious symptoms of neurological deficit were observed; '1' when the right forepaw could not be fully stretched; '2' when the rat had a tendency to rotate to the right; '3' when the rat tended to show dumping to the right side while walking; '4' when the rat cannot walk independently or loss of consciousness occurs.

HE staining

Brain tissue samples were fixed in 4% paraformaldehyde for 72 h. Conventional dehydration and paraffin embedding were performed. Continuous coronal paraffin sections 5 μm in thickness were prepared, dewaxed and then dried. Conventional HE staining was conducted. The cell arrangement and morphology of the hippocampus were observed. The number of surviving neurons was counted. These cells had round or oval nuclei without shrinking or edema. The number of pyramidal neurons was counted in every mm segment. Four continuous segments were counted, and the average value was calculated.

TUNEL fluorescence staining

Apoptosis was detected using a one-step TUNEL cell apoptosis detection kit (Beyotime

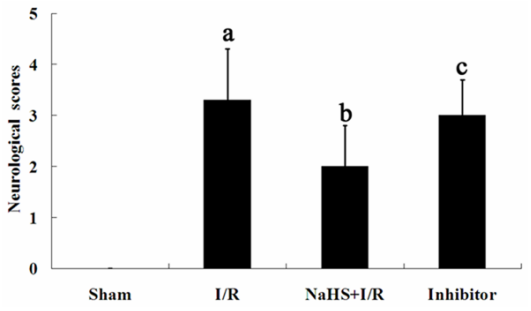

Figure 1. Neurological scores of rats in different groups (n = 12). [a]P < 0.05 versus the sham group; [b]P < 0.05 versus the I/R group; [c]P < 0.05 versus the NaHS+I/R group.

Institute, China). Brain tissue paraffin sections were dewaxed and hydrated. A proteinase K working solution was added, and PBS was used to rinse the sections. The confining liquid was used to seal the samples. Subsequently, each sample was added with 50 μL of TUNEL detection liquid, incubated for 60 min away from light, mounted and then immediately observed under a fluorescence microscope. Four fields of view were randomly selected under 200× magnification. The number of apoptotic cells with green fluorescence was counted, and the average value was calculated. The apoptotic rate (%) is the ratio of the number of apoptotic cells to the total number of cells under the field of vision multiplied by 100%.

Western blot

The left hippocampus of each rat was removed. The protein lysate was added, and the total protein was extracted via centrifugation. The protein concentration was detected using a microplate reader in accordance with the Bradford colourimetric method. The sample buffer was added to the EP tube containing the protein extract and then boiled for 5 min to denature the proteins completely. The sample was loaded at 30 g per well for electrophoresis. The sample protein was transferred on a nitrocellulose membrane. The rabbit-anti-rat p-Akt (Ser473), caspase-9, caspase-3 and β-actin primary antibodies were added after closing. The kit for Western blot analysis was provided by Beijing Zhongshan Biotechnology Co., Ltd. (Beijing, China). The second antibody was added after shaking overnight on the table concentrator at 4°C, followed by shaking at 37 C for 1 h before the cells were stained for 5 min away from light. The staining step was terminated with tap water. After blotting out the excess water, the stained membranes were kept away from light and photographed. The integrated optical density (IOD) of positive bands on the Western blot was measured with a gel image analysis system. The relative expression levels of the two proteins were shown with the IOD ratios of pAkt and Akt, as well as the IOD ratios of caspase-9, caspase-3 and the corresponding β-actin control.

Statistical analysis

Data were analysed with SPSS 18.0 statistical software (SPSS Inc, Chicago, IL, USA). The non-parametric Mann-Whitney U test was used for neurological scoring. The other data were analysed by one-way ANOVA. Results were expressed as $\bar{x} \pm s$. Values with P < 0.05 were considered statistically significant.

Results

Neurological scores

Experimental results showed that no obvious neurological deficit occurred in the rats after cerebral I/R for 24 h. The neurological score significantly increased in the sham group after I/R (P < 0.05). Compared with the I/R group, the NaHS group had a significantly lower neurological score (P < 0.05). The neurological score in the inhibitor group was significantly higher than that in the NaHS group (P < 0.05), suggesting that NaHS could significantly inhibit brain injury and that the PI3K/Akt signal mediated the neuroprotective effect of NaHS on I/R injury (**Figure 1**).

Morphological changes in the hippocampus

HE staining showed the structure and arrangement of pyramidal neurons in the hippocampal region (**Figure 2**). The neurons in the sham group were arranged regularly and closely. The boundaries, nuclear membranes and nucleoli were clear, and large nuclei were observed. The staining was light, and the cytoplasm was rich. By contrast, the neurons in the I/R group were sparse. The nuclei were deeply stained, but vacuoles were present in the cytoplasm. The cell peripheral space significantly broadened. The number of neurons significantly decreased. However, several blank non-tissue structures

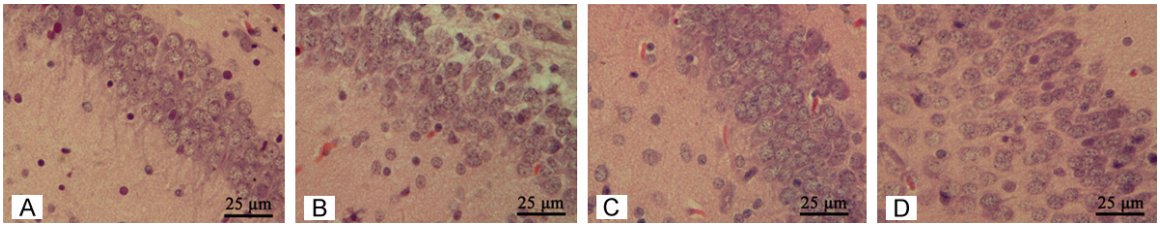

Figure 2. Morphological changes in the hippocampus of the (A) sham, (B) I/R, (C) NaHS+I/R and (D) inhibitor groups. Magnification, 400×; HE staining.

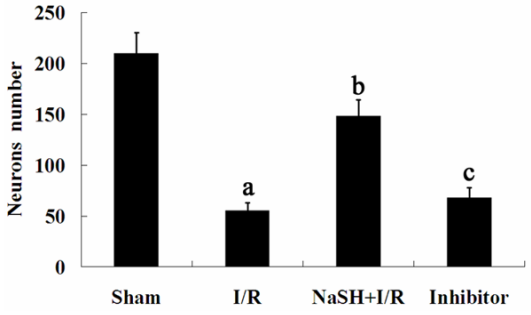

Figure 3. Cell count of the surviving neurons in the hippocampus. [a]$P < 0.05$ versus the sham group; [b]$P < 0.05$ versus the I/R group; [c]$P < 0.05$ versus the NaHS+I/R group.

were present. The outline of the pyramidal neurons in NaHS+I/R group was clear. The shape of the nucleus was regular, and the necrosis of neurons significantly decreased. Finally, the neurons were sparse and the intercellular space broadened in the inhibitor group.

Cell counts of surviving neurons in the hippocampus

The counts of the surviving neurons in all groups showed that the number of neurons in the I/R group significantly decreased. The NaHS+I/R group had significantly more neurons than the I/R group ($P < 0.05$), and the inhibitor group had significantly fewer neurons than the NaHS+I/R group ($P < 0.05$). These results suggest that NaHS can decrease the damage to hippocampal neurons in rats and that the Akt inhibitor can block the protective effect of NaHS on I/R brain injury in rats (**Figure 3**).

TUNEL fluorescence staining

TUNEL fluorescence staining showed that the number of positive apoptotic cells was less in the sham group. The number of positive cells was significantly greater in the I/R group than in the sham group ($P < 0.05$). The number of positive cells was significantly less in the NaHS+I/R group than in the I/R group ($P < 0.05$). The number of positive cells was significantly greater in the inhibitor group than in the NaHS+I/R group ($P < 0.05$). These data indicate that NaHS can protect the neurons of rats from I/R injury and that the Akt inhibitor can block the protective effect of NaHS (**Figure 4**).

Effect of NaHS on Akt activation

Western blot analysis showed that pAkt was up-regulated in the I/R injury group when compared with the sham group, but the difference was not significant ($P > 0.05$). The ratio of pAkt to Akt was significantly higher in the NaHS group than in the I/R group ($P < 0.05$). Wortmannin significantly decreased the increase in pAkt levels induced by NaHS ($P < 0.05$) (**Figure 5**).

Effects of NaHS on caspase-9 and caspase-3 cleavage

Western blot analysis showed that the expression levels of caspase-9 and caspase-3 were significantly higher in the I/R group than in the sham group ($P < 0.05$). Compared with the I/R group, the NaHS group had significantly lower expression levels of caspase-9 and caspase-3 ($P < 0.05$). The expression levels of caspase-9 and caspase-3 were significantly higher in the Akt inhibitor group (caspase-9) than in the NaHS+I/R group ($P < 0.05$). These results indicate that NaHS can inhibit the expression of pro-apoptotic proteins and reduce the apoptosis of neurons. Akt inhibitors blocked the inhibition of pro-apoptotic proteins by NaHS (**Figure 6**).

Discussion

Cerebral ischemic injury induced by local or whole cerebral blood flow decrease is the main

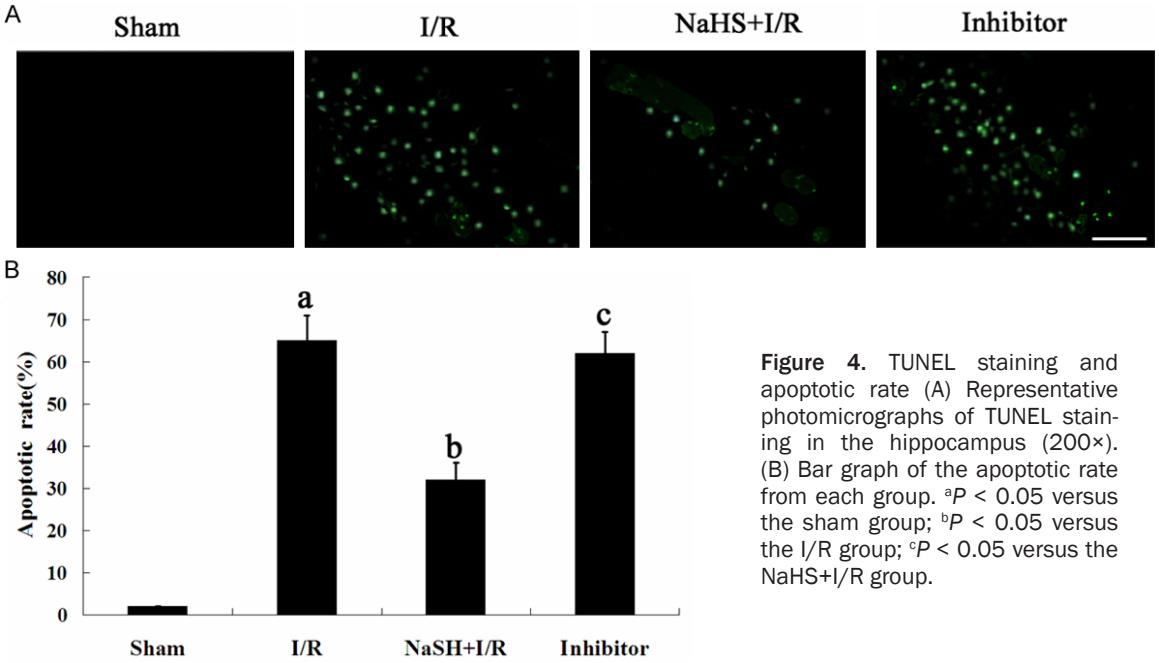

Figure 4. TUNEL staining and apoptotic rate (A) Representative photomicrographs of TUNEL staining in the hippocampus (200×). (B) Bar graph of the apoptotic rate from each group. [a]$P < 0.05$ versus the sham group; [b]$P < 0.05$ versus the I/R group; [c]$P < 0.05$ versus the NaHS+I/R group.

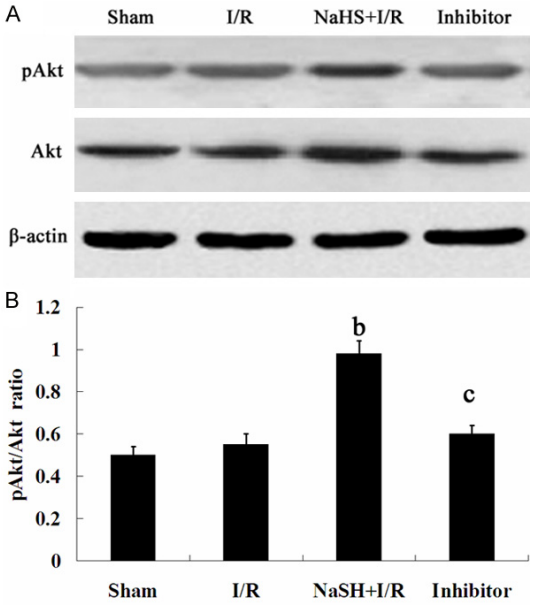

Figure 5. A. Western blot analysis of phosphorylated and total Akt from each group. B. Bar graph shows quantitative data for phosphorylated Akt from each group. [a]$P < 0.05$ versus the Sham group; [b]$P < 0.05$ versus the I/R group; [c]$P < 0.05$ versus the NaHS+I/R group.

cause of death or disability in adults. I/R-induced cell damage could lead to ATP depletion, ion loss of homeostasis, protease activation and mitochondrial dysfunction, among others [10]. H_2S is a gas with a strong smell of rotten eggs [11]; its production is catalysed by cystathionine-β-synthetase (CBS), 3-mercaptopyruvate trans-sulphurase (3-MST) and cystathionine-γ-lyase. Among these enzymes, CBS and 3-MST are expressed, which trigger the nervous system to produce endogenous H_2S [12, 13].

Previous studies showed that sulphuretted hydrogen can inhibit the inflammatory reaction and resist apoptosis to protect against whole-brain I/R damage by preventing oxidative stress [14]. In the cultured hippocampal neurons of the oxygen glucose deprivation model, H_2S can inhibit neuronal death in vitro [15]. NaHS can improve the synaptic plasticity of hippocampal neurons in cerebral ischemia rats and inhibit the oedema and nuclear shrinkage of pyramidal neurons after ischemia [16]. Another study found that the H_2S donor compound NaHS (25 μmol/kg) exerts a protective effect on neuronal injury induced by cerebral I/R [7].

The present study investigated the effect of exogenous H_2S on the neurological score in rats with I/R brain injury, the hippocampal neuronal structures and their quantity, the neuronal apoptotic rate and the protein expression of pAkt, caspase-3 and caspase-9 in the hippocampus. Neurological scoring showed no obvious neurological deficit in rats of the sham group. Neurological deficits were obvious only

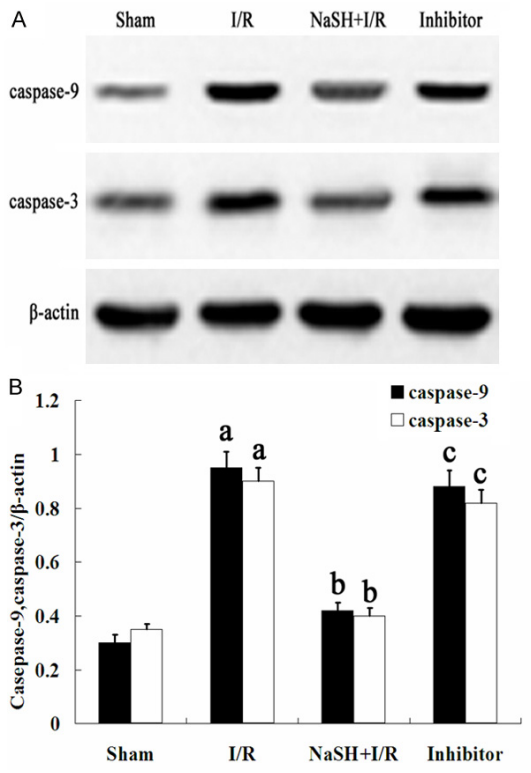

Figure 6. A. Western blot analysis of caspase-9, caspase-3 and β-actin from representative sham, I/R, NaHS+I/R and Inhibitor groups. B. Bar graph shows quantitative data for caspase-9, caspase-3 from each group. [a]$P < 0.05$ versus the Sham group; [b]$P < 0.05$ versus the I/R group; [c]$P < 0.05$ versus the NaHS+I/R group.

after I/R occurred. However, NaHS could significantly inhibit the brain damage. The PI3K/Akt signaling pathway mediated the neuroprotective role of NaHS on I/R, which could be inhibited by the Akt inhibitor wortmannin.

Previous studies on various animal models of ischemic hypoxic brain damage showed that the hippocampus is the most sensitive position to ischemic damage [17]. In the present experiment, the structure and arrangement of pyramidal neurons in the hippocampus were observed by HE staining, and the number of surviving neurons was counted. The neurons in the sham group were normal and arranged regularly and compactly. The neurons in the I/R group were sparse; the peripheral space significantly broadened, and the number of neurons significantly decreased. The outline of neurons was clear in the rats of the NaHS+I/R group. The shapes of the nuclei were regular, whereas neuronal necrosis significantly decreased. The neurons were sparse in the Akt inhibitor group; the peripheral space broadened, and the number of surviving neurons decreased, thereby implying that the protective effect of NaSH on I/R neurons was blocked.

Cell apoptosis is a programmed and self-initiated cell death controlled by various genes mainly via the activation of signal transduction pathways and the expression of apoptotic genes. The PI3K/Akt pathway is an important anti-apoptosis signaling pathway that has been studied in recent years [18-20]. When the PI3K/Akt pathway is activated, Akt is activated into pAkt, and the release of CytC in the mitochondria is blocked (CytC and apaf-1, as well as procaspase-9, constitute the apoptosome); furthermore, caspase-9 and caspase-3 are inhibited, thereby inhibiting cell apoptosis [21]. In the present study, cell apoptotic rate increased in the I/R cells, and the expression of caspase-9 and caspase-3 increased. The apoptotic rate decreased in the NaSH+I/R group; the expression of p-Akt significantly increased whereas the expression levels of caspase-3 and caspase-9 decreased. Compared with the NaHS+I/R group, the expression of p-Akt decreased, whereas the expression levels of caspase-3 and caspase-9 increased. These results indicate that NaSH exerts an anti-apoptotic effect by down-regulating the expression levels of caspase-9 and caspase-3, which are related to the activation of the PI3K/Akt signaling pathway.

This study showed that I/R damage in rats promoted apoptosis. The exogenous NaHS can activate the PI3K/Akt signaling pathway, increase the expression of pAkt, inhibit the expression of apoptotic protease and relieve apoptosis in I/R injury to protect the brain.

Acknowledgements

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Disclosure of conflict of interest

None.

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