Hydrogen Sulfide Improves Functional Recovery in Rat Traumatic Spinal Cord Injury Model by Inducing Nuclear Translocation of NF-E2-Related Factor 2

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Hydrogen sulfide (H2S), an important gaseous messenger, is known to have neuroprotective effects in many neurological disorders. This study examined the neuroprotective effects and the associated mechanisms of H2S in the model Sprague–Dawley (SD) rats with spinal cord injury (SCI). We found that H2S showed neuroprotective effects in SCI model rats, improved the symptoms of neurological impairment, reduced the secretion of inflammatory factors, nerve cell apoptosis, and endoplasmic reticulum (ER), and oxidative stresses. Moreover, these effects were produced by activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) protein. Our results suggest that H2S supplementation could be a potential therapeutic strategy to promote SCI recovery.

Key words hydrogen sulfide (H2S); spinal cord injury (SCI); inflammatory response; oxidative stress; endoplasmic reticulum (ER) stress

INTRODUCTION

Spinal cord injury (SCI), a common clinical disease of the spinal cord, is often accompanied by limbs and nerve dysfunction and even paralysis sequelae in severe cases. Moreover, the high disease incidence with expensive and poor therapeutic outcomes leads to high morbidity. This significantly affects the QOL and longevity of patients causing a great social burden. The pathogenesis of SCI includes primary and secondary injuries. Primary injury refers to the segmental injury in which a direct mechanical force leads to irreversible damage of spinal cord tissues, axons, and blood vessels. The secondary SCI may involve a variety of mechanisms, including oxidative stress, apoptosis, autophagy, endoplasmic reticulum (ER) stress, and inflammation. In contrast to the primary injury, secondary injury can certainly be therapeutically targeted to treat SCI.

Nuclear factor (NF)-Nrf2-related factor 2 (Nrf2), widely existing in tissues and organs, is an important regulator of cellular reduction-oxidation (REDOX) response. A stress injury can activate the Nrf2 signaling pathway and the activated Nrf2 enters the nucleus to activate the downstream protein molecules that play an antioxidant role. Under stable physiological conditions, Nrf2 is ubiquitinated in the cytoplasm and subsequently degraded. Nrf2/antioxidant response element (ARE) signaling pathway has been linked to anti-apoptosis, anti-inflammatory, and especially the anti-oxidative functions. Notably, Nrf2 transcriptionally regulates multiple detoxifying enzymes and antioxidant proteins, such as glutathione S-transferases (GSTs), nicotinamide adenine dinucleotide (NAD) (P)H quinone oxidoreductase 1 (NQO1), and heme oxygenase-1 (HO-1). These molecules protect multiple organ systems from various stresses. Moreover, Nrf2 plays an important connecting role in such stress regulatory responses; therefore, in-depth exploration of the Nrf2 signaling pathway in the pathogenesis of various diseases can aid in the development of potential drugs. Especially, the role and the underlying mechanism of Nrf2 in SCI are still largely unknown.

Hydrogen sulfide (H2S), an important gaseous messenger, is involved in various biological processes. Notably, H2S level in the body has been associated with several diseases, such as atherosclerosis, diabetes, hypertension, and inflammation. It is suggested that exogenous H2S could be a potential therapeutic strategy for the treatment of such diseases. Notably, H2S plays an important role in regulating redox balance mainly through two molecular mechanisms, first, by facilitating the conversion of cysteine thiols to persulfides via post-translational modification of the thiol redox proteome and second, by remodeling of redox metabolome, targeting the electron transfer chain to disrupt the major redox nodes. Fascinatingly, a study showed that H2S plays an important role in the regulation of key longevity-related hormones in mice lacking cystathionine γ-lyase. In the oxidative stress-induced mouse skin injury model, mesenchymal stem cell (MSC)-derived exosomes attenuated injury by adaptive regulation of the Nrf2 defense system. Another study showed that sodium hydrosulide (sodium hydrosulide, NaHS), an exogenous H2S donor, relieved oxidative stress and restored ischemic reperfusion-mediated cardioprotection by regulating the expression of the Survivin gene. Recently, a study showed that H2S-mediated Keap1 -sulfhydration alleviates liver damage by activating Nrf2. Although several studies have shown the protective effects of H2S in multiple organ injuries, it has yet not been examined concerning SCI.
MATERIALS AND METHODS

Spinal Cord Injury Rat Model  Sprague–Dawley (SD) rats (eight-week-old, male, Shanghai Laboratory Animal Center, Chinese Academy of Science, Shanghai, China) were housed under suitable conditions. The protocol for the care and use of animals followed the guidelines of the National Institutes of Health. All experiments were approved by the Research Ethics Committee of Zhejiang University. To establish the SCI rat model, the animals were anesthetized with 2% (w/v) sodium pentobarbital (40 mg/kg, intraperitoneally (i.p.)). Rat’s back hairs were shaved and the site was disinfected with iodophor. The T8–11 segment lamina was removed and the T9 lamina was removed to fully expose the spinal cord while ensuring the integrity of the dura. The T9 spinal cord was clamped with a 30 g vascular clamp (Oscar, China) for 1 min. In the control group, only the spinal cord was exposed without arterial clamp injury. The rats were i.p. injected with NaHS (5.6 mg/kg, Aladdin, Shanghai, China) daily for 28 d. The control group was treated with an equivalent volume of saline for the same duration. After the surgery, each rat was maintained (fed) in a single cage and was artificially assisted for urination twice a day until the bladder urination function recovered.

Locomotion Recovery Assessment  The Basso Beattie Bresnahan (BBB) scale, ranging from 0 (complete paralysis) to 21 (normal locomotion), was used to evaluate the locomotion recovery. The experiment was conducted in a double-blind manner. The motor function of both hind limbs was examined on 1, 3, 7, and 14 d after the operation, and the physiological changes, such as body balance, range and number of joint movements, coordination of the hind and forelimbs, and weight-bearing, were recorded.

Cell Culture and Oxygen–Glucose Deprivation (OGD)  Pheochromocytoma-derived 12 cells (PC12, the Cell Bank weight-bearing, were recorded.

RESULTS

NaHS Treatment Attenuates SCI-Induced Neurofunctional Deficits  To examine the neuroprotective protective effect of NaHS on postoperative SCI model rats, the animals were graded using the BBB scale. We found that compared to the untreated SCI model rats, after 7 and 14 d, the BBB score of NaHS treated SCI model rats was markedly higher (Figs. 1A–C). Likewise, H&E staining showed that compared to the untreated SCI group, treatment with NaHS reduced the formation of cavities in the spinal cord tissue (Fig. 1D). To further verify the inhibitory effect of NaHS on the apoptosis of spinal cord tissue cells after SCI, we examined the levels of apoptosis-related proteins. We found that NaHS treatment downregulated the protein levels of active-caspase3 and Bax but upregulated Bcl-2 (Figs. 1E–I).

NaHS Suppressed SCI-Induced Oxidative Stress and Inflammatory Response  Next, we examined the NaHS effect on oxidative stress and inflammatory response induced by SCI. For this, we used the corresponding kits to measure the spinal cord tissue levels of MDA, GPx, SOD, TNF-α, IL-6, and IL-1β. As shown in Figs. 2A–C, compared to the Sham...
Fig. 1. NaHS Treatment Attenuates SCI-Induced Neurofunctional Deficits

(A–C) Basso Beattie Bresnahan (BBB) score. (D) H&E staining of spinal cord tissue of the respective groups. (E) Immunofluorescence co-staining of NeuN and c-caspase3 of the spinal cord tissue of the respective groups. (F) The immunofluorescence intensity of c-caspase3. (G–I) The protein expression and densitometric quantification of Bax and Bcl-2. (n = 5 per group; ***p < 0.001; **p < 0.01; *p < 0.05 versus Sham group; *p < 0.05 versus SCI group).
group, the level of MDA was elevated in the SCI group, which was reversed by NaHS treatment. Also, the levels of SOD and GPx (important anti-oxidant enzymes) were lower in the SCI group than the Sham group, which were elevated by NaHS treatment. Furthermore, NaHS abolished the SCI-induced secretion of inflammatory cytokines, TNF-α, IL-6, and IL-1β (Figs. 2D–I).

NaHS Accelerated Nuclear Translocation of Nrf2 and Inhibited ER Stress in SCI Model Rats

We showed that NaHS has anti-inflammatory and antioxidant effects in the SCI rat model. To further elaborate the underlying mechanism, we examined the nuclear and cytoplasmic levels of Nrf2. We found that compared to the other experimental groups, NaHS treatment remarkably promoted the nuclear translocation of Nrf2 (Figs. 3A–C). Next, we examined the expression of Nrf2 downstream genes by qRT-PCR (Figs. 3D, E). We found that the mRNA expression of HO-1 and NQO-1 was upregulated after SCI than in the Sham group. Also, the expression of ER stress-related proteins, glucose-regulated protein (GRP)78 and CHOP, were higher in the SCI model rats. Notably, these were significantly reduced after NaHS treatment (Figs. 3F–H).

NaHS Treatment Suppressed OGD-Induced Injury in Cells

To further validate our findings, we conducted in vitro cell experiments to test the efficacy of NaHS (Figs. 4A–C). We found that cell viability of the OGD model group was significantly lower than the control group; however, treatment with 0, 10, 20, 40, 80, and 100 µM NaHS reversed this trend. Moreover, NaHS treatment enhanced the nuclear levels of Nrf2 causing a sharp drop in the cytoplasmic levels in the OGD group (Figs. 4I–K). Like earlier, NaHS treatment also upregulated Nrf2 downstream factors, NQO-1 and HO-1 (Figs. 4G, H), and improved cell anti-oxidative ability.
thermore, compared with the OGD group, NaHS treatment downregulated the protein levels of GRP78 and CHOP; however, the effect was not so prominent in the Nrf2 knocked-out cells (Figs. 4L–N).

**DISCUSSION**

Finding an effective treatment for SCI is one of the major concerns of the medical field. Its main pathogenic factors are compression injury or traffic accidents, especially in young and middle-aged people, affecting a large number of people every year. Moreover, the process of ischemia–reperfusion after SCI causes necrosis and apoptosis of tissue cells, while the neurons are non-regenerative. Therefore, once cell injury and necrosis occur, there will be irreversible and serious consequences that are difficult to treat. Besides, the blood–brain and blood–spinal cord barriers are a serious challenge to the bioavailability of drugs limiting their usage in the treatment of SCI. For the anti-inflammatory treatment of acute SCI, the U.S. Food and Drug Administration (FDA) has approved the only hormone drug methylprednisolone. Although the clinical trials showed that methylprednisolone is effective only if administered within 8h of acute SCI, and a higher dose can lead to serious side effects. Therefore, a safe and effective...
Fig. 4. NaHS Treatment Regulates OGD-Induced Injury in Vitro

(A–C) CCK8 proliferation analysis. (D–F) The ELISA analysis of SOD, MDA, and GPx levels in the spinal cord tissue of the respective groups. (G–I) The protein expression and densitometric quantification of Nrf2 in the respective groups. (J, K) The qRT-PCR analysis of HO-1 and NQO-1 levels in the respective groups. (L–N) The protein expression and densitometric quantification of GRP78 and CHOP in the respective groups. (n = 5 per group; ** p < 0.01 versus CON group; * p < 0.05 versus OGD group; # p < 0.05 versus OGD + NaHS group).
treatment strategy for SCI is urgently needed.

The widely existing gaseous signaling molecules such as nitric oxide (NO), molecular hydrogen (H₂), and hydrogen sulfide (H₂S) play important anti-inflammatory, anti-oxidant, and anti-cancer activities. Biogas, with a small molecular weight, can easily transverse biological barriers than a chemical drug. Studies showed that gas molecules have neuroprotective effects in central nervous system (CNS) diseases with higher efficiency of administration. Notably, H₂S can diffuse freely across the cell membrane and can activate a variety of cell targets without having a specific receptor. Besides, a study showed that H₂S supplementation can effectively inhibit apoptosis of experimental glaucoma retinal ganglion cell (RGC), which is related to the preservation of mitochondrial function, reduction of oxidative stress, inhibition of glial activation, inflammatory pathways, and autophagy. Therefore, we hypothesized that H₂S can also have a protective effect after spinal cord injury. Accordingly, here, we showed that in the SCI model of SD rats, NaHS treatment significantly improved the neurological function deficit in the injured mice.

Activated Nrf2 can enhance the oxidative stress resistance of neuronal cells and thereby exert a neural protection function. Studies showed that in mouse models of traumatic brain injury (TBI), activation of the Nrf2/ARE signaling pathway effectively increased the activities of antioxidant enzymes and reduced cell apoptosis. In general, Nrf2, anchored to the cytoplasm by Keap1 (Kelch ECH associating protein 1), is degraded in the cytoplasm, while in response to a stimulus, it gets transported to the nucleus to transcriptionally regulate the downstream antioxidant stress elements exerting an anti-oxidant role. Keap1 has five domains: N-terminal region (NTR), BTB, intervening region (IVR), double glycine repeat (DGR), and C-terminal region (CTR). The DGR domain binds to Nrf2, while the cysteine-rich IVR domain is receptive to oxidants and electrophiles. Hydrogen sulfide anion-induced electrophile sulhydration is a unique mechanism to control electrophile-mediated redox signaling. Also, hydrogen sulfide is a unique nucleophile and the nucleophilic reaction of persulfides depends on the presence of cysteine. Endogenous H₂S affects the cysteine sulfation in the IVR region of Keap1 that triggers the conformational change in the protein. Some studies, using a mouse model of diabetes and cellular senescence, showed that H₂S induces Nrf2 nuclear translocation via S-sulhydration of Keap1. Based on these findings, we also speculate that NaHS promoted Nrf2 nuclear translocation in SCI rats (at least in part) via H₂S induce S-sulhydration of Keap1. This abolished the inhibitory effect on Nrf2 promoting its transport to the nucleus activating the downstream proteins. Moreover, Nrf2 has been linked to anti-inflammatory processes by regulating the expression of antioxidant response genes and the recruitment of inflammatory cells. Here, we showed that NaHS promoted Nrf2 activation and increased its translocation to the nucleus, producing an antioxidant and anti-inflammatory effect in SCI rats. In addition, ER stress plays an important role in SCI, especially in the secondary injury. Activation of the Nrf2/ARE pathway was shown to effectively regulate the ER stress (ERR) alleviating acetaminophen-induced Liver damage. Interestingly, H₂S treatment eased ventilator-induced lung injury via regulating ER stress. Based on these findings, we speculate that NaHS mediated inhibition of ER stress in SCI could be related to the activation of Nrf2. We showed that the inhibitory effects of NaHS on ER stress were significantly hampered in the Nrf2 knockout cells.

In conclusion, NaHS activates Nrf2 signaling molecules to increase the nuclear translocation of Nrf2, which then activates the transcription of downstream target genes to exert the anti-inflammatory, antioxidant and neuroprotective effects in SCI rats. Also, NaHS can inhibit ER stress potentially via the activation of Nrf2. Our study provides a feasible basis for the clinical usage of NaHS in SCI.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1. McDonald JW, Sadowsky C. Spinal-cord injury. Lancet, 359, 417–425 (2002).
2. Altura BS, Wilson JR, Nori S, Kotter MRN, Druschel C, Curt A, Fehlings MG. Traumatic spinal cord injury. Nat. Rev. Dis. Primers, 3, 17018 (2017).
3. Li Z, Wu F, Xu D, Zhi Z, Xu G. Inhibition of TREM1 reduces inflammation and oxidative stress after spinal cord injury (SCI) associated with HO-1 expressions. Biomed. Pharmacother., 109, 2014–2021 (2019).
4. He M, Ding Y, Chu C, Tang J, Xiao Q, Luo ZG. Autophagy induction stabilizes microtubules and promotes axon regeneration after spinal cord injury. Proc. Natl. Acad. Sci. U.S.A., 113, 11324–11329 (2016).
5. Chen Z, Guo H, Lu Z, Sun K, Jin Q. Hyperglycemia aggravates spinal cord injury through endoplasmic reticulum stress mediated neuronal apoptosis, gliosis and activation. Biomed. Pharmacother., 112, 108672 (2019).
6. Orr MB, Gensel JC. Spinal cord injury scarring and inflammation: therapies targeting glial and inflammatory responses. Neurotherapeutics, 15, 541–553 (2018).
7. Bellezza I, Giambanco I, Minelli A, Donato R. Nrf2–Keap1 signaling in oxidative and reductive stress. Biochim. Biophys. Acta Mol. Cell Res., 1865, 721–733 (2018).
8. Tonelli C, Chio IIC, Tuveson DA. Transcriptional Regulation by Nrf2. Antioxid. Redox Signal., 29, 1727–1745 (2018).
9. Lu MC, Ji JA, Jiang ZY, You QD. The Keap1–Nrf2–ARE pathway as a potential preventive and therapeutic target: an update. Med. Res. Rev., 36, 924–961 (2016).
10. Olas B. Hydrogen sulfide in signaling pathways. Clin. Chim. Acta, 439, 212–218 (2015).
11. Powell CR, Dillon KM, Matson JB. A review of hydrogen sulfide (H₂S) donors: chemistry and potential therapeutic applications. Biochem. Pharmacol., 149, 110–123 (2018).
12. Hine C, Harputlugil E, Zhang Y, Ruckenstuhl C, Lee BC, Brace L, Longchamp A, Treviño-Villarreal JH, Mejia P, Ozaki CK, Wang R, Gladysz VNE, Macheo F, Mair WB, Mitchell JR. Endogenous hydrogen sulfide production is essential for dietary restriction benefits. Cell, 160, 132–144 (2015).
13. Wang T, Jian Z, Baskys A, Yang J, Li J, Guo H, He Y, Xian P, He Z, Li Z, Li N, Long Q. MSC-derived exosomes protect against oxidative stress-induced skin injury via adaptive regulation of the NRF2 defense system. Biomaterials, 257, 120264 (2020).
