Pharmacological Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

# Hydrogen sulfide attenuates hypoxia-induced neurotoxicity through inhibiting microglial activation

Qun Zhang [a], Lin Yuan [a], Dexiang Liu [b], Jianmei Wang [a], Shuanglian Wang [a], Qingrui Zhang [a], Yanfen Gong [a], Hongda Liu [a], Aijun Hao [c], Zhen Wang [a,c,*]

[a] Department of Physiology, Shandong University School of Medicine, Jinan, Shandong 250012, PR China
[b] Department of Medical Psychology, Shandong University School of Medicine, Jinan, Shandong 250012, PR China
[c] Key Laboratory of the Ministry of Education for Experimental Teratology, Shandong Provincial Key Laboratory of Mental Disorders, Department of Histology and Embryology, Shandong University School of Medicine, 44#, Wenhua Xi Road, Jinan, Shandong 250012, PR China

ARTICLE INFO

*Article history:*
Received 22 November 2013
Received in revised form 28 March 2014
Accepted 17 April 2014

*Keywords:*
Hydrogen sulfide
Hypoxia
Microglial activation
Neurotoxicity
Nuclear factor κB

ABSTRACT

Endogenously produced hydrogen sulfide ($H_2S$) may have multiple functions in the brain including potent anti-inflammatory effects. Activated microglia can secrete various pro-inflammatory cytokines and neurotoxic mediators, which may contribute to hypoxic injuries in the developing brain. The aim of this study is to investigate the potential role of $H_2S$ in altering hypoxia-induced neurotoxicity *via* its anti-inflammatory actions as examined *in vitro* and *in vivo* models. Using the BV-2 microglial cell line, we found that sodium hydrosulfide (NaHS), a $H_2S$ donor, significantly inhibited hypoxia-induced microglial activation and suppressed subsequent pro-inflammatory factor release. In addition, treating murine primary cortical neurons with conditioned medium (CM) from hypoxia-stimulated microglia induced neuronal apoptosis, an effect that was reversed by CM treated with NaHS. Further, NaHS inhibited phosphorylation of the p65 subunit of NF-κB, phosphorylation of ERK and p38 but not JNK MAPK in these hypoxia-induced microglia. When administered *in vivo* to neonatal mice subjected to hypoxia, NaHS was found to attenuate neuron death, an effect that was associated with suppressed microglial activation, pro-inflammatory cytokines and NO levels. Taken together, $H_2S$ exerts neuroprotection against hypoxia-induced neurotoxicity through its anti-inflammatory effect in microglia. This effect appears to be attributable to inhibition of iNOS, NF-κB, ERK and p38 MAPK signaling pathways. Our results suggest a potential therapeutic application of $H_2S$ releasing drugs in hypoxic brain damage treatment.



## Introduction

Due to the high oxygen and energy requirements of the developing brain it is susceptible to hypoxic damage. Hypoxia-induced inflammatory responses have been recognized as one of the main perpetrators of hypoxic brain injury. In this regard, a hallmark feature of such injury is microglial activation which results in overproduction of inflammatory cytokines. These inflammatory cytokines, which include agents like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), glutamate, nitric oxide (NO) and reactive oxygen species can collectively result in neuronal and oligodendrocyte death, axonal degeneration as well as disruption of the immature blood brain barrier. Recent evidence has indicated that TNF-α, IL-6, monocyte chemoattractant protein-1 and macrophage colony stimulating factor which are produced by activated microglia are linked to the pathogenesis of periventricular white matter damage in the hypoxic brain. Thus, inhibition of microglial activation may have a therapeutic benefit in the treatment of hypoxic damage.

Hydrogen sulfide ($H_2S$) has been classified as a novel gasotransmitter signaling molecule, like that of nitric oxide and carbon monoxide [1]. Endogenous $H_2S$ is generated in mammalian tissues

*Abbreviations:* CBS, cystathionine β synthase; CNS, central nervous system; CM, conditioned medium; CSE, cystathionine γ lyase; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; $H_2S$, hydrogen sulfide; iNOS, inducible nitric oxide synthase; IL-6, interluekin-6; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide sodium; MAPK, mitogen-activated protein kinase; NaHS, sodium hydrosulfide; NF-κB, nuclear factor κB; NO, nitric oxide; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α.

* Corresponding author at: Institute of Physiology, Shandong University School of Medicine, 44#, Wenhua Xi Road, Jinan, Shandong 250012, PR China.
Tel.: +86 531 88383902; fax: +86 531 88382039.
*E-mail address:* wangzhen@sdu.edu.cn (Z. Wang).

http://dx.doi.org/10.1016/j.phrs.2014.04.009
1043-6618/

by two pyridoxal-5′ phosphate-dependent enzymes, cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). Both enzymes use L-cysteine as substrate. CBS and CSE are widely distributed throughout the body, however, CBS activity is 30-fold greater than CSE in brain while CSE expression and activity are much higher than CBS in the cardiovascular system. It has been proposed that CBS was responsible for $H_2S$ production in the brain [2]. $H_2S$ concentrations ranging between 50 and 160 μM have been detected in human, rat and bovine brains [3]. A number of functions have been associated with $H_2S$. Physiological concentrations of $H_2S$ can potentiate the activity of the N-methyl-D-aspartate (NMDA) receptor and enhance the induction of hippocampal long-term potentiation [4], which is associated with learning and memory. $H_2S$ can also induce $Ca^{2+}$ waves and increase intracellular concentrations of $Ca^{2+}$ in both astrocytes and microglia [5,6].

Interestingly, accumulating evidence has been garnered which suggests that exogenous $H_2S$ can function as a powerful neuroprotective agent. Kimura et al. were first to demonstrate that $H_2S$ protected primary rat cortical neurons from oxidative stress-induced injury [3]. $H_2S$ also protects PC12 cells against cobalt chloride-induced chemical hypoxia injuries; and, $H_2S$ has been shown to protect against cytotoxicity induced by beta amyloid, the 1-methy-4-phenylpyridinium ion, peroxynitrite, and hypochlorous acid in PC12 [7,8] and SH-SY5Y cells [9,10]. In addition, $H_2S$ has protective effects against lipopolysaccharide-induced inflammation in microglia [11], attenuates rotenone-induced apoptosis in SH-SY5Y cells [12], and inhibits $H_2O_2$ induced cytotoxicity in astrocytes [13]. In addition, $H_2S$ may be beneficial in various types of neurological disorders, such as Parkinson's disease [16], Alzheimer's disease [17] and brain hypoxia-ischemia injury [18,19].

The key mechanisms of $H_2S$ neuroprotection appear to involve its antioxidant, anti-inflammatory, and anti-apoptotic effects [8,13–15]. Therefore, considering that inflammation is involved in the pathophysiology of hypoxia brain damage, and there exist no studies examining the effects of $H_2S$ on these targets, the present study was aimed at investigating the possible anti-inflammatory effects of $H_2S$ in mice exposed to hypoxia insult. To this end, we tested the effects of sodium hydrosulfide (NaHS), a $H_2S$ donor, on hypoxia-induced neurotoxicity and microglial activation as assessed within *in vitro* and *in vivo* models along with the molecular mechanisms regulating microglial production of pro-inflammatory mediators such as TNF-a, IL-6 and NO. In addition, two putative pathways including mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) activation, which may be involved in these anti-inflammatory effects of NaHS, were also investigated.

## Materials and methods

### *BV2 microglial cell culture and hypoxic exposure*

BV-2 cells in a 5% $CO_2$ incubator were maintained in Dulbecco's modified Eagle medium (DMEM, Hyclone Co., Logan, UT, USA) with 10% fetal bovine serum (FBS, Hyclone Co.), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA). For all experiments, BV-2 cells were used at 75–80% confluency. Prior to use in the experiment, plated cells were incubated with serum-free DMEM for 1 h. The medium was then replaced with serum-free DMEM containing NaHS (Sigma-Aldrich) for the various time intervals and concentrations as indicated below. The NaHS was initially dissolved in normal saline. For most experiments, BV-2 cells were treated with NaHS at concentrations ranging from 1–100 μM while controls were treated with the vehicle (normal saline) except where indicated differently.

BV-2 cells were challenged to hypoxia by placing them in a chamber (Model: Heraeus HERAcell 240i; Thermo Scientific, USA) filled with 3% oxygen, 5% $CO_2$ and 92% nitrogen at 37 °C for the time intervals indicated below. BV-2 cells serving as controls were incubated at 37 °C with 95% air and 5% $CO_2$ [20].

### *Cell viability assay*

Cell viability was determined by the MTT assay. BV2 cells were plated into 96-well culture plates at a density of $5 \times 10^4$ cells/ml with 200 μl culture medium per well. Following exposure to hypoxia with or without differing concentrations of NaHS, 20 μl MTT solution (5 mg/ml) was added to each well and incubated for 4 h. The medium was then aspirated and 200 μl dimethyl sulfoxide was added. The absorbance value was measured using a multiwell spectrophotometer (Bio-Rad, USA) at 490 nm. Cell viability was expressed as a percent of viable cells obtained relative to that of controls.

### *Measurement of TNF-α and IL-6*

The Enzyme-Linked ImmunoSorbent Assay (ELISA, R&D Systems Inc., Minneapolis, MN, USA) kits were used to quantitatify cytokine levels in BV-2 cell culture supernatants. BV-2 cells ($3 \times 10^5$ cells per well in a 12-well plate), were exposed to hypoxia with or without differing concentrations of NaHS for 24 h. The supernatant of the culture medium from the various treatments was then collected and levels of cytokines, TNF-α and IL-6 in culture medium were measured using ELISA kits according to manufacturers' instructions.

### *Assay of NO production*

NO production was assessed by the Griess reaction. Homogenates of the whole-brain or the supernatant of the culture medium were mixed with an equal volume of Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid, Sigma-Aldrich) in a 96-well plate and incubated at room temperature for 10 min. Absorbance was measured at 550 nm on a microplate reader (Bio-Rad Labs). Sodium nitrite, diluted in culture medium at concentrations ranging from 10 to 100 μM, was used to generate a standard curve.

### *Immunofluorescence imaging*

The cells, exposed to normoxia or hypoxia for 60 min in the presence or absence of NaHS (10 μM), were fixed in 4% paraformaldehyde for 20 min and blocked with 10% goat serum in PBS. Slides were incubated overnight in a humidified chamber at 4 °C with the following primary antibody (ionized calcium-binding adapter molecule-1, Iba-1, 1:200, rabbit polyclonal, Abcam, Cambridge, MA, USA). After primary antibody incubation, samples were washed and incubated with the appropriate fluorescent-conjugated secondary antibody (1:500 dilution, Sigma-Aldrich) for 1 h. Images were captured using a Nikon TE2000U microscope.

### *Preparations of nuclear and cytosolic cellular fractions*

After treatment, cells were washed with ice-cold PBS, collected and centrifuged at 1000 × *g* for 5 min at 4 °C. Pellets were resuspended in 300 μl of lysis buffer and incubated on ice for 15 min. Then 20 μl of 10% NP-40 was added and the tube was vortexed for 10 s. After centrifugation at 13,000 × *g* for 1 min at 4 °C, supernatants (cytosolic fractions) were collected and stored at −80 °C, while the pellets were further processed to obtain nuclear extracts. The pellets were resuspended in extraction buffer (5 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM



Please cite this article in press as: Zhang Q, et al. Hydrogen sulfide attenuates hypoxia-induced neurotoxicity through inhibiting microglial activation. Pharmacol Res (2014), http://dx.doi.org/10.1016/j.phrs.2014.04.009