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L-CYSTEINE PROMOTES THE PROLIFERATION AND DIFFERENTIATION OF NEURAL STEM CELLS VIA THE CBS/H_2S PATHWAY

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Abstract—Growing evidence has suggested that hydrogen sulfide (H_2S) acts as a novel neuro-modulator and neuroprotective agent; however, it remains to be investigated whether H_2S has a direct effect on neural stem cells (NSCs). We report here that NSCs expressed cystathionine β synthase (CBS) and addition of exogenous H_2S donor, L-cysteine, stimulated proliferation and increased the differentiation potential of NSCs to neurons and astroglia. Moreover, pre-treatment with aminooxyacetic acid, the inhibitor of CBS or knockdown of CBS in using siRNA, significantly attenuated the effects of L-cysteine on elevated H_2S levels and the cell proliferation; it also effectively suppressed L-cysteine-induced neurogenesis and astrocytogenesis. Further analysis revealed that L-cysteine-induced proliferation was associated with phosphorylation of extracellular signal-regulated kinases 1/2 and differentiation with altered expression of differentiation-related genes. Taken together, the present data suggest that L-cysteine can enhance proliferation and differentiation of NSCs via the CBS/H_2S pathway, which may serve as a useful inference for elucidating its role in regulating the fate of NSCs in physiological and pathological settings. **© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: L-cysteine, neural stem cells, proliferation, differentiation.

INTRODUCTION

Hydrogen sulfide (H_2S) has been classified as the third novel gasotransmitter signaling molecule alongside nitric oxide and carbon monoxide (Wang, 2010). Endogenous H_2S is generated in mammalian tissues by two pyridoxal-5′ phosphate-dependent enzymes, cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). Both of these enzymes use L-cysteine as substrate. CBS and CSE are widely distributed in tissues; however, CBS activity is 30-fold greater than CSE in the brain, whereas CSE expression and activity are much higher than CBS in the cardiovascular system. It was also reported that CBS was responsible for H_2S production in the brain (Abe and Kimura, 1996). In the human, rat and bovine brain, H_2S concentrations between 50 and 160 μM were detected (Kimura and Kimura, 2004). Physiological concentrations of H_2S can potentiate the activity of the *N*-methyl-D-aspartate receptor and enhance the induction of hippocampal long-term potentiation (Eto et al., 2002), 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 (Nagai et al., 2004; Lee et al., 2006).

Interestingly, accumulating evidence suggested that H_2S acted as a powerful neuroprotective agent. Kimura and Kimura (2004) firstly demonstrated that H_2S protected primary rat cortical neurons from oxidative stress-induced injury. H_2S also protects PC12 cells against cobalt chloride-induced chemical hypoxia injuries (Lan et al., 2011). It was also documented that H_2S protects against cytotoxicity induced by beta amyloid and 1-methy-4-phenylpyridinium ion, peroxynitrite, and hypochlorous acid in PC12 (Tang et al., 2008, 2011a) and SH-SY5Y cells (Whiteman et al., 2004, 2005). Additionally, H_2S has protective effects against lipopolysaccharide-induced inflammation in microglia (Hu et al., 2007), attenuates rotenone-induced apoptosis in SH-SY5Y cells (Hu et al., 2009), and inhibits H_2O_2-induced cytotoxicity in astrocytes (Lu et al., 2008). The key mechanisms involved in neuroprotection by H_2S are its antioxidant, anti-inflammatory, and anti-apoptotic effects (Hu et al., 2007; Lu et al., 2008; Tang et al., 2008; Lan et al., 2011). In addition, H_2S may be beneficial in various types of neurological disorders,

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Abbreviations: AOAA, aminooxyacetic acid; bFGF, basic fibroblast growth factor; bHLH, basic helix–loop–helix transcription factors; CBS, cystathionine β synthase; CSE, cystathionine γ lyase; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinases; GFAP, glial fibrillary acidic protein; Hes, hairy and enhancer of split; H_2S, hydrogen sulfide; Id, inhibitor of DNA-binding; MAP2, microtubule associated protein 2; Mash, mammalian achaete-scute homolog; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Nng1, Neurogenin1; NNDPD, *N*,*N* dimethyl-p-phenylenediamine sulfate; NSCs, neural stem cells; PBS, phosphate-buffered saline; RT–PCR, reverse transcription–polymerase chain reaction.

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such as Parkinson's disease (Kida et al., 2011), Alzheimer's disease (Liu and Bian, 2010), brain ischemia (Li et al., 2011).

In embryonic central nervous system, CBS gene is expressed at an especially high level in the whole neural tube and brain vesicles (Quere et al., 1999). Early expression of CBS gene in neural development suggests that endogenous H_2S may play critical roles in neural lineage restriction. Recently, it has been suggested that H_2S is a novel inducer of neuronal differentiation in NG108-15 cells, as characterized by neuritogenesis (Nagasawa et al., 2009; Tarui et al., 2010). Arising from this, we hypothesized endogenous H_2S might regulate neurogenesis via stimulating proliferation and differentiation of neural stem cells (NSCs).

In this study, we investigated the effects and mechanism of endogenous H_2S on the *in vitro* proliferation and differentiation of NSCs. We showed that aminooxyacetic acid (AOAA), the inhibitor of CBS or knockdown of CBS in using siRNA, significantly attenuated the effects of L-cysteine on elevated H_2S levels and the cell proliferation, also effectively suppressed L-cysteine-induced neurogenesis and astrocytogenesis. Further analysis revealed that L-cysteine-induced proliferation was associated with phosphorylation of extracellular signal-regulated kinases (ERK) 1/2 and differentiation with altered expression of differentiation-related genes.

EXPERIMENTAL PROCEDURES

Primary NSCs' culture and expansion

In the handling and care of all animals, the International Guiding Principles for Animal Research, as stipulated by the World Health Organization (1985) and as adopted by the Laboratory Animal Center, Shandong University, were followed. During the study, the number of animals used and their suffering were minimized. Brains were removed from mouse embryos at E13.5 according to a previously described method (Liu et al., 2009). Following this, the telencephalon was trypsinized for 5 min at 37 °C. After three times washing with Dulbecco's modified Eagle medium – DMEM/F12 (1:1) medium (Gibco, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, cells were resuspended in DMEM/F12 (1:1) medium and cell number was counted. Cells were seeded at 2×10^5 cells/ml in DMEM/F12 (1:1) medium supplemented with 2% B27 (Gibco BRL, Carlsbad, CA, USA), plus 100 U/ml penicillin, 100 μg/ml streptomycin, and basic fibroblast growth factor (bFGF) (20 ng/ml; R&D, Minneapolis, MN, USA), epidermal growth factor (EGF) (20 ng/ml; R&D, USA). The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The culture medium was changed every 2 days and the cells grew into floating neurospheres. After 7 days in vitro, primary neurospheres were dissociated into single cells and the cells grew into neurospheres again. Secondary or tertiary neurospheres were used for subsequent experiments and all experimental procedures were carried out using monolayers of NSCs.

To examine the proliferation of NSCs, the dissociated cells were cultured in reduced growth medium (DMEM/F12 (1:1) medium supplemented with 2% B27, 5 ng/ml of bFGF and 20 ng/ml EGF, plus 100 U/ml penicillin, 100 μg/ml streptomycin). For differentiation of NSCs, neurospheres were transferred into differentiation medium (DMEM/F12 (1:1) medium, plus 100 U/ml penicillin, 100 μg/ml streptomycin, containing 2% fetal bovine serum (FBS) without the growth factor) and cultured for 3–7 days.

Cell viability assay

Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Dissociated NSCs were plated into 96-well culture plates at a density of 5×10^4 cells/well with 200-μl reduced growth medium per well in triplicate. Following this, the cells were incubated with test substances or vehicle, for 24 and 48 h, respectively. Then, 20-μl MTT solution (5 mg/ml, Sigma–Aldrich, St.Louis, MO, USA) was added to each well and incubated at 37 °C for 4 h. The culture medium was aspirated and followed by addition of 200-μl dimethyl sulfoxide. The absorbance value was measured in a microplate reader (Bio-Rad Labs, Hercules, CA, USA) at 490 nm. Values were expressed as a percentage relative to those obtained in controls.

Immunocytochemistry

The cells were fixed in 4% paraformaldehyde for 20 min, and blocked with 10% goat serum in phosphate-buffered saline (PBS). Slides were incubated overnight in a humid chamber at 4 °C with the following primary antibody: anti-glial fibrillary acidic protein (GFAP) (1:500, mouse monoclonal, Millipore Corporation, Billerica, MA, USA); anti-nestin (1:200; rabbit polyclonal, Sigma–Aldrich); anti-microtubule-associated protein 2 (MAP2) (1:200; mouse monoclonal, Millipore); anti-CBS (1:250; rabbit polyclonal, Santa Craz Biotech, CA, USA). After primary antibody incubation, samples were washed again and incubated in the appropriate fluorescent-conjugated secondary antibody (goat anti-mouse/rabbit IgG, 1:500 dilution, Sigma–Aldrich) for 1 h. The cells were counterstained by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma–Aldrich). Images were captured with a Nikon TE2000U microscope.

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was extracted from induced cell cultures using the Trizol reagent (Gibco, Invitrogen) according to the manufacturer's instructions. RNA concentration was determined by a spectrophotometer (Bio-Rad. Labs) at 260 nm. Identical amounts of RNA (1 μg) were reverse transcribed into cDNA by using a commercial RT–PCR kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. cDNA was subsequently amplified by PCR with specific primers (Table 1). PCR products separated on a 1.2% agarose/TAE gel were visualized by staining with ethidium bromide. The densitometric analysis of the data was normalized to β-actin. The intensity of bands was determined using the Image-Pro Plus 6.0 software. Results were mean ± SD from four separate experiments for each group.

Western blot analysis

Cells were washed with cold PBS and lysed in ice-cold RIPA buffer containing protein inhibitors. Cell lysates were incubated at 4 °C for 20 min. The sample was centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentration in the supernatants of cell extract was determined using a BCA protein assay kit (Pierce Biotechnology, Inc. Rockford, IL, USA). A quantity of 30 μg total proteins was loaded onto a 4–20% gradient polyacrylamide gel, electrophoretically transferred to polyvinylidene difluoride membrane and probed with the following primary antibodies: GFAP (1:1000; Millipore), MAP2 (1:1000; Millipore), ERK1/2

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