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Invited Perspective

Hydrogen sulfide protects against endoplasmic reticulum stress and mitochondrial injury in nucleus pulposus cells and ameliorates intervertebral disc degeneration

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ABSTRACT

It has been suggested that excessive apoptosis in intervertebral disc cells induced by inflammatory cytokines, such as interleukin (IL)-1β, is related to the process of intervertebral disc degeneration (IVDD). Hydrogen sulfide (H_2S), a gaseous signaling molecule, has drawn attention for its anti-apoptosis role in various pathophysiological processes in degenerative diseases. To date, there has been no investigation of the correlation of H_2S production and IVDD or of the effects of H_2S on IL-1β-induced apoptosis in nucleus pulposus (NP) cells. Here, we found that the expression levels of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), two key enzymes in the generation of H_2S, were significantly decreased in human degenerate NP tissues as well as in IL-1β-treated NP cells. NaHS (H_2S donor) administration showed a protective effect by inhibiting the endoplasmic reticulum (ER) stress response and mitochondrial dysfunction induced by IL-1β stimulation *in vitro*, the effect was related to activation of the PI3K/Akt and ERK1/2 signaling pathways. Suppression of these pathways by specific inhibitors, LY294002 and PD98059, partially reduced the protective effect of NaHS. Moreover, in the percutaneous needle puncture disc degeneration rat tail model, disc degeneration was partially reversed by NaHS administration. Taken together, our results suggest that H_2S plays a protective role in IVDD and the underlying mechanism involves PI3K/Akt and ERK1/2 signaling pathways-mediated suppression of ER stress and mitochondrial dysfunction in IL-1β-induced NP cells.

1. Introduction

Intervertebral disc degeneration (IVDD) is a spinal disorder whose pathogenesis has not been fully elucidated. It has been suggested that nucleus pulposus (NP) cells which produce cartilage-specific extracellular matrix components play an important role in IVDD [1]. Aberrant apoptosis [2–4] of NP cells has been considered to be the major cellular process associated with IVDD. Studies have demonstrated that several pathological conditions may lead to apoptosis of NP cells, among which abnormally increased inflammatory cytokines, such as IL-1β and tumor necrosis factor-alpha, were considered to play a crucial role in the process of IVDD. Through a series of signaling networks, inflammatory cytokines may induce NP cell apoptosis, which further result in progressive IVDD [5].

The endoplasmic reticulum (ER) is an important organelle responsible for cellular homeostasis. When exposed to prolonged or strong intracellular and extracellular stimuli, the ER stress-related proteins such as caspase 12 will be activated, which may ultimately lead to apoptotic cell death, therefore ER stress is closely related to apoptosis. Another major event in apoptosis is mitochon-

Abbreviations: IL-1, βinterleukin (IL)-1β; IVDD, intervertebral disc degeneration; IVD, intervertebral disc; H2S, hydrogen sulfide; NP, nucleus pulposus; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; ER, endoplasmic reticulum; siRNA, small interfering RNA; MRI, magnetic resonance imagings; mPTP, mitochondrial permeability transition pore; MMP, mitochondrial transmembrane potential.

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drial process, and the Bcl-2 protein family is involved not only in the alteration of mitochondrial membrane potential but also in the release of mitochondrial apoptotic factors [6]. Recently, intervertebral disc cell apoptosis was shown to be mediated by both the endoplasmic reticulum (ER) and the mitochondrial pathways in rats [7]. Zhang et al. reported that annular cell apoptosis induced by cyclic stretch was partially reversed by the inhibition of ER stress in rats [8]. And, there is evidence showing that mitochondrial pathway contribute to fas-mediated apoptosis of human lumbar disc cells [9]. These studies suggest an emerging picture of the importance of the mitochondria and ER in IVDD.

Hydrogen sulfide (H_2S) is synthesized from L-cysteine primarily by two key enzymes: cystathionine-c-lyase (CSE) in the peripheral tissues and cystathionine-b-synthetase (CBS) in the central nervous system. Recognized as the third endogenously produced gaseous messenger, along with nitric oxide and carbon monoxide, H_2S regulates a variety of biological functions including anti-inflammatory [10] and anti-apoptotic [11] effects, affecting several key mechanisms and pathways both *in vitro* and *in vivo*. Recent studies have suggested possible roles for H_2S in modulating ER stress-induced cardiovascular disease. In a rat model of hyperhomocysteinemia-induced cardiomyocytic ER stress injury and in doxorubicin-induced H9c2 cells, H_2S supplementation was shown to antagonize cytotoxicity and apoptosis through inhibiting ER stress-associated proteins [12,13]. Similarly, a study on formaldehyde-induced neurotoxicity in PC12 cells showed that H_2S pre-treatment significantly attenuated the increases in the expression levels of ER stress markers [14]. Additionally, it was recently demonstrated that H_2S may reconcile Akt2 knockout-induced myocardial contractile defects *via* attenuation of mitochondrial injury and apoptosis [15]. However, to our knowledge, whether the production of H_2S is correlated with IVDD and whether H_2S can ameliorate ER stress and mitochondrial dysfunction-induced apoptosis in human NP cells remain unanswered.

This study aimed to illustrate the role of H_2S in IVDD as well as its working mechanism. Our results suggest that H_2S protects against apoptosis in NP cells *in vitro* and ameliorates disc degeneration in rats *in vivo*. We believe that this study may help us to better understand the role of H_2S in pathogenesis of IVDD.

2. Materials and methods

2.1. Ethics statement

All surgical interventions, treatments and postoperative animal care procedures were performed in strict accordance with the Animal Care and Use Committee of Wenzhou Medical University (wydw2014-0129).

2.2. Nucleus pulposus cell isolation and culture

The study was approved by the Second Affiliated Hospital of Wenzhou Medical University Ethics Committee. Patients, between 11 years and 76 years old (A total of 43 disc samples were collected from 38 patients, in total of 17 female and 21 male patients with scoliosis or disc herniation or vertebral fracture), signed an informed consent form allowing the researchers to use NP tissues obtained during spinal surgery. 30 disc samples (Pfirrmann < grade III, and 4 were defined as grade I) were classified as non-degenerative IVD tissue samples according to the Pfirrmann grading scale [16] determined by magnetic resonance imaging (MRI), total 17 disc samples were used in Pfirrmann grading scale(2 male and 2 female, age from 19 to 45, average 34.3 ± 10.9 in grade I; 3 male and 3 female, age from 25 to 46, average 35.8 ± 7.2 in grade III; 4 male and 3 female, age from 38 to 47, average 42.2 ± 4.8 in grade V), which were only used for gene analysis and western blot. And there was no significant difference among the three Pfirrman grade groups in age or sex. In this study, no other complications related to IVDD, such as systemic disease (Diabetes, *etc.*) were found in patients that consented to donate their NP tissues. The gel-like NP tissues were digested in 0.25% trypsin and 0.2% type II collagenase (Gibco) for approximately 3 h at 37 °C. Then, the digested tissues were transferred as explants to Dulbeccomodified Eagle medium (DMEM; Gibco, Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific, Logan, UT) and antibiotics (1% penicillin/streptomycin) in a 5% CO_2 incubator. When confluent, the cells were passaged by using 0.25% Trypsin-EDTA (Gibco, Invitrogen), and subcultured in 60-mm culture dish at the appropriate density. During passaging, no significant changes in morphology of cells between primary cells (passage 0) and later passage cells (passage 2) were noticed.

2.3. Cell culture treatment protocols

To determine whether degeneration induced changes in H_2S-generating and -metabolizing enzymes, NP cells were treated with IL-1β (10 ng/ml) (PeproTech, Rocky Hill, USA) for 24 or 48 h. In order to evaluate the effect of NaHS (Sigma-Aldrich, St. Louis, MO) on the viability of NP cells, cells were incubated for 24 h with increasing concentrations (0.06–1 mM) of NaHS. To establish the apoptosis model of NP cells, different concentrations of IL-1β (10–100 ng/ml) were added into the culture medium for 24 h. Cells were pre-treated with different concentrations of NaHS for 1 h before the addition of IL-1β (75 ng/ml) to investigate its effect on cell apoptosis. To study the role of PI3K/Akt and ERK1/2 signal pathways in NaHS-induced cell protection, NP cells were pre-treated with 1 mM NaHS and 50 μM LY294002 (PI3K/Akt inhibitor; Cell Signaling Technology, MA, USA) or PD98059 (ERK1/2 inhibitor; Beyotime, Shanghai, China) for 1hr before they received IL-1β administration.

2.4. Cell viability assay

Cell viability was assayed with the cell counting kit-8 (CCK-8; Dojindo Co, Kumamoto, Japan) according to the manufacture's protocol. NP cells were treated with NaHS, IL-1β, LY294002 and PD98059 as described above. After treatment the cells were washed with PBS, then 100 μl of DMEM containing 10 μl of CCK-8 solution was added to each well, and the plate was incubated for an additional 1 h. The absorbance of wells was then measured at 450 nm by a micro-plate reader.

2.5. Western blot assay

The human NP tissues and cells were lysed in ice-cold radioimmuneprecipitation assay (RIPA) buffer, supplemented with phosphatase inhibitors and protease inhibitor cocktail (Biotech Well, Shanghai, China). Protein concentration was measured by the BCA protein assay kit (Beyotime, Shanghai, China), and equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidenedifluoride (PVDF) membrane (BIO-RAD, USA). Then the protein was probed with antibody specific to CBS, CSE, CHOP, cytochrome c (Santa Cruz Biotechnology, CA, USA), ERK, p-ERK, Akt, p-Akt, cleaved-caspase3, Bax, Bcl-2 (Cell Signaling Technology, Beverly, MA, USA), ATF-6 (Bioworld, MN, USA), GAPDH (Arigo, Taiwan, ROC), GRP78 and Caspase12 (Abcam, Cambridge, UK) followed by ECL signal detection (Invitrogen).