Nitric Oxide 46 (2015) 114–122

Contents lists available at ScienceDirect

Nitric Oxide

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

Hydrogen sulfide improves glucose metabolism and prevents hypertrophy in cardiomyocytes

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ARTICLE INFO

Article history:
Available online 15 December 2014

Keywords:
Hydrogen sulfide
Cardiac hypertrophy
Glucose transporter

ABSTRACT

Introduction: Hydrogen sulfide (H_2S) has been reported to inhibit myocardial hypertrophy in a cell model of cardiomyocyte hypertrophy. Our previous study also shows an H_2S-induced increase in glucose metabolism in insulin-targeting cells. The present study aims to examine the hypothesis that H_2S attenuates myocardial hypertrophy and promotes glucose utilization in cardiomyocytes.
Methods: The cell model of cardiomyocyte hypertrophy was induced by application of phenylephrine and cardiomyocyte hypertrophy was examined using leucine incorporation assay. Protein levels were measured using Western blot analysis. The activity of related enzymes was measured with enzyme-linked immunosorbent assay (ELISA).
Results: NaHS (an H_2S donor) treatment increased the activity of cultured cardiomyocytes and reduced hypertrophy in cultured cardiomyocytes at concentrations ranging from 25 to 200 μmol/L. NaHS treatment increased glucose uptake and the efficiency of glycolysis and the citric acid cycle. The key enzymes in these reactions, including lactate dehydrogenase and pyruvate kinase and succinate dehydrogenase, were activated by NaHS treatment (100 μmol/L). Some intermediates of glycolysis and the citric acid cycle, including lactic acid, cyclohexylammonium, oxaloacetic acid, succinate, L-dimalate, sodium citrate, cis-aconitic acid, ketoglutarate and DL-isocitric acid trisodium also showed anti-hypertrophic effects in cardiomyocytes.
Conclusions: H_2S improves glucose utilization and inhibits cardiomyocyte hypertrophy.

1. Introduction

Hydrogen sulfide (H_2S) is a colorless gas with the typical odor of rotten eggs. Because of its broad biological effects found in recent years, it has been considered as a new gaseous messenger [1]. We have previously reported that H_2S inhibited cardiomyocyte hypertrophy by up-regulating miR-133a and reducing ROS generation [2]. H_2S can also inhibit hypertrophy and fibrosis by inhibiting the activity of Angiotensin-II and by modifying the expression of Cx43 in the cardiomyocytes [3].

Overexpression of glucose transporter 1 (GLUT1) promotes glucose uptake and glycolysis, increases glucose utilization and prevents cardiomyocyte hypertrophy in a model of chronic pressure overloading [4]. GLUT1 upregulation also protects cells against agonist-induced cardiomyocyte hypertrophy [5]. On the other hand, GLUT4 null mice are more likely to suffer from cardiac hypertrophy [6,7]. A reduction of GLUT4 in a degree of less than 5% induces cardiac hypertrophy in GLUT4-Lox transgenic mice [8]. It is also reported that cytosolic oxidative stress can induce cardiac hypertrophy in mice with specific GLUT4 deletion in the heart [9].

Here we aim to examine the effects of H_2S on cardiomyocyte hypertrophy, glucose metabolism (including glycolysis and the Krebs cycle), the expression of GLUT1 and 4.

2. Methods

2.1. Animal

The animals were treated under the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH) of the United States and were approved by the Ethic Committee of Experimental Research, Shanghai Medical College, Fudan University.

2.2. Reagents

Lactic acid (LD), 2, 3-Diphospho-D-glyceric acid penta (cyclohexylammonium) salt, oxaloacetic acid, succinate, L-dimalate,

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http://dx.doi.org/10.1016/j.niox.2014.12.007
1089-8603/

cis aconitic acid, ketoglutarate and DL-isocitric acid trisodium were purchased from Sigma (St. Louis, MO). Sodium pyruvate and sodium citrate were purchased from Genebase Gene-Tech (Shanghai, China).

2.3. Cell culture

Primary neonatal rat cardiac ventricular myocytes (NRCMs) were collected as previously described with some modifications [10]. Briefly, cardiac ventricles were dissected from new born Sprague-Dawley rats and the tissues were put in ice-cold phosphate-buffered-buffered saline (PBS). After rinsing for 3 times, the cardiac ventricles were cut and digested with 0.125% trypsin. Isolated cardiomyocytes were cultured in DMEM/F12 (Dulbecco's modified Eagle's medium/F-12, Life Technologies Corporation, California, USA) supplemented with 10% FBS (Thermo Fisher Hyclone, MA, USA), penicillin/streptomycin (100 units) and 0.1 mmol/L 5-bromo-2′-deoxyuridine. Phenylephrine (PE, 10 or 100 μmol/L, Sigma) was applied to induce cardiomyocyte hypertrophy in serum-free medium. NRCMs were pretreated with different concentrations of NaHS before PE application to induce cardiomyocyte hypertrophy.

2.4. Assessment of cardiomyocyte hypertrophy with [^{3}H]-leucine incorporation and cell surface measurement

Protein synthesis was detected by [^{3}H]-leucine (Perkin Elmer Life Sciences, MA, USA) incorporation assay in the cardiomyocytes. The NRCMs were cultured in 24-well plates, treated with PE or vehicle for 24 h. Then [^{3}H]-leucine (1 μCi/ml) was added into the medium, and the NRCMs were cultured for another 24 h. The cells were washed three times with ice-cold D-PBS and incubated with 10% trichloroacetic acid for 30 min at 4 °C. Then the cells were washed three times and homogenized in 1 M NaOH. Radioactivity of cell homogenate was counted using liquid scintillation counter (Beckman LS6000, Fullerton, CA, USA).

The NRCMs were plated at the density of 1.5×10^5 cells/ml. The cells were treated with vehicle (control), PE (10 μmol/L) or NaHS (100 μmol/L) for 24 h. Then cells were stained with hematoxylin-eosin and micrograph were captured with an inverted fluorescence microscope (Leica TCS SP2, Wetzlar, Germany) equipped with a CCD camera. Cell surface area was measured with ImageJ software. 15 individual cells were randomly selected in each field, six random fields were examined by an observer blinded to the treatment regimen. Values were normalized to the controls.

2.5. Western blotting

The NRCMs were harvested in lysis buffer. Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, China). Equal amounts (70 μg) of protein were added and separated on a 12% SDS-polyacrylamide gel. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA), blocked in TBS with 5% milk/0.1% Tween 20, and incubated overnight at 4 °C with polyclonal anti-GLUT1 (1:1000, Abcam, Cambridge, UK), anti-GLUT4 (1:1000, Abcam), anti-insulin receptor (Abcam 1:3000), anti-p-insulin receptor (Abcam 1:1000) or anti-beta Actin (Beyotime 1:3000) in blocking buffer. The membranes were washed in 0.1% Tween/TBS and incubated in horseradish peroxidase-conjugated anti-rabbit (Beyotime) or anti- mouse secondary antibodies (Beyotime) for 2 h at room temperature, followed by detection of chemiluminescence. The objective bands were tested with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific-Pierce, Waltham, MA, USA).

2.6. Cell viability assays

The viability of NRCMs was measured by using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan). The cells were cultured in 96-well plates, and then incubated with 10 μl CCK-8 in 90 μl DMEM/F12 without phenol red for 4 h at 37 °C. The absorbance of CCK-8 was obtained with a microplate reader at 450 nm. The values were normalized to the controls. Each experiment was repeated for six times.

2.7. Assessment of lactic acid and glucose content in supernatant

NRCMs plated on 35 mm dishes were treated with vehicle (control), PE (10 μmol/L) or NaHS (50 and 100 μmol/L) for 48 h. 10 μl supernatant was collected and applied to the Lactate Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Another 4 μl sample was used to measure protein concentrations for standardization.

NRCMs plated on 35 mm dishes were treated with vehicle (control), PE 10 μmol/L or NaHS for 48 h. 5 μl diluted supernatant (1:10 dilution) was applied to the Glucose Assay Kit (Applygen Technologies Inc, Beijing, China) according to the manufacturer's instructions.

2.8. Assessment of the activity of LDH, PK and SDH

NRCMs plated on 35 mm dishes were treated with vehicle (control), PE (10 μmol/L) or NaHS (100 μmol/L) for 48 h. Cardiomyocytes were harvested in 100 μl WB&IP buffer (Beyotime). 1 μl diluted sample (1:30 dilution) was applied to LDH Assay Kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Another 5 μl sample was used to measure protein concentration for standardization.

For PK activity analysis, the NRCMs were seeded and treated as LDH analysis. Cardiomyocytes were harvested in 60 μl WB & IP buffer (Beyotime). 4 μl sample was applied to the PK Assay Kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Another 5 μl sample was used to measure protein concentration for standardization.

For SDH activity analysis, the NRCMs were seeded, treated and harvested as LDH analysis. 10 μl sample was applied to the SDH Assay Kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Another 5 μl sample was used to measure protein concentration for standardization.

2.9. Assessment of ATP and cAMP contents in the NRCMs

The NRCMs plated on 35 mm dishes were treated with vehicle (control), PE (10 μmol/L) or NaHS (100 μmol/L) for 48 h. Cardiomyocytes were harvested in 200 μl buffer supplied with the ATP Assay Kit (Beyotime). 10 μl sample was applied to the ATP Assay Kit according to the manufacturer's instructions. Another 5 μl sample was used to measure protein concentration for standardization.

The NRCMs plated on 35 mm dishes were treated with vehicle (control), PE (10 μmol/L) or NaHS (100 μmol/L) for 48 h. The cells were harvested in 60 μl WB and IP buffer (Beyotime). 10 μl sample was applied to the cAMP Assay Kit (R&D systems, Minneapolis, USA) according to the manufacturer's instructions.

2.10. 2-Deoxyglucose uptake assay

Uptake of 2-Deoxyglucose (2-DG) by NRCMs was measured at the end of treatment protocols. The NRCMs were rinsed with KRP buffer (128 mmol/L NaCl, 4.7 mmol/L KCL, 1.25 mmol/L $CaCl_2$, 1.25 mmol/L $MgSO_4$, 5 mmol/L NaH_2PO_4, 5 mmol/L Na_2HPO_4, and 10 mmol/L HEPES, pH 7.4) containing 0.1% (w/v) BSA and 5 mmol/L