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Research article

Inhibition of hydrogen sulfide on the proliferation of vascular smooth muscle cells involved in the modulation of calcium sensing receptor in high homocysteine

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ABSTRACT

Hyperhomocysteinemia induces the proliferation of vascular smooth muscle cells (VSMCs). Hydrogen sulfide (H_2S) inhibits the phenotype switch of VSMCs and calcium-sensing receptor (CaSR) regulated the production of endogenous H_2S. However, whether CaSR inhibits the proliferation of VSMCs by regulating the endogenous cystathionine-gamma-lyase (CSE, a major enzyme that produces H_2S) pathway in high homocysteine (HHcy) has not been previously investigated. The intracellular calcium concentration, the concentration of H_2S, the cell viability, the proliferation and the expression of proteins of cultured VSMCs from rat thoracic aortas were measured, respectively. The results showed that the $[Ca^{2+}]_i$ and the expression of p-CaMK and CSE increased upon treatment with CaSR agonist. In HHcy, the H_2S concentration decrease, the proliferation and migration rate increased, the expression of Cyclin D1, PCNA, Osteopontin and p-Erk1/2 increased while the α-SM actin, $P21^{Cip/WAK-1}$ and Calponin decreased. The CaSR agonist or exogenous H_2S significantly reversed the changes of VSMCs caused by HHcy. In conclusion, our results demonstrated that CaSR regulate the endogenous CSE/H_2S is related to the PLC-IP_3 receptor and CaM signal pathways which inhibit the proliferation of VSMCs, and the latter is involved in the Erk1/2 dependent signal pathway in high homocysteine.

1. Introduction

Hyperhomocysteinemia is a pathological condition characterized by an increase in plasma concentration of total homocysteine (Hcy) [1,2]. Numerous studies have indicated that hyperhomocysteinemia is an independent risk factor for cardiovascular diseases and the latter is a major cause of death and disability in patients [3,4]. During vascular injury, vascular smooth muscle cell proliferation is a critical factor and a noted characteristic. Recent studies have suggested that vasodilators play a major role in modulating the proliferation of VSMCs [5].

Hydrogen sulfide (H_2S) is a member of the gaseous transmitter family. It is a strong reducing agent [6,7] and vasodilator [8] and is endogenously synthesized from L-cysteine. In mammalian cardiovascular tissues, the biosynthesis of H_2S is mainly catalyzed by cystathionine-gamma-lyase (CSE). Recent studies have shown that H_2S is involved in vasorelaxation [8], cardioprotection [9] and the inhibition of proliferation of VSMCs [10]. A deficiency in the CSE/H_2S pathway plays an important role in the development of certain cardiovascular diseases [11,12]. However, whether H_2S protects the periphery vasculature against high homocysteine (HHcy)-induced injury remains unclear.

The calcium-sensing receptor (CaSR) belongs to the transmembrane G-protein coupled receptor family. Extracellular calcium binds CaSR to cause phosphatidylinositol biphosphate (PIP_2) to be cleaved into diacylglycerol (DAG) and inositol 1,4,5-trisphoshpate (IP_3) through the PLC (phospholipase C) pathway. IP_3 binds IP_3 receptor on the sarcoplasmic reticulum, causes calcium ion to be released and thus increases the calcium ion concentration in the cytoplasm [13,14]. Evidence has suggested that a

Abbreviations: HHcy, high homocysteine; VSMCs, vascular smooth muscle cells; H_2S, hydrogen sulfide; CaSR, calcium-sensing receptor; CSE, cystathionine-gamma-lyase; $[Ca^{2+}]_i$, intracellular calcium ion; PIP_2, phosphatidylinositol biphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphoshpate; PLC, phospholipase C; A7r5, vascular smooth muscle cells of rat thoracic aorta; FI, fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; BrdU, 5-bromo-2′-deoxyuridine; p-CaMK, phosphorylation calmodulin kinase

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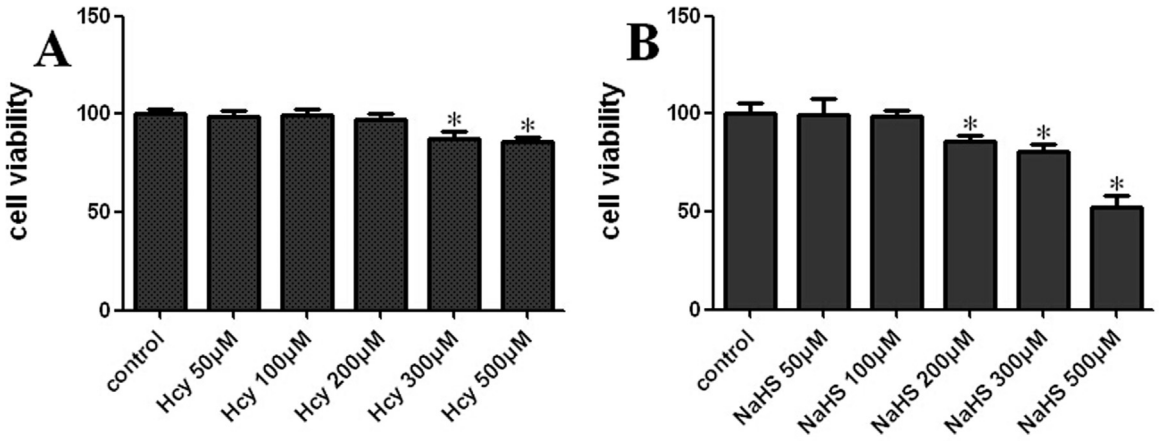

Fig. 1. The proper concentration of treatment liquid (Hcy or NaHS) on the VSMCs by MTT assay (The viability of A7r5 cells was measured by the MTT assay, A**:** Hcy vs control, $^{*}p < 0.05$; B**:** NaHS vs control,$^{*}p < 0.05$; n=3).

functional CaSR is expressed in VSMCs [15]. A recent study found that increasing the $[Ca^{2+}]_i$ increases endogenous H_2S production in smooth muscle cells [16].

In the present study, we determined that the reduction of H_2S production in a VSMCs high homocysteine model stimulated SMCs phenotype changes. CaSR could regulate H_2S production and arrest the progression of VSMCs proliferation in high homocysteine.

2. Materials and methods

2.1. Materials

The primary antibodies for *anti*-CaSR, anti-CSE, anti-PCNA, $P21^{Cip/WAK-1}$, α-smooth muscle actin, calponin, OPN, p-CaMK, p-Erk1/2, actin and MTT, BrdU kits were from Santa Cruz (Bergheimer, Germany). NaHS (exogenous H_2S donor), Calindol (CaSR agonizts), U73122 (PLC-specific inhibitor), Calhex231 (CaSR-specific inhibitor), 2-APB (IP_3 receptor inhibitor), TG (Ca^{2+}-ATP inhibitor), PD98095 (Erk inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). H_2S ELISA kit was purchased from Kamiya Biomedical Co. (Seattle, WA, USA). All other chemicals were from Sigma or Santa Cruz.

2.2. Vascular smooth muscle cells of rat thoracic aorta (A7r5) culture and the experimental protocols

Vascular smooth muscle cells of rat thoracic aorta (A7r5) were maintained in DMEM containing 10% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD), penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified chamber containing 5% CO_2 incubator. The experiments were performed when the cells reached 80–90% confluence. In all studies, cells were incubated in the low glucose (5.56 mM) medium. The cultured cells were randomly divided into the normal part experiment groups and HHcy part experiment groups and treatments. The normal part experiment groups including: control (5.56 mM glucose), Calindol (2 μM, CaSR agonizts), calhex231 (3 μM, CaSR-specific inhibitor)+Calindol, 2-APB (75 μM, IP_3 receptor inhibitor)+Calindol, U73122 (10 μM, PLC-specific inhibitor)+Calindol and TG (10 μM, Ca^{2+}-ATP inhibitor)+Calindol; The HHcy part groups including: control, HHcy (200 μM), HHcy+NaHS (100 μM), HHcy+Calindol (2 μM) and HHcy+PD98095 (10 μM, Erk inhibitor). In HHcy part experiment groups, cells were subsequently incubated in the homocysteine (200 μM) medium for 48 h. In all experiment groups, drugs were added directly to the medium for 24 h. Each group included 3–4 samples (n=3–4).

2.3. Measurement of $[Ca^{2+}]_i$ with Fluo-4/AM

The VSMCs were placed onto coverslips, which were covered in 12-well culture plates. After 72 h at 37 °C, the VSMCs were washed with PBS and incubated with 5 μM Fluo-4/AM for 30 min at 37 °C in the dark. The cells were rinsed three times with Tyrode's solution to remove the remaining dye. During experiment, FI (fluorescence intensity) of fluo-4/AM in VSMCs was recorded using a laser-scanning confocal microscope (Olympus, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

In positive and negative group, cells were exposed to CaSR agonizts (Calindol, 2 μM) recorded for 330 s at 3 s intervals. In Calindol experiments, the VSMCs preincubated with specific inhibitor, U73122 (10 μM), Calhex231 (3 μM), 2-APB (75 μM) and TG (10 μM) for 20 min before Calindol treatment. Image analysis was performed off-line using Fluoview-FV300 (Olympus, Japan) to select cell regions from which FI was extracted, and further analysis was conducted with Excel (Microsoft) and Origin Version 7.5 software (OriginLab Corporation). $[Ca^{2+}]_i$ changes were expressed as fluorescence intensity representing FI and normalized to initial fluorescence intensity.

2.4. Measurement of H_2S production

H_2S production rate was measured with rat hydrogen sulfide ELISA kit for the quantitative determination. The coated well immunoenzymatic assay utilizes a monoclonal anti-H_2S antibody and a H_2S-HRP conjugate. Briefly, cell culture fluid and buffer are incubated together with H_2S-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are decanted and washed three times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450 nm in a microplate reader.

2.5. Cell viability, proliferation and scratch wound repair assay

Vascular smooth muscle cells of rat thoracic aorta were cultured in 96-well tissue culture plates (1×10^4 cells/well) with 10% FBS for 24 h. Then the serum-free medium was used and cells were exposed to different reagents for another 24 h. Cell viability, proliferation and migration rate were measured respectively by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 5-bromo-2′-deoxyuridine (BrdU) and scratch wound repair incorporation assays.