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Calcium sensing receptor initiating cystathionine-gamma-lyase/hydrogen sulfide pathway to inhibit platelet activation in hyperhomocysteinemia rat

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ABSTRACT

Hyperhomocysteinemia (HHcy, high homocysteine) induces the injury of endothelial cells (ECs). Hydrogen sulfide (H_2S) protects ECs and inhibits the activation of platelets. Calcium-sensing receptor (CaSR) regulates the production of endogenous H_2S. However, whether CaSR inhibits the injury of ECs and the activation of platelets by regulating the endogenous cystathionine-gamma-lyase (CSE, a major enzyme that produces H_2S)/H_2S pathway in hyperhomocysteinemia has not been previously investigated. Here, we tested the ultrastructure alterations of ECs and platelets, the changes in the concentration of serum homocysteine and the parameters of blood of hyperhomocysteinemia rats were measured. The aggregation rate and expression of P-selectin of platelets were assessed. Additionally, the expression levels of CaSR and CSE in the aorta of rats were examined by western blotting. The mitochondrial membrane potential and the production of reactive oxygen species (ROS) were measured; the expression of phospho-calmodulin kinases II (p-CaMK II) and Von Willebrand Factor (vWF) of cultured ECs from rat thoracic aortas were measured. We found that the aggregation rate and the expression of P-selectin of platelets increased, and the expression of CaSR and CSE decreased in HHcy rats. In the ECs of HHcy group, the ROS production increased and the mitochondrial membrane potential decreased markedly, the expression of CSE and the p-CaMK II increased after treatment with CaSR agonist while decreased upon administration of U73122 (PLC-specific inhibitor) and 2-APB (IP_3 Receptor inhibitor). CaSR agonist or NaHS significantly reversed the ECs injured and platelet aggregation caused by hyperhomocysteinemia. Our results demonstrate that CaSR regulates the endogenous CSE/H_2S pathway to inhibit the activation of platelets which concerts the protection of ECs in hyperhomocysteinemia.

1. Introduction

Hyperhomocysteinemia (HHcy) is a pathological condition characterized by an increase in plasma concentration of total homocysteine (Hcy) [1,2]. Numerous clinical and epidemiological studies have indicated that HHcy is an independent risk factor for ischemic heart disease, stroke, peripheral vascular disease and atherothrombotic disease [3–5]. Studies using animal models of genetic- and diet-induced HHcy have recently demonstrated a causal relationship between HHcy and endothelial dysfunction [6]. Recent studies have also demonstrated that HHcy causes cell dysfunction and induces apoptotic cell death in cell types which relevant to atherothrombotic disease, including endothelial cells (ECs) [7,8] and smooth muscle cells (SMCs). There is growing interest for treatment of HHcy as a strategy for prevention of atherothrombotic disease.

Hydrogen sulfide (H_2S) is a member of the gaseous transmitter family. It is a strong reducing agent [9,10] and vasodilator [11] and is endogenously synthesized from L-cysteine which is originated from homocysteine [1]. 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 [11], protection of ECs [12] and the mediation of anti-thrombotic [13]. A deficiency in the CSE/H_2S pathway plays an important role in the development of certain cardiovascular and atherothrombotic diseases,

Abbreviations: HHcy, hyperhomocysteinemia; ECs, endothelial cells; H_2S, hydrogen sulfide; CaSR, calcium-sensing receptor; CSE, cystathionine-gamma-lyase; [Ca2+]i, intracellular calcium ion; PIP2, phosphatidylinositol biphosphate; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphoshpate; PLC, phospholipase C; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; p-CaMK, phosphorylation calmodulin kinase; vWF, Von Willebrand Factor; ROS, reactive oxygen species

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such as atherosclerosis and thrombosis [13,14]. However, whether H_2S protects ECs against hyperhomocysteinemia-induced activation of platelets 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 the secondary messengers diacylglycerol (DAG) and inositol 1,4,5-trisphoshpate (IP_3) through the PLC (phospholipase C) pathway. IP_3 binds the IP_3 receptor on the sarcoplasmic reticulum, which causes calcium to be released and thus increases the calcium concentration in the cytoplasm [15,16]. Some evidence suggests that a functional CaSR is expressed in aortic endothelial cells (AECs) and smooth muscle cells (SMCs) [17,18]. A recent study found that increasing the $[Ca^{2+}]_i$ increases endogenous H_2S production in SMCs [18].

The role of H_2S in inhibiting the aggregation of platelets has been investigated previously [19]. However, the effects of the CSE/H_2S pathway on platelet aggregation have not been characterized, and, identifying an interaction between CaSR and the CSE/H_2S pathway may uncover the mechanism of endogenous H_2S protection on ECs involved in the activation of platelets.

In the present study, we have determined that the reduction of H_2S production in a methionine-induced HHcy rat model stimulates platelets aggregation. CaSR can influence CSE expression and H_2S production and arrest the activation of platelets in hyperhomocysteinemia.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–250 g) were obtained from the Experimental Animal Center of Harbin Medical University (Harbin, People's Republic of China). All animal experimental protocols complied with the 'Guide for the Care and Use of Laboratory Animals' published by the United States National Institutes of Health. All animals were housed at the animal care facility of Harbin Medical University at 25 °C with 12/12-h light/ dark cycles and allowed free access to normal rat chow and water throughout the study period. Rats were randomly assigned to different treatment groups.

2.2. HHcy model and experimental groups

HHcy was induced by gastragavage of L-methionine (2.0 g/kg/d) dissolved in 2.5% starch, whereas the control group was gastragavaged 2.5% starch alone by the same route. Total Hcy (tHcy) levels of the rat serum were measured every week after gastragavage of methionine, and blood tHcy levels over 16.0 μM were accepted as HHcy. The HHcy rats were divided into 4, 8, and 12 weeks HHcy groups, HHcy + NaHS (H_2S donor, 5.6 mg/kg/d) and HHcy + Calindol (CaSR agonist, 10 μM/kg/d in saline) treatment groups [20]. NaHS was given by intraperitoneal injection in the NaHS treatment groups and Calindol was given by subcutaneous injection in the Calindol treatment groups each day.

2.3. Clinical laboratory testing

The blood of rats was drawn from aorta abdominalis by vacuum blood collection tube containing EDTA or sodium citrate. Immediately after the collection, blood samples were transferred to the hospital laboratory, where routine blood tests and standard coagulation tests were performed using automatic blood cell analyzer and blood coagulation analyzer.

2.4. Preparation of platelet-rich plasma and platelet aggregation assay

Whole fresh blood was drawn from the aorta abdominalis into anticoagulant tubes containing 3.8% sodium-citrate (9:1, v/v). The collected blood was centrifuged at 180×*g* for 10 min at room temperature, and the supernatant was used as platelet-rich plasma (PRP). The pellet was further centrifuged at 1500×*g* for 10 min at room temperature to obtain the platelet-poor plasma (PPP). The number of plate-lets was adjusted to 3×10^8 cells/L by diluting PRP with PPP.

Agonist (ADP 5.0 μM or Collagen 5.0 μg/mL)-induced platelet aggregation was determined by using a light transmission aggregometer. Briefly, PRP was used to set 0% light transmission and PPP was used to set 100% light transmission. Then, in each cuvette, 200 μl of PRP was dispensed and incubated at 37 °C under constant stirring at 1000 rpm for 1 min. Subsequently, the platelet aggregation agonist was added to initiate platelet aggregation. The maximum aggregation was recorded. All aggregation tests were performed within 3 h of blood collection.

2.5. Flow cytometry

The surface expression of CD62P (P-selectin) binding to activated platelets was studied by flow cytometry in a FACS Calibur flow cytometer (BD, San Jose, CA) using antibodies from BD Biosciences. Briefly, an aliquot of 100 mL pretreated PRP was incubated with anti-CD62P-FITC for 30 min in the dark at 37 °C. Following labeling, platelets were fixed in 1% para-formaldehyde and diluted with PBS for further analysis. Platelet specific antigen, CD61, was used in all tests for platelet gating. Besides, anti -IgG1-FITC was used as isotype control to exclude nonspecific binding. Data from 10,000 events were collected in each sample for the evaluation of mean fluorescence intensity (MFI).

2.6. Ultrastructural changes in the ECs of aortas and platelets of rats

Ultrastructural changes in the ECs of aortas and platelets of rats were detected by transmission electron microscopy (TEM). Samples were taken at the aortas in six rats from each group. Samples for TEM were cut into pieces less than 1 mm^3 and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 4 h. Tissues were post-fixed in 1% osmium tetroxide in 1% $K_4Fe(CN)_6$ buffered with 0.1 M sodium cacodylate, dehydrated through graded concentrations of ethanol and propylene oxide and subsequently embedded in Epon 812. Ultrathin sections were cut from blocks and mounted on copper grids. Then, the grids were counterstained with lead citrate and uranyl acetate. Photomicrographs were obtained using Zeiss Axiophot microscopes.

2.7. Enzyme-Linked Immuno sorbent Assay (ELISA)

Plasma vWF levels were assessed using commercial ELISA Kit and following the manufacturer's procedures. Each ELISA was carried out in duplicate for at least three separate experiments.

2.8. Endothelial cells of rat thoracic aorta culture

Endothelial cells of rat thoracic aorta were maintained in DMEM containing 10% fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, 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 medium. In certain selective experiments, cells were subsequently incubated in the homocysteine (200 μM) medium for 48 h.

2.9. Cell viability assay

Endothelial 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