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Original article

Hypercysteinemia promotes atherosclerosis by reducing protein S-nitrosylation

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ABSTRACT

Protein S-nitrosylation plays important role in the regulation of cardiovascular functions in nitric oxide (NO) Pathway. Hypercysteinemia (HHcy) is an independently risk factor for atherosclerosis. We hypothesized that HHcy promotes atherosclerosis by reducing level of vascular protein S-nitrosylation. The aim of present study is to investigate effect of HHcy on vascular protein S-nitrosylation. A total of 45 male apoE−/− mice were randomly divided into three groups. The control group was fed a Western-type diet. The HHcy group was fed a diet containing 4.4% L-methionine, and the HHcy + NONOate group was fed a diet containing 4.4% L-methionine and administrated NONOate (ip). Human umbilical vein endothelial cells were performed for in vitro experiment. Plasma lipids were measured every 4 weeks. After 12 weeks, aortic atherosclerotic lesion areas were detected as well as cellular components. The levels of plasma homocysteine (Hcy) and NO were measured. S-nitrosylation was detected using immunofluorescence, and further confirmed by biotin switch method. We found that compared with the control group, Hcy levels, and atherosclerotic plaque, and content of vascular smooth muscle cells and macrophages in lesions significantly increased, and levels of NO significantly decreased in the HHcy group. However, NONOate reverses this effect. In addition, Hcy significantly reduced protein S-nitrosylation in human umbilical vein endothelial cells. This reduction of protein S-nitrosylation was accompanied by reduced levels of NO. Our results suggested that Hcy promoted atherosclerosis by inhibiting vascular protein S-nitrosylation.

1. Introduction

S-nitrosylation is a covalent post-translational modification that occurs in proteins in virtually all biological systems. The process involves the coupling of a nitric oxide (NO) moiety to the reactive thiol of an adjacent cysteine residue to form an S-nitrosothiol (SNO) [1]. S-nitrosylation is similar to photophosphorylation which can modulate the biological activity of a multitude of proteins.

S-nitrosylation plays an important role in NO signal transduction, such as ion channel activity [2], anti-oxidative stress [3], anti-apoptotic mechanisms [4], and anti-inflammatory responses [5]. These functions were attributed to the protein S-nitrosylation [2–5]. It has been shown that many factors are involved in the regulation of protein S-nitrosylation in endothelial cells. Tumor necrosis factor-α [5], oxidized low density lipoprotein [5], and high glucose [6] decrease protein S-nitrosylation, whereas shear stress [7], 17β-estradiol [8], and acute hypoxia [9] up-regulate protein S-nitrosylation in endothelial cells.

Homocysteine is a sulfur-containing amino acid that functions as a key intermediate in methionine metabolism. It is produced as a

Abbreviations: Hcy, homocysteine; HDL-C, high-density lipoprotein cholesterol; HHcy, hypercysteinemia; HUVECs, human umbilical vein endothelial cells; Mϕ, macrophage; NF-κB, nuclear factor κB; NO, nitric oxide; ROS, reactive oxygen species; SMC, smooth muscle cell; SNO, S-nitrosothiol; TC, total cholesterol; TG, triglycerides.

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byproduct of methyl-transfer reactions, which are important for the synthesis of DNA, methylated proteins, neurotransmitters, and phospholipids [10]. High plasma levels of homocysteine, termed hyperhomocysteinemia (HHcy), numerous studies have demonstrated that HHcy is an independent risk factor for cardiovascular disease [11]. It has been reported that Hcy reduced level of NO [12]. Our team has demonstrated that Hcy impairs endothelial function by inhibiting endothelial protein S-nitrosylation [13], However, it remains unclear whether Hcy impairs protein S-nitrosylation in aorta by mediating the level of NO to induce the development of atherosclerosis.

Thus, we hypothesized that Hcy may promote the development of atherosclerosis by reducing the levels of protein S-nitrosylation in aorta. In the present study, we examined the effects of homocysteine on mice aortas and human umbilical vein endothelial cells (HUVECs). We found that Hcy significantly reduced the aortas and cellular protein S-nitrosylation. These findings provide new insights into the understanding of the pathway for Hcy-mediated vascular damage.

2. Materials and methods

2.1. Animals

Forty-five male homozygous apoE$^{-/-}$ mice (25–27 g), obtained from Vital River Laboratory Animal Technology Co. Ltd. and fed western diet containing 21% vegetable fat, 0.15% cholesterol, 1.1% L-methionine (L-Met, Fluka/Sigma–Aldrich, St. Louis, MO, USA), and 0.001% folic acid at 6 weeks of age. After a week, the mice were divided into three groups: control group (western diet, $n = 15$), HHcy group (additional 3.3% L-Met in the western diet, $n = 15$), HHcy + NONOate group [NONOates (Cayman Chemical Company, Ann Arbor, MI, USA), 0.4 mg/kg/day, intraperitoneal injection, additional 3.3% L-Met in the diet, giving a total of 4.4% L-Met, $n = 15$)] for 12 weeks. The mice were housed in the temperature room with a 12-h light/12-h dark cycle with free access to food and water. The animal experiments in this study were approved by the Laboratory Animal Administration Committee of Xi'an Jiaotong University and performed according to the Guidelines for Animal Experimentation of Xi'an Jiaotong University and the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996).

2.2. Plasma lipids, NO and Hcy assay

Blood was collected via the retinal vein plexus after fasting overnight every 4 weeks. At the end of study, after fasting overnight the mice were anesthetized and exsanguinated by withdrawing the maximum amount of blood from the right ventricle. The tubes of blood containing EDTA anticoagulant were stored on ice and centrifuged (2000 rpm for 15 min at 4 °C) to obtain plasma and the plasma was stored at −80 °C. Plasma total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) were analyzed using commercial kits (Biosino Bio-technology & Science Inc., Beijing, China). Total plasma Hcy concentrations were measured using an ELISA kit (R&D Systems Inc., Minneapolis, MN, USA).

The level of NO of the plasma was determined by estimating the total concentration of nitrate/nitrite using a nitrate/nitrite colorimetric assay kit (Jiancheng Technology Ltd., Nanjing, China).

2.3. Analysis of histology of atherosclerotic lesions

After blood sampling, the mice were flushed (20 ml 0.01 M PBS) from the right ventricle. The aortic tree was isolated, opened longitudinally, fixed in 10% neutral buffered formalin, and stained with oil red O. The area of the atherosclerotic lesion (oil red O area) was measured using image analysis software (Mitani Co., Tokyo, Japan).

For the microscopic quantification of the lesion area, half of the hearts were cut transversely, the heart including the aortic root embedded in OCT compound and frozen for quantification of plaque areas, the aortic root was cut into 5 mm thick serial sections, stained with hematoxylin and eosin (H&E), and oil red O. For microscopic evaluation of the cellular components, serial frozen sections were immunohistochemically stained with antibodies (Abs) against mac-2 (Abcam, Cambridge, MA, USA), α-actin (Abcam, Cambridge, MA, USA) [14]. Macrophage (Mϕ) and smooth muscle cell (SMC) were measured in the lesions by quantifying the area positive for immunohistochemical staining using an image analysis system.

2.4. Cell culture

HUVECs were isolated from human umbilical cords using 0.1% (w/v) collagenase (Gibco/Invitrogen, Carlsbad, CA, USA). The protocol was approved by the Xi'an Jiaotong University Second Affiliated Hospital Ethics Committee, and the investigation also conformed to the principles outlined in the Declaration of Helsinki for the use of human tissue or subjects. HUVECs were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in M199 medium (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Life Technologies, Carlsbad, CA, USA), 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA), 100 μg/ml heparin sodium (Fluka/Sigma–Aldrich, St Louis, MO, USA), and 1% (v/v) endothelial cell growth supplement (Sciencell Research Laboratories, Carlsbad, CA, USA). The growth medium was changed every three days until the cells reached confluence. The identity of the HUVECs was performed by positive staining with an anti-von Willebrand factor antibody (Thermo Fisher Scientific Pierce, Rockford, IL, USA) [15]. After the HUVECs became confluent, they were incubated in M199 medium containing 2% FBS for 16 h prior to different treatments [16].

2.5. Immunofluorescence

The aortic sinuses frozen sections which placed on glass slides fixed in acetone for 10 min. The sections were permeabilized with 5% (v/v) Trion X-100 for 10 min, and blocked with 5% goat serum for 60 min, and incubated with rabbit polyclonal anti-nitrosocysteine antibody (Abcam, Cambridge, MA, USA) at 4 °C. After incubation for overnight, sections were treated with a FITC-conjugated goat anti-rabbit IgG (H + L) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for 5 h at room temperature. The results were visualized using fluorescence microscopy (Olympus, Tokyo, Japan). The mean fluorescence intensity was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA) [8].

The HUVECs were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were permeabilized with 0.1% (v/v) Triton X-100 for 15 min, and blocked with 5% horse serum for 30 min, and incubated with anti-nitrosocysteine rabbit polyclonal antibody (Abcam, Cambridge, MA, USA) at 4 °C. After incubation for overnight, Cell was treated with a FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for 30 min at room temperature. The results were visualized using fluorescence microscopy (Olympus, Tokyo, Japan). The mean fluorescence intensity was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA) [15].