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Effects of allicin on hyperhomocysteinemia-induced experimental vascular endothelial dysfunction

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ABSTRACT

This study was designed to investigate the effect and mechanism of allicin on hyperhomocysteinemia-induced experimental vascular endothelial dysfunction in rats. Fifty male Wistar rats were randomly divided into five groups: the normal control rats (NC), the high-methionine-diet rats (Met), the high-methionine-diet rats treated with folic acid, vitaminB_6 and vitaminB_{12} (Met+F), or with low-dose allicin (Met+L), or with high-dose allicin (Met+H). After 6 weeks, we collected blood samples of all groups to determine plasma endothelin (ET), serum homocysteine (Hcy), nitric oxide (NO), superoxide dismutase (SOD), malondialdehyde (MDA), and detected the expression of basic fibroblast growth factors (bFGF), transforming growth factor beta (TGF-β), tumor necrosis factor-alpha (TNF-α), and intercellular adhesion molecule-1 (ICAM-1) in the aorta. The Hcy and the expression of TGF-β in both the Met+L and Met+H groups were significantly lower than the Met and Met+F groups. The ET, ET/NO ratio and the MDA levels of the Met+L and Met+H groups were significantly lower than the Met group. The SOD and NO levels and the expression of bFGF, TNF-α and ICAM-1 of the Met+L and Met+H groups were significantly higher than the Met group. Our data indicate that allicin inhibits lipid peroxidation induced by hyperhomocysteinemia and regulates the excretion and equilibrium of ET and NO, and suggest that allicin might be useful in the prevention of endothelial dysfunction caused by hyperhomocysteinemia.

1. Introduction

Elevated serum Hcy has been considered as an independent risk factor for atherosclerotic vascular disease (Homocysteine Studies Collaboration, 2002; Bautista et al., 2002; Cui et al., 2008). As it has been demonstrated by many studies (Antoniades et al., 2009) that serum Hcy levels in patients with vascular disease are significantly higher than those of normal subjects, it is no doubt that Hcy plays an important role in the development of atherosclerotic lesion. The pathological mechanisms that lead to the atherogenic propensity associated with hyperhomocysteinemia demonstrate that a key target of elevated Hcy levels is the vascular endothelium, where it produces endothelial dysfunction and structural endothelial injury (Weiss et al., 2002, 2003). For these conclusions, supplementation with clinical medication to improve vascular endothelial dysfunction caused by hyperhomocysteinemia may prevent the development and progression of atherosclerosis.

Garlic has been used as a general food and a natural Oriental medicine for a long time. Studies have shown that garlic and its extract are modulators of multiple cardiovascular risk factors (Steiner et al., 1996), such as blood pressure, platelet aggregation and adhesion, total cholesterol, LDL, HDL, LDL oxidation, and improve endothelial function, inhibit endothelial cell damage and directly suppress atherosclerosis (Campbell et al., 2001; Rahman and Billington, 2000; Pittler and Ernst, 2005; Budoff, 2006; Kaul et al., 2006). Especially allicin, a main component of freshly prepared garlic homogenate, an organosulfur compound derived from alliin catalyzed by allinase, has attracted researchers' attention for its anti-atherosclerotic effect. There is preliminary evidence from clinical studies that allicin has shown many beneficial effects in the prevention of coronary heart disease, cerebrovascular disease and other atherosclerotic vascular diseases (Banerjee and Maulik, 2002; Nie et al., 2002; Zhang et al., 2003). Recently, our previous studies demonstrate that allicin significantly reduced serum Hcy levels and vascular endothelial cells (VECs) injury in coronary heart disease patients with hyperhomocysteinemia, which is useful for the treatment of atherosclerotic vascular disease (Hao et al., 2007; Li et al., 2009). However, only a few studies have investigated the effect of allicin on hyperhomocysteinemia-induced experimental vascular endothelial dysfunction. In this study, blood

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samples were collected from experimental animals to determine the levels of serum SOD, MDA, NO and plasma ET. The expression of bFGF, TGF-β, TNF-α, and ICAM-1 in the aorta were detected by the immunohistochemical method simultaneously. To our knowledge, this is the first study where effect of allicin on vascular endothelial dysfunction caused by hyperhomocysteinemia in an animal model is evaluated.

2. Materials and methods

2.1. Animals and induction of hyperhomocysteinemia

Eight-week-old male Wistar rats of specific pathogen-free (SPF) grade weighting between 190 g and 220 g were obtained from the Animal Center of Shandong University and housed in the Animal Laboratory of Shandong Academy of Medical Science. The above mentioned rats were raised with sufficient food and water at an ambient temperature of 22 ± 1 °C, a relative humidity of $48 \pm 2\%$ and under a 12 h light/dark cycle. All rats received humane care, and the study protocol was approved by the Ethics Committee of Qilu Hospital of Shandong University.

Hyperhomocysteinemia was induced in rats by feeding a high-methionine diet, which consisted of a regular diet plus 3% methionine (wt/wt). In the previous study (Chen et al., 2004), after being fed 3% methionine forage, the concentration of serum Hcy of all rats swiftly stepped up and achieved a higher level at the fourth week, and then, gradually increased. Many studies have suggested that a high-methionine diet for 4 weeks is sufficient to induce hyperhomocysteinemia (Morita et al., 2001; Wang et al., 2002; Chen et al., 2004).

2.2. Groups and administration

Fifty healthy, 8-week-old, male Wistar rats were employed in this experiment. Hyperhomocysteinemia was induced in 40 rats with high-methionine forage as described by Chen et al. (2004). All experimental animals were divided into five groups ($n=10$ for each group): the normal control rats (NC), the high-methionine-diet rats (Met), the high-methionine-diet rats treated with folic acid, vitaminB_6 and vitaminB_{12} (Met+F), the high-methionine-diet rats treated with low-dose allicin (Met+L), and the high-methionine-diet rats treated with high-dose allicin (Met+H). The Met+L and Met+H groups were treated with allicin (Shanghai Harvest Pharmaceutical Co., Ltd., China) by intraperitoneal injection at a dose of 6 mg/kg and 10 mg/kg once per day, respectively. This dose was determined with reference to the description of Du et al. (2002) and Liu et al. (2012) and in accordance with the method of dose conversion between humans and animals. The Met+F group was treated with folic acid 0.45 mg/kg B.W. (Shandong Luoxin Pharmaceutical Co., Ltd., China), vitaminB_6 2.7 mg/kg B.W. (Qingdao Huanghai Pharmaceutical Co., Ltd., China) and vitaminB_{12} 6.75 μg/kg (Beijing Zizhu Pharmaceutical Co. Ltd., China), which were mixed with distilled water for once daily intraperitoneal injection. The same amount of normal saline was given to the NC and the Met groups. The NC group was fed a regular diet, which was mixed without 3% methionine. The above operations continued for 6 weeks.

2.3. Sample collection

At the sixth week, blood was collected in a common test tube and a special tube containing disodium ethylenediamine tetra-acetic acid (1 mg/ml) and aprotinin (500 U/ml; Sigma, St. Louis, MO), and then centrifuged immediately at 956g for 15 min at 4 °C; serum and plasma were stored at −80 °C before assays.

The aorta was removed immediately from the animals after sacrifice and rinsed in phosphate-buffered saline (PBS). The tissue samples were fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, and embedded in paraffin. Thereafter, paraffin block samples were sectioned with a 5 μm thickness.

2.4. Biochemical analysis

2.4.1. Hcy assay

The concentration of serum Hcy was assayed with a Waters 2695 high efficiency liquid chromatograph and a Waters 2475 fluorescence detector (Waters Corp., Milford, MA), using Millennium ^{32} software. The Waters C18 column (3.9 mm × 150 mm) was used. Mobile phase: A was 0.05 mol/l acetate buffer (PH6.8); B was acetonitrile, with gradient elution; the flow rate was 1.3 ml/min, and fluorescence detected at excitation/emission wavelengths ex: 335/em: 455. Results showed that serum Hcy was well-separated. The DL-homocysteine, acetonitrile, β-mercaptoethanol and o-phthalaldehyde were purchased from Sigma Chem. Co (St. Louis, MO).

2.4.2. ET-1 assay

The concentration of plasma ET-1 was measured using a commercially available ET-1 radioimmunoassay kit (supplied by Beijing East Asia Immunology Institute, China) according to the manufacturer's specifications. In brief, samples were incubated with 100 μl of rabbit anti-ET-1 antibody for 24 h at 4 °C. Then 100 μl of [^{125}I] ET-1 were added and incubated for 24 h at 4 °C. After separated binding and free antigen by 500 μl of the PR reagent for 20 min at room temperature, the mixture was centrifuged at 1301g for 20 min; [^{125}I] ET-1 in the precipitate was measured by an auto-gamma counter of SN-682B type (Shanghai Hefu Electronic Instruments Co., China). The sensitivity of the assay was 5 pg/ml and the intra-assay coefficient of variation (CV) of the radioimmunoassay system was below 5%.

2.4.3. Serum NO assay

Since NO is unstable and is rapidly converted into nitrates and nitrites, the NO concentration was determined indirectly as the concentrations of nitrate and nitrite as previously described (El-Latif et al., 2006). Briefly, nitrate was converted to nitrite by the enzyme nitrate reductase. The total nitrite was measured with the Griess reagent (Green et al., 1982), and the absorbance was determined at 550 nm. This assay was performed using a colorimetric assay kit (supplied by Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Results were expressed as μmol/l of serum.

2.4.4. Serum total SOD activity assays

Serum total SOD activity was determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by the xanthine/xanthine oxidase method according to the manufacturer's instructions. In brief, a xanthine/xanthine oxidase complex is used to produce superoxide anions, which oxidize hydroxylamine to nitrite. The nitrite formreacts with a chromogenic reagent to form a purple-colored product, and the absorbance was measured spectrophotometrically at 550 nm.

2.4.5. Serum MDA activity assays

Serum MDA activity was determined using a spectrophotometric diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Briefly, MDA was allowed to react with thiobarbituric acid (TBA), which yielded red-colored products. These were spectrophotometrically quantified by measuring the maximum absorption peaks at 532 nm.