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Geraniol improves endothelial function by inhibiting NOX-2 derived oxidative stress in high fat diet fed mice

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ABSTRACT

Endothelial dysfunction occurs in obese patients and high-fat diet (HFD) fed experimental animals. While geraniol has been reported to ameliorate inflammation and oxidative stress, inhibit tumor cell proliferation, and improve atherosclerosis, its direct effect on endothelial function remains uncharacterized. The present study therefore investigated the effect of geraniol on endothelial function in HFD mice and its underlying mechanisms. C57 BL/6 mice were fed an HFD (n = 40) or a normal diet (n = 20) for 8 weeks. HFD fed mice then were randomized to intraperitoneal treatment with geraniol (n = 20) or vehicle (n = 20) for another 6 weeks. Acetylcholine (Ach)-induced endothelial dependent vasorelaxation was measured on wire myography; reactive oxygen species (ROS) generation was assessed by fluorescence imaging, and NADPH oxidases (NOXs) and adhesive molecules VCAM-1 and ICAM-1 protein expression by western blotting. Geraniol improved endothelial function in HFD fed mice, as evidenced by its: 1. restoring endothelial dependent vasorelaxation induced by Ach, and reversing increased VCAM-1 and ICAM-1 expression; 2. attenuating HFD induced increased serum TBARS and aortic ROS generation; and 3. downregulating aortic NOX-2 expression in both HFD fed mice and in palmitic acid treated endothelial cells. Geraniol therefore protects against endothelial dysfunction induced by HFD through reducing NOX-2 associated ROS generation.

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1. Introduction

In both obese humans and animals, a high fat diet impairs endothelial function [1,2]. Impaired endothelial dysfunction plays a critical role in the development of atherosclerosis, preceding severe cardiovascular diseases, and in metabolic disorders is associated with a rise in oxidative stress, mainly manifesting as ROS overproduction in the vasculature [3]. NADPH oxidases (NOXs) are the predominant source of ROS in the vasculature, participating in normal cell function and triggering injury development in pathological settings [4], and NOXs thus have emerged as a promising target for endothelial function and vascular diseases associated with metabolic disorders [5]. Geraniol, a monoterpene extracted from plants, has been reported to be an effective antioxidant in various metabolic disorders, attenuating atherosclerosis, hyperlipidemia, oxidative stress, inflammation and fibrosis in multiple organs, and increased serum NO levels in hamsters [6–8]; reversing raised systolic blood pressure (SBP), uric acid level and glucose associated index in rats with fructose induced metabolism syndrome [9]; and ameliorating methionine-choline deficient diet-induced nonalcoholic steatohepatitis (NASH) in rats by attenuating oxidative stress and apoptosis [10]. However, geraniol's direct effect on endothelial function and its underlying mechanisms beyond lipid lowering had remained elusive, and therefore the subject of the present study is on impaired endothelial function in HFD-fed mice.

2. Methods and materials

2.1. Animals and experimental groups

Animal procedures in the present study were approved by the Animal Care and Use Committee of Harbin Medical University, and

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conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85–23, revised 2011). Sixty male C57BL/6 mice (18–20 g) were obtained from Vital River (Beijing, China), housed in a temperature-controlled setting under 12-h light/dark cycles, and divided to receive either normal chow diet (NCD, AIN-93G diet for 8 weeks; n = 20) or high fat diet (HFD for 8 weeks; n = 40). HFD fed mice then were randomized to receive treatment with geraniol (purchased from Sigma, USA, and administered intraperitoneally [ip] at 100 mg/kg/d after dissolution in corn oil; n = 20) or corn oil vehicle only (HFD controls; n = 20) for 6 weeks. After 6 weeks, systolic blood pressure was measured using tail cuff plethysmography (IITC Life Science MRBP Blood Pressure System). The mice were deeply anaesthetized with sodium pentobarbital (60 mg/kg ip) and the following procedures were carried out after absence of response to corneal stimulation; blood samples were collected from the abdominal aorta; and tissues were sampled, and immediately weighed and frozen at −80 °C for subsequent analysis.

2.2. Vascular reactivity studies

Thoracic aortas were dissected and cut into 2–3-mm-long segments into cold (4 °C) modified physiological salt solution (PSS) containing NaCl, 130 mM; KCl, 4.7 mM; MgSO47H2O, 1.17 mM; KH2PO4, 1.18 mM; NaHCO3, 14.9 mM; CaCl2, 1.6 mM; D-glucose, 5.5 mM (PH7.35–7.45). The aortic rings were mounted on 2 triangle-shaped stainless-steel holders, which were connected to a force transducer (Taimeng Instruments, Chengdu, China), and were incubated in organ chambers containing 10 ml of PSS gassed with 95% oxygen and 5% carbon dioxide at 37 °C to record isometric tension. The resting tension was adjusted to 0.5 g. After a 60-min equilibration period, precontraction was caused by exposure to a high-K^+ PSS (60 mmol/L KCl). After wash, a sustained contraction was induced by 1 μmol/L phenylephrine. Cumulative concentration relaxations caused by acetylcholine (Ach; 10^{-9}–10^{-5} mol/L; Sigma, USA) and sodium nitroprusside (SNP; 10^{-9}–10^{-7} mol/L) were performed.

2.3. In situ detection of vascular ROS content

In situ vascular ROS production was measured by dihydroethidium (DHE, Sigma, USA) fluorescence. Fresh frozen sections of the aortas prepared in 10 μm thickness using a cryostat were incubated in PSS containing 5 μM DHE for 15 min, and then washed twice in PSS. Fluorescence images were obtained with ZEISS fluorescence microscope at 515 nm excitation and 585 nm emission.

2.4. Cell culture and CCK8 assay

In the present study, the in vivo diet induced obese setting was reproduced in vitro by exposing HUVECs to palmitic acid (PA), the most common circulating free fatty acid, with or without geraniol. Human umbilical vein endothelial cells (HUVECs) were purchased from ALLCELLS Company (Shanghai, China). The third to sixth passages were used in this study. For CCK8 tests, cells were cultured in endothelial complete media containing endothelial cell growth factors. HUVECs (5.0×10^3/well) were plated in 96-well plates (three wells per group) and treated with geraniol (0–1000 μM) for 24 h, respectively. 10 μL of CCK8 (Dojindo, Japan) was added to the cells, and the viability of the cells was measured at 490 nm with an ELISA reader (BioTek, USA) according to manufacturer's instructions. Cells were pretreated with geraniol (50 μM) for 30 min before exposure to PA (300 μM). Fatty acid free BSA (Equitech-Bio) solutions were added as control.

2.5. Western blot

Tissues were homogenized in an ice-cold RIPA lysis buffer containing 1 mM PMSF (Beyotime, Shanghai, China). Cell lysates were centrifuged at 16,000 g for 20 min at 4 °C. Extracted protein concentration was determined using the BCA method (Beyotime, Shanghai, China). Protein samples were denatured by boiling for 10 min. Equal amount of protein samples together with the prestained protein molecular weight marker were electrophoresed on a 10% SDS-polyacrylamide gel. The resolved proteins were electrophoretically transferred onto an polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% BSA in 0.05% Tween-20 tris-buffered saline (TBST), and incubated overnight at 4 °C with primary antibodies against NOX-2 (Abcam, UK), NOX-4 (Abcam, UK), NOX-1 (Santa Cruz, USA) and VCAM-1 (Abcam, UK), ICAM-1 (Santa Cruz, USA), and GAPDH (Kangcheng, Shanghai, China). The membrane was washed thrice in TBST, and the corresponding secondary antibodies were added. After another wash, the membranes were developed with an ECL kit and detected by ChemiDoc XRS gel documentation system (Bio-Rad, Hercules, CA, USA). The protein bands were analyzed with Image Lab. GAPDH, as housekeeping protein, was used to normalize loading amounts across samples.

2.6. Serum determinations

Serum was collected by centrifugation at 4000 *g* for 15 min at 4 °C. Serum FFA, TBARS and IL-6 levels were measured with ELISA kits (Bioassay, USA, Blue Gene, Shanghai, China) according to the manufacturer's instructions.

2.7. Statistical analysis

Variables are presented as mean ± SD, except for relaxation which is presented as percentage reduction in phenylephrine contraction. Comparisons among groups were made using ANOVA followed by Newman–Keul tests. The results were considered statistically significant at a P value of <0.05.

3. Results

3.1. Geraniol improved endothelial function of HFD fed mice

As shown in Fig. 1, compared with mice fed a normal diet, endothelial dependent vasorelaxation induced by Ach was impaired in aortas from HFD fed control mice, and geraniol treatment restored the impaired endothelial function, while endothelial independent relaxation response to SNP was not significantly different among groups.

3.2. Geraniol inhibited ROS production in aortas from HFD fed mice

ROS production was measured by DHE staining. The fluorescence intensity in aortas from HFD fed control mice was enhanced, and geraniol treatment reduced ROS generation (Fig. 2).

3.3. Geraniol downregulated NOX-2 expression in vivo and in vitro

Because NADPH oxidase is the major source of ROS in the vasculature, we investigated the effect of geraniol on the expression of NOX-1, NOX-2, and NOX-4. HFD resulted in NOX-2 expression upregulation in the aorta, which was reversed by geraniol treatment. However, NOX-1 and NOX-4 expression appeared not to be affected by HFD or geraniol intervention (Fig. 3 A–C).

To assess the effect of geraniol in vitro, we exposed HUVECs to

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