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Dietary ellagic acid improves oxidant-induced endothelial dysfunction and atherosclerosis: Role of Nrf2 activation

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ABSTRACT

Background: Oxidative stress-induced vascular endothelial cell injury is a major factor in the pathogenesis of atherosclerosis. Several evidences indicate that ellagic acid (EA), a phenolic compound, contributes to cardiovascular health. This study was to investigate the effects of EA on endothelial dysfunction and atherosclerosis via antioxidant-related mechanisms.
Methods: In animal studies, wild-type (WT) C57BL/6 mice and apolipoprotein E-deficient mice ($ApoE^{-/-}$) mice were fed: a high-fat (21%) diet (HFD) or a HFD plus with EA (HFD + EA), for 14 weeks. Vascular reactivity was studied in mice aortas. The effect of EA in human umbilical vein endothelial cells (HAECs) exposed to hypochlorous acid (HOCl) was also investigated.
Results: Compared with animals on HFD alone, EA attenuated atherosclerosis in WT mice. In aortic rings from two mice models, EA significantly improved endothelium-dependent relaxation and attenuated HOCl-induced endothelial dysfunction. Besides, EA significantly improved nitric oxide synthase activity, antioxidant capacity and markers of endothelial dysfunction in plasma. Western blot analysis showed that EA increased NF-E2-related factor 2 (Nrf2) and heme oxygenase-1(HO-1) expression in the aortas ($P < 0.05$). In a separate experiment, EA did not protect against HOCl-induced endothelial dysfunction in arteries obtained from Nrf2 gene knockout mice compared with WT mice. In HAECs, EA prevented HOCl-induced cellular damage and induced HO-1 protein expression, and these effects markedly abolished by the siRNA of Nrf2.
Conclusions: Our results provide further support for the protective effects of dietary EA particularly oxidant-induced endothelial dysfunction and atherosclerosis partly via Nrf2 activation.
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1. Introduction

Atherosclerosis is one of the leading causes of coronary heart disease. Endothelial dysfunction and oxidative stress are associated with the pathogenesis of atherosclerosis [1]. Endothelial dysfunction appears early in the process of atherosclerosis, which is an independent predictor of cardiovascular disease outcomes [2]. Endothelial dysfunction is thought to arise due to a reduction in the bioavailability of NO [3], although the precise mechanisms remain unknown. Nitric oxide (NO) produced by endothelial nitric oxide synthase is an important regulator of vascular function [4]. Under normal conditions, NO regulates the diameter of blood vessels and maintains an anti-proliferative and anti-inflammatory environment in the vessel wall. Oxidative stress, characterized by an imbalance between pro-oxidants and antioxidants, is a key factor in the pathogenesis of atherosclerosis and other cardiovascular diseases [5]. Their normal functions include host defense and surveillance through regulated generation of diffusible radical species, reactive oxygen or nitrogen species, and hypochlorous acid (HOCl).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox sensitive master regulatory transcriptional factor. Nrf2 plays an important role in maintaining the atheroprotective capacity of vascular endothelial cells by regulating endothelial redox balance [6]. It binds to ARE and activates ARE-dependent transcription of phase II and antioxidant defense enzymes, including heme oxygenase-1 (HO-1). In particular, HO-1 has been recognized as an important factor protecting vascular tissue against atherosclerosis by exerting antioxidant, anti-inflammatory, antiapoptotic and vasodilatory effects on the vasculature [7]. It has been demonstrated that Nrf2 activators decrease the probability of developing atherosclerotic lesions by decreasing oxidative stress [8].

Abbreviations: ACh, acetylcholine; $ApoE^{-/-}$, apolipoprotein E gene knockout ellagic acid; HAECs, human aortic endothelial cells; HFD, high-fat diet; HO-1, heme oxygenase-1; HOCl, hypochlorous acid; ND, normal diet; NO, nitric oxide; Nrf2, NF-E2-related factor 2; $Nrf2^{-/-}$, nrf2-deficient; EA, ellagic acid; ROS, reactive oxygen species; siRNA, small interfering RNA; WT, wild-type.

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Epidemiological evidence suggests that the antioxidant activity by polyphenols could be mediated by the stimulation of Nrf2 and the activation of the antioxidant response element [9]. Increased intake of dietary polyphenols in fruits and vegetables has long been recognized as being protective against atherosclerosis [10], such as oxidative stress and endothelial dysfunction [11,12]. Ellagic acid (EA) is a naturally occurring phenolic constituent present in fruits and nuts with the highest levels found in raspberries. EA-rich foods may be protective against certain chronic diseases in human [13]. EA is able to reduce plasma and macrophage lipid peroxidation, prevents endothelial inflammation and blocks oxLDL induced proliferation of vascular smooth muscle cells [14,15]. In streptozotocin-induced diabetic rats, EA was shown to block atherosclerotic process suppressing oxidative stress and inflammation [16]. However, mechanisms for anti-atherosclerosis by which EA exerts its anti-oxidative effects are not completely understood. The phenolic nature of EA makes itself a powerful antioxidant and in cells or animals, EA could protect against oxidative stress with up regulation of the Nrf2 [17,18]. In context of epigenetics and the developmental origins of atherosclerosis, it is of interest to determine whether chronic consumption of EA could improve endothelial function and attenuate atherosclerosis by increasing NO bioavailability, as well as inducing Nrf2.

In this study, we sought to investigate protective effects of chronic EA consumption on vascular dysfunction and atherosclerosis development in models of wild-type (WT) C57BL/6 mice and apolipoprotein E-deficient ($ApoE^{-/-}$) mice fed with a high-fat diet (HFD). Furthermore, we investigated the mechanisms of EA on Nrf2-mediated antioxidant effect in human umbilical vein endothelial cells (HUVECs).

2. Methods

2.1. Animals

As susceptibility to atherosclerosis is determined by both environmental and genetic factors, we used two mice models of atherosclerosis. WT and $ApoE^{-/-}$ mice (male, 4 weeks old, the genetic background of $ApoE^{-/-}$ was C57BL/6) were provided by the Experimental Animal Center of Beijing University Medical College (Beijing, China) and the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). The experiments were performed in adherence with the institutional guidelines on the use and care of laboratory animals. Only male mice were studied to exclude the possible confounding effects of the estrous cycle in females. Mice were housed in an environmentally controlled vivarium with unlimited access to water and a controlled photoperiod (12-h light: 12-h dark). Body weight was recorded weekly.

Mice (male, 4 weeks old) were randomized to receive HFD (a high-fat diet containing 21% fat and 0.2% cholesterol) or HFD plus EA (Sigma, CAS: 476-66-4, purity ≥ 95%) (HFD + EA) for 14 weeks. Mice were given 0.5 g EA/kg of diet for a dose of about 30 mg/kg body weight/day. The dose of EA was chosen based upon previous studies [19,20]. At the end of the experimental period, a 24-h urine sample was collected from mice placed in a metabolic cage. Mice were anesthetized with a bolus injection of pentobarbitone sodium and blood was collected from the abdominal aorta. Then, their aorta was removed and dissected free of adherent connective tissues. The plasma and urine were stored at −80 °C until further analysis.

2.2. Analysis of aortic lesions

The aortic arch and thoracic aortas were stripped of any surrounding tissues and fixed overnight in phosphate-buffered formaldehyde (4% by volume, pH 7.0). Paraffin-embedded aortic arch and thoracic aorta specimens were sectioned at a thickness of 4 μm. Atherosclerosis was determined using hematoxylin and eosin (HE)-stained sections as described previously [21]. At the aortic arch lesion size was determined as a 3-mm-long segment along the inner curvature between the opening of the brachiocephalic artery and left subclavian artery. At the thoracic aorta serial cross-sectional sections were taken 20 mm apart centered at the 4th, 5th, and 6th pair of intercostal arteries. The lesion area in the transverse section of the aorta was expressed as a percentage of the lesion to the total area of the tissue.

2.3. Determination lipids, antioxidant capacity and markers of endothelial dysfunction in plasma

The plasma from mice (18 weeks) with a normal diet (ND) was used as normal control. Total plasma cholesterol (TC) and triglyceride (TG) concentration were determined using commercially available kits (Zhong Sheng Bei Kong, Peking, China) following the manufacturer's instruction. Endothelial dysfunction [soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin (sE-selectin)] was determined by cytokine enzyme-linked immunosorbent assay (ELISA) kits (Transhold Co. Ltd, China). The ferric reducing ability of plasma (FRAP) was determined in fasted plasma collected on EDTA using the Benzie and Strain method with slight modifications [24]. Systemic oxidative stress was determined by measuring plasma F2-isoprostane with gas chromatography–mass spectrometry (GC/MS) method [25].

2.4. Assessment of ex vivo endothelial function

The aorta from WT and $ApoE^{-/-}$ mice was excised, loose connective and adipose tissues were removed. All arterial rings were incubated for approximately 30 min in Krebs–Henseleit-modified buffer aerated with 95% O_2/5% CO_2 at 37 °C. The rings were primed with 60 mM KPSS buffer (pH 7.4) consisting of (mM): KCl 123.7, $MgSO_4$ 1.17, KH_2PO_4 1.18, $CaCl_2$ 2.5, $NaHCO_3$ 25, EDTA 0.03, and glucose 5.5 and norepinephrine (1–30) to cause approximately 50% of the predetermined maximal constriction. Endothelial-dependent relaxation was assessed in response to increasing doses of acetylcholine (ACh) endothelium-dependent. Endothelial-independent relaxation which was assessed in response to increasing doses of papaverine (nitric oxide-independent) was examined. Each assessment was performed in duplicate.

2.5. Ex vivo endothelial functional studies with arterial rings from $Nrf2^{-/-}$ mice

In a separate study, endothelial function was assessed with arterial rings from nrf2-deficient ($Nrf2^{-/-}$) and WT mice (25–28 g) provided by the Experimental Animal Center of Beijing University Medical College (Beijing, China) and the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). Arterial rings were collected as described above. Rings were incubated with ellagic acid or vehicle for 6 h, and then pretreated with or without 100 μM HOCl (for WT mice) or 20 μM HOCl (for $Nrf2^{-/-}$ mice) for 1 h. Prior to assessment of relaxation to Ach, different concentrations of HOCl were used to achieve a similar degree of endothelial dysfunction to assessment of relaxation to Ach. Each assessment was performed in duplicate.

2.6. Determination of NOS activity and urinary nitrite

NOS activity in aortic homogenates was determined by monitoring the conversion of L-[3H] arginine to L-[3H] citrulline using an NOS activity assay kit (Cayman Chemical, USA) [22]. Nitrite concentrations in urine were determined using a previously published gas chromatographic–mass spectrometric method [23]. Results were corrected for creatinine levels.

2.7. Measurement of EA concentration in plasma

A modified published LC–MS/MS method for human plasma was used to analyze the plasma and aorta content of the intact form of EA [24]. The analysis was performed using an Agilent 1200 series HPLC and an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA). Chromatographic separation was performed on a Diamonsil C18 column (Dikma, China). The mobile phase consisted of a methanol and 0.2% phosphoric acid water solution (20:80, v/v). The negative ion transitions monitored for EA were 301 to 200. Under negative ion conditions, the cone voltage and collision energy were 40 V and 30 eV.

2.8. Cell culture

Human umbilical venous endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in ATCC formulated with F-12 K medium, supplemented with 0.1 mg/mL heparin, 0.05 mg/mL endothelial cell growth supplement (Sigma Aldrich Co., USA), 10% fetal bovine serum (Gibco, USA), and 1% penicillin–streptomycin (Gibco, USA). Cells were grown in T-25 flasks and passaged every 3–4 days as necessary. All experiments were performed on confluent cells between passages 3 and 9 in 6-well culture plates.

2.9. Nrf2 silencing

Prior to transfection, 250 μL of Prime Fect diluent was mixed with 5 μL of Prime Fect transfection reagent and incubated at room temperature for 15 min. Nrf2 siRNA (Santa Cruz) or control RNA was added to the transfection solution for a final concentration of 50 nM and incubated at room temperature for 15 min. The transfection solution was applied to cells grown to 70–80% confluence in antibiotic free medium, along with 1.25 mL antibiotic free growth medium. The volume of transfection reagent used caused minimal distress to the cells as assessed by minimal changes to cell morphology. Experiments were performed on day 2 after the transfection, when gene silencing was optimal. Cells were treated with EA for 16 h for Western blot analyses.

2.10. MTT measurement of cell viability

To determine the preventive effect of EA on HOCl-induced cell toxicity, confluent cells were pretreated with EA for 16 h, washed with phosphate-buffered saline (PBS), and exposed to HOCl (100 μM) for 6 h. Cell viability was detected using an MTT assay according to the reported method [25]. Additionally, to determine the involvement of Nrf2 in oxidative stress induced cell viability, cells were pretreated for 16 h with EA after transfection with Nrf2 siRNA, and then exposed to HOCl for 6 h.

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