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ScienceDirect

Journal of Nutritional Biochemistry xx (2015) xxx–xxx

Journal of Nutritional Biochemistry

Effects of dietary resveratrol on excess-iron-induced bone loss via antioxidative character

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Received 3 September 2014; received in revised form 29 April 2015; accepted 12 May 2015

Abstract

Estrogen deficiency has been considered to be a major cause of osteoporosis, but recent epidemiological evidence and mechanistic studies have indicated that aging and the associated increase in reactive oxygen species (ROS) are the proximal pathogenic factors. Through ROS-mediated reactions, iron can induce disequilibrium of oxidation and antioxidation and can cause bone loss in mice. Therefore, we investigated the effects of resveratrol (RES) on bone mineral density, bone microstructure and the osteoblast functions under iron-overload conditions. Excess iron disrupted the antioxidant/prooxidant equilibrium of the mice and induced the defect and the lesion of the bone trabecula as well as disequilibrium between bone formation and bone resorption in iron-overload mice. Oral administration of RES significantly prevented bone loss in the osteoporotic mice. RES reversed the reduction of Runx2, OCN and type I collagen from excess iron; up-regulated the level of FOXO1; and maintained the antioxidant/prooxidant equilibrium in the mice. RES also reduced the ratio of OPG/RANKL in MC3T3-E1 cells and in mice and significantly inhibited subsequent osteoclastogenesis. These results provide new insights into the antiosteoporosis mechanisms of RES through antioxidative effects, suggesting that RES can be considered a potential natural resource for developing medicines or dietary supplements to prevent and treat osteoporosis.
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Keywords: Osteoporosis; Iron overload; Resveratrol; Antioxidant effect; Sirt 1

1. Introduction

Osteoporosis is a progressive bone disease caused by aging, estrogen deficiency and genetic factors and is characterized by bone mass decline and trabecular architecture deterioration that results in fragility fractures [1]. Estrogen deficiency has been considered to be a major cause of osteoporosis in both women and men. Even though hormone replacement therapy is partially effective at slowing bone loss in postmenopausal women, a substantial number of women have discontinued its use because of the concerns about the potential risks (especially breast cancer) [2,3].

From the view of osteoporosis pathogenesis, women experience not only estrogen deficiency but also iron accumulation as a result of ceasing menstruation [4]. Men older than 40 years are at high risk for iron loading [5]. Osteoporosis and fractures occur frequently in disorders associated with iron overload, such as thalassemias and hereditary hemochromatosis [6].

Previous studies have suggested that increased oxidative levels are associated with aging in both elderly people and OVX rats and that increased iron plays a causal role in osteopenic development [7]. Iron is critical for cell growth, oxygen utilization, various enzymatic activities and responses of immune systems, but abnormal iron uptake causes widespread organ damage including liver, adrenal glands, heart, pancreas and bone [8,9]. Iron occurs in the +II and +III oxidative states, and the ferrous ions are unstable in aqueous media and tend to catalyze the generation of damaging reactive free radicals via Fenton reaction [8]. Through reactive oxygen species (ROS)-mediated reactions, iron can disrupt the antioxidant/prooxidant equilibrium of cells and can cause indirect DNA damage, lipid peroxidation and protein modification. Therefore, oxidative damage and the dysfunction of antioxidant system are the proximal pathogenesis of male and female osteoporosis. In an iron-overload male mice model, administration of iron dextran increased the level of ROS and the phosphorylation of $p66^{shc}$ (an amplifier of H_2O_2 generation in mitochondria) in bone and caused trabecular and cortical thinning, while the bone loss was largely prevented by treatment with the antioxidant *N*-acetyl-L-cysteine (NAC) [6]. $p66^{shc}$ is a crucial mediator of the effects of oxidative stress on osteoblast apoptosis, NF-κB activation and production of cytokines, such as TNF-α and IL-6 [10]. Upon oxidative stress, $p66^{shc}$ is phosphorylated at Ser36, contributing

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http://dx.doi.org/10.1016/j.jnutbio.2015.05.009
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to inactivation of the winged forkhead transcription factors FOXO1, FOXO3, FOXO4 and FOXO6, which maintain skeleton genesis. Extensive research has shown that FOXOs control the regulation of many genes involved in the cell cycle, stress response, cell death, modulation of inflammation, metabolism, protection from oxidative stress and cell survival, and FOXO1 has been demonstrated to be a major regulator of osteoblast function [11].

Resveratrol (RES) is a member of the stilbene family of phenolic compounds. The major dietary sources of RES include peanuts, pistachios, berries, dark chocolate and grapes as well as their derivatives. Grapes have the highest content, while red wine is the most notable dietary source [12]. Many reports have shown that RES can prevent or slow the progression of a wide variety of illnesses, including skeletal diseases, cancer, cardiovascular disease and ischemic injuries, as well as can enhance the resistance to stress and extend the lifespan of various organisms. The major impacts are antioxidative, antiinflammatory, cardioprotective, antiaging as well as anticancer and chemopreventive [13]. Sirtuin1 (Sirt1), an NAD^{+}-dependent deacetylase and a key player in aging and metabolism, has been shown to regulate bone mass [14]. As a natural Sirt1 agonist, RES can upregulate the level of FOXOs and increase the activities of antioxidant enzymes in C2C12 mouse myoblasts cells and human monocytic THP-1 cells under oxidative stress conditions [15,16]. Researchers have demonstrated that RES supplementation can promote osteogenic differentiation of modulation of Sirt1/Runx2 mediated by mesenchymal stem cell, increase the levels of osteocalcin and alkaline phosphatase (ALP) in plasma and ameliorate the loss of femur strength in hindlimb-suspended old male rats [17,18]. However, the relevant molecular mechanisms of RES and the regulation of FOXOs on bone and osteoblasts damage induced by iron overload remain unclear. In this study, we investigated the effect of dietary RES on excess-iron-induced bone loss and the potential proximate mechanisms.

2. Materials and methods

2.1. Chemicals

The mouse antiosteocalcin (OCN) antibody was purchased from Millipore (Bedford, MA). The mouse anti-FOXO1 antibody was purchased from Cell Signaling Technology (Beverly, MA), and other antibodies were purchased from Abcam (Cambridge, MA). RES, NAC, ferric ammonium citrate (FAC) and all other chemicals were purchased from Sigma.

2.2. Animals

2.2.1. Ethics

All of the experimental procedures in this study were approved by the Animal Ethics Committee of the Shanghai Jiao Tong University. The care and use of animals were conducted under the Guidelines for Animal Experiment of the Shanghai Jiao Tong University (Approval No. SYXK 2012-0017, Shanghai, China), and all efforts were made to minimize suffering.

2.3. In vivo study design

Sixty 2-month-old C57/BL6 male mice were randomly divided into six groups with ten mice each group: control group; model group; positive drug (NAC, 100 mg/kg, ig per day) group; low-dose RES group (30 mg/kg, ig per day); middle-dose RES group (60 mg/kg, ig per day); high-dose RES group (90 mg/kg, ig per day). The experimental animals were housed in hygienic plastic cages in a clean well-ventilated room and were given free access to food and water with normal light and dark cycles. The mice in the model group and the groups administered RES were treated once a week for 3 months with intraperitoneal iron dextran (100 mg/kg) or placebo (in the normal group). Three months later, the mice were killed. An hour after the last RES or placebo treatment, blood was collected to measure the cytokines and the plasma levels of RES. The femurs and livers were collected for micro-computed tomography (micro-CT) scanning, mechanical testing or an antioxidant enzyme assay.

2.3.1. Micro-CT

After removing the soft tissues, the left femurs of the mice were placed in a phosphate-buffered saline (PBS) buffer with 10% formaldehyde. The femurs were placed with gauze in the sample holder and were scanned using the GE Healthcare Locus SP micro-CT (GE Healthcare, USA) using a resolution of 6 μm, 80 kV, 80 μA, 400 number of views and exposure of 5 h [19]. The explore reconstruction utility software (GE Healthcare, USA) was used for three-dimensional reconstruction and data processing. A global threshold was defined as the lowest mineral density. Calculation methods of bone parameters have been previously described [20]. The volumetric parameters of bone volume fraction (BVF), trabecular thickness (μm), trabecular number (no./mm), trabecular spacing (μm) and the thickness and area of cortices were assessed to investigate the effect of NAC and RES on the microarchitecture of the cortical bone at mid-diaphysis femur and trabecular bone from distal femur.

2.3.2. Mechanical testing

Three-point bending testing was performed using a Dynamic Mechanical Analyzer (Shimadzu, Japan) to determine the material properties (elastic load, maximum load, elastic stress, maximum stress and modulus of elasticity) of the bones. The right femurs of the mice were thawed to room temperature and kept moist in PBS. The right femurs were loaded to failure in three-point bending with a span length of 6 mm at a rate of 0.1 mm/s until the moment of fracture [21]. The obtained load–time curve was converted into a load displacement curve, and elastic load, maximum load, elastic stress, maximum stress and elastic modulus were calculated according to formulas [22].

2.3.3. Antioxidant enzyme assay

Oxidative stress was defined as an imbalance between antioxidant systems in the body and free radical production caused the lipid peroxidation in lipid bilayers of cells. Recent clinical, epidemiological and mechanistic evidence has indicated that aging and the associated increase in ROS are the proximal culprits of osteoporosis [23]. The activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and the concentration of malondialdehyde (MDA) in liver reflect the conditions of oxidative stress and oxidative damage caused by iron overload. Liver samples were quickly excised and washed in ice-cold PBS, dried using filter paper and weighed. They were then homogenized in 4 volumes of Tris–HCl buffer and then centrifuged at 5000*g* for 15 min. The supernatant was collected and determined within 2 h. SOD activity was determined by inhibiting autocatalytic adrenochrome formation at 480 nm. CAT activity was determined by the changes in absorbance that were recorded at 240 nm, calculated in terms of micromoles of H_2O_2 consumed per minute per milligram of protein. GPx activity was assayed by measuring the glutathione disulfide reduction (per minute per milligram of protein) mediated by NADPH oxidation at 340 nm. Lipid peroxidation was assayed by measuring the level of MDA using the method of Ruiz-Larrea *et al.* [24].

2.4. Cell experiments

2.4.1. Cell lines and culture conditions

MC3T3-E1 cells derived from newborn mouse calvaria were purchased from the typical Culture Collections Committee cell library of the Chinese Academy of Sciences (Shanghai, China). MC3T3-E1 cells were maintained in dishes (55 cm^2) in α-MEM containing 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 in air at 37°C.

2.4.2. Cell treatment

Cells were harvested when they reached approximately 80% confluence and were randomly divided into the following six groups: a normal control group, a FAC group, three RES groups (2, 10 and 50 μM) and NAC (1 mM) group. To prove the relationship between activation of Sirt1 and promotion of RES on bone formation, MC3T3-E1 cells were treated with 50 μM RES and 5 μM EX-527 (a Sirt1 inhibitor, IC_{50} of 38 nM) [25].

2.4.3. Cell proliferation

To evaluate the effect of iron overload on osteoblast proliferation, the MC3T3-E1 cells were suspended in complete medium and plated in 96-well culture plates (Nunc, Denmark) at a density of 10^4 cells per well and incubated overnight in 10% FBS medium. Then, the cells were subjected to FAC, and cell proliferation was measured using an MTT assay after incubation for 24, 48 and 96 h at 37°C [26]. The toxicity of RES (2–1000 μM, for 48 or 72 h) was determined by MTT assay. After each cell group establishment, 20 μl MTT solution (5 mg/ml) diluted in PBS was added to each well of culture plates, and the cultures were immediately incubated for 4 h at 37°C. The medium was then removed, and 200 μl of DMSO was added to each well. Later, the plates were shaken for 10 min at room temperature and read on a microplate reader at a wavelength of 570 nm.

2.4.4. ALP assay

ALP activity was determined according to a method reported previously. MC3T3-E1 cells were suspended in complete medium and plated in 24-well culture plates at a density at a density of 5×10^4 cells per well and incubated overnight. The cells were cultured with NAC (1 mM) and RES (2, 10 and 50 μM). After 2 h, the cells were subjected to FAC (500 μM) for 3 days. After incubation, the medium was removed, and the cells were gently washed twice with PBS, scraped into 0.2% Triton X-100 and incubated at 37°C for 30 min. The lysed cells were centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was used to measure intracellular ALP activity and total protein content according to a method reported previously [27]. ALP activity was measured using an ALP activity assay kit (Jiancheng, Nanjing, China).

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