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Rice bran enzymatic extract restores endothelial function and vascular contractility in obese rats by reducing vascular inflammation and oxidative stress☆

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Abstract

Background: Rice bran enzymatic extract (RBEE) used in this study has shown beneficial activities against dyslipidemia, hyperinsulinemia and hypertension. Our aim was to investigate the effects of a diet supplemented with RBEE in vascular impairment developed in obese Zucker rats and to evaluate the main mechanisms mediating this action.

Methods and results: Obese Zucker rats were fed a 1% and 5% RBEE-supplemented diet (O1% and O5%). Obese and their lean littermates fed a standard diet were used as controls (OC and LC, respectively). Vascular function was evaluated in aortic rings in organ baths. The role of nitric oxide (NO) was investigated by using NO synthase (NOS) inhibitors. Aortic expression of endothelial NOS (eNOS), inducible NOS (iNOS), tumor necrosis factor (TNF)-α and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits and superoxide production in arterial wall were determined. Endothelial dysfunction and vascular hyperreactivity to phenylephrine in obese rats were ameliorated by RBEE treatment, particularly with 1% RBEE. Up-regulation of eNOS protein expression in RBEE-treated aortas should contribute to this activity. RBEE attenuated vascular inflammation by reducing aortic iNOS and TNF-α expression. Aortas from RBEE-treated groups showed a significant decrease of superoxide production and down-regulation of NADPH oxidase subunits.

Conclusion: RBEE treatment restored endothelial function and vascular contractility in obese Zucker rats through a reduction of vascular inflammation and oxidative stress. These results show the nutraceutical potential of RBEE to prevent obesity-related vascular complications.

Keywords: Obesity; Rice bran; Vascular dysfunction; Vascular inflammation; Vascular oxidative stress

1. Introduction

Occidental dietary habits have contributed to increased prevalence of obesity, type 2 diabetes mellitus and other pathologies included in the metabolic syndrome [1]. Obesity, in particular abdominal obesity, has been established as a primary contributor to acquired insulin resistance, as increasing adiposity is correlated with impaired insulin action. Endothelial dysfunction, an independent predictor of cardiovascular events [2], has been consistently associated with obesity and the metabolic syndrome [3] in a complex interplay with insulin resistance [4]. Nevertheless, it has been reported that vascular dysfunction of obesity is not only limited to the endothelium but also involves the smooth muscle cell layer, leading to an increased oxidative stress in the vascular wall and the subsequent deregulation of the main control mechanisms providing vascular homeostasis [5]. Vascular function impairment in the metabolic syndrome mainly implies an unbalance between the vasoprotective effect of endothelial nitric oxide (NO) and the unfavorable action of vasoconstrictor factors [e.g., endothelin-1 and reactive oxygen species (ROS)] and proinflammatory mediators [e.g. tumor necrosis factor (TNF)-α] [5]. The pathophysiology of obesity-related vascular dysfunction is therefore an important target for developing new therapeutic approaches aimed to ameliorate cardiovascular risk factors related to metabolic syndrome.

Numerous studies suggest that the first strategy in the prevention of disorders associated with obesity consists of including in the diet food or dietary components with functional properties [6,7]. Rice, and particularly rice bran, is an excellent nutritional source of bioactive compounds, including high-healthy-value proteins and phytochemicals such as γ-oryzanol, sterols and tocols [8,9]. Besides, phenolic compounds contained in rice bran, such as γ-oryzanol and ferulic acid, are known to provide strong antioxidant activities [10,11]. The

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hypolipidemic, antioxidant and anti-inflammatory properties of the mixture of phytosteryl ferulates contained in γ-oryzanol make it a good candidate for health food [12,13]. However, the therapeutic use of rice bran is limited because of the insolubility of its proteins and the integrity of its nutraceutical compounds. For this reason, rice bran oils have become commonly utilized in order to study its properties, despite the high risk of rancidity that it involves [14]. These limitations have been counteracted by the recent production of a water-soluble rice bran enzymatic extract (RBEE) [15] that provides numerous advantages over other rice bran derivatives regarding water solubility, increased content in nutraceutical compounds and lack of rancidity.

Our recent investigations have evidenced that a diet supplemented with RBEE is able to ameliorate cardiometabolic risk factors in obese Zucker rats, showing a remarkable action on dyslipidemia, moderate hypertension, insulin resistance and adiponectin levels [16]. However, the effect of RBEE on vascular dysfunction associated with obesity and the main mechanisms by which this rice bran derivative induces its beneficial action on cardiometabolic risk factors remain unknown. The potential of rice bran extracts on vascular alterations has only been recently suggested in a few *in vitro* investigations which evaluated the effects of γ-oryzanol and a rice bran ethyl acetate extract on adhesion molecules expression in vascular endothelial cells or hypertrophy in smooth muscle cells, respectively [17,18].

Considering the beneficial effect of an RBEE-enriched diet on cardiometabolic parameters developed in obese rats, it is expected that RBEE will be able to restore endothelial dysfunction and vascular inflammation and oxidative stress associated with obesity in an animal model of metabolic syndrome. Thus, the aim of our study was to determine the capacity of a diet supplemented with RBEE to modify vascular dysfunction in obese Zucker rats and to identify the main pathways that could be implicated in the RBEE bioactivity. Elucidation of these vascular mechanisms might partially explain the beneficial action of RBEE treatment in cardiometabolic parameters of obese animals found in our previous investigations.

2. Materials and methods

2.1. Preparation and composition of RBEE

RBEE was prepared according to an enzymatic process previously described [15]. Briefly, RB was modified by enzymatic hydrolysis by using an endoproteases mixture as hydrolytic agent in a bioreactor with controlled temperature (60°C) and pH (pH 8) and using the pH-stat method. The processing of this product follows different steps including centrifugation, filtration and concentration. The final product is brown syrup completely soluble in water. RBEE was chemically characterized by using Association of Official Analytical Chemists standard protocols.

The chemical composition of RBEE has been previously characterized by Parrado et al. [15]. Briefly, protein is the major component (38%), in the form of peptides and free amino acids, due to the use of proteases for RB stabilization and with the aim of extracting, solubilizing and hydrolyzing the initial insoluble proteins. The fat components present in RBEE (30%) are mainly soluble because of protein interactions. Minor functional components of lipid fraction in RBEE include phytosterols (4084 mg/kg), γ-oryzanol (1260 mg/kg), tocopherols (99 mg/kg) and tocotrienols (174 mg/kg).

2.2. Animals and diets

Obese Zucker rats and their littermate controls, lean Zucker rats (8 weeks old; Charles River Laboratories, Barcelona, Spain) were fed standard diet and water *ad libitum*. Obese rats were divided into three groups (n=7) and treated daily with 1% RBEE supplementation (O1%), 5% RBEE supplementation (O5%) or standard diet (obese control group, OC). A group of lean Zucker rats (n=7) was also fed a standard diet (lean control group, LC). RBEE treatment was administered for 20 weeks in syrup form included in the standard diet supplemented with the concentrations indicated above. RBEE was extracted and supplied by the Enzymatic Production Technology group of the Department of Biochemistry and Molecular Biology (University of Seville, Spain). The selected doses of RBEE have shown beneficial effects in our previous study [16].

Body weight, food and water intake, and systolic blood pressure were evaluated weekly. At the end of treatment, animals were kept during 12 h fasting and were anesthetized with chloral hydrate 12% intraperitoneally. The protocol for animal handling and experimentation agreed with the European Union European Community guidelines for the ethical treatment of animals (EEEC Directive of 1986; 86/609/EEC) and was approved by the Ethical Committee for Animal Research of the University of Seville.

2.3. Tissue preparation

Thoracic aortas were dissected, cleaned and placed in cold modified Krebs–Henseleit solution (KHS) (in mmol/L: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 1.8, KH_2PO_4 1.18 and glucose 11). Aortic rings to be used for detection of superoxide anion ($O_2^{\bullet-}$) were maintained in KHS containing 30% sucrose overnight, placed into cryomolds containing Tissue-Tek OCT embedding medium (Sakura Finetek Europe, the Netherlands) and immediately frozen in liquid nitrogen for storage at −80°C [19]. For immunofluorescence studies, aortas were fixed in 4 % phosphate-buffered paraformaldehyde (pH 7.4) for 1 h and then washed in phosphate-buffered saline (PBS). Afterwards, arterial segments were placed in 30% sucrose PBS overnight, transferred to cryomolds with Tissue Tek OCT embedding medium, frozen in liquid nitrogen and kept at −80°C until analysis [20].

2.4. Reactivity experiments

Aortic rings were disposed in organ baths, and mechanical activity was measured as previously described [19]. Contractile capacity of the vessels was assessed with either KCl 80 mmol/L or phenylephrine (Phe) 0.3 μmol/L prior to contraction or relaxation experiments, respectively. The presence of functional endothelium was evaluated by the ability of acetylcholine (ACh) 1 μmol/L to induce more than 50% relaxation of precontracted vessels (8 of 130 aortic rings were excluded).

For the experiments, aortic segments were exposed to cumulative concentrations of Phe (0.001–10 μmol/L) to obtain concentration–response curves. Endothelium-dependent and -independent vasodilatations were studied by evaluating the relaxation induced by ACh (0.001–10 μmol/L) in endothelium-intact [E(+)] rings and sodium nitroprusside (SNP, 0.1–100 nmol/L) in endothelium-denuded [E(−)] rings in Phe-precontracted arteries. Concentration–response curves were constructed in the absence or presence of the nonselective NO synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L) and the selective inducible NOS (iNOS) inhibitor 1400W (30 μmol/L).

2.5. Western blot analysis

Protein fraction was purified from aortas after guanidine hydrochloride extraction of RNA and DNA. After isolation by ethanol precipitation, proteins were dissolved in sodium dodecyl sulfate (SDS) 4%–urea 1 M. Protein (40 μg) was resolved by electrophoresis on SDS polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose membranes. Immunoblotting was performed using a specific primary endothelial NOS (eNOS) antibody (1:800 dilution; Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. Bands were visualized by enhanced chemiluminescence assay (Pierce Chemical Company, Rockford, IL, USA) and evaluated by densitometry. The sample loading was verified by immunostaining of smooth muscle β-actin.

2.6. Real-time polymerase chain reaction (PCR)

Total RNA was isolated from frozen aortic tissue using TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the protocol of the manufacturer. Integrity of total RNA was evaluated by agarose gel electrophoresis. Total RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm (NanoDrop 2000; Thermo Scientific, Waltham, MA, USA). Reverse transcription (RT) was performed using random hexamers primers, 4 μg of total RNA as template in 50-μl reaction volume and the High-Capacity cDNA RT Archive Kit (Applied Biosystems, Carlsbad, CA, USA). Aortic gene expression of iNOS and TNF-α was determined by quantitative real-time PCR (qRT-PCR) using commercial TaqMan probes. mRNAs for eNOS and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits (NOX-1, $p47^{phox}$ and $p22^{phox}$) were quantified by qRT-PCR based on SYBR Green fluorescence using a LightCycler 480 II PCR system (Roche, Spain) [21]. Expression levels of cDNA were compared to the internal standard β-actin to normalize the data. The specific primer sequences were the following: β-actin (Rn00667869, Applied Biosystems), iNOS (Rn00561646, Applied Biosystems), TNF-α (Rn00562055_m1, Applied Biosystems), eNOS (forward 5′-TGTCACTATGGCAACCAGCGT-3′ and reverse 5′-GCGCAATGTGAGTCCGAAAA-3′), NOX-1 (forward 5′-CCTTCCATAAGCTGGTGGCAT-3′ and reverse 5′-GCCATGGATCCCTAAGCAGAT-3′), $p22^{phox}$ (forward: 5′-GGCCATTGCCAGTGTGATCTA-3′ and reverse 5′-TGCTTGATGGTGCCTCCAA-3′) and $p47^{phox}$ (forward 5′-AGGAGATGTTCCCCATTGAGG-3′ and reverse 5′-CAGTCCCATGAGGCTGTTGAA-3′). Threshold cycle (Ct) values obtained for each gene were referenced to β-actin (ΔCt) and converted to the linear form using $2^{-\Delta Ct}$ as a value directly proportional to the copy number of cDNA and initial quantity of mRNA [21].