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 (0% and 0.5%). 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.

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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].
2.2. Animals and diets

Briefly, protein is the major component (38%), in the form of peptides and free amino acids, including centrifugation, filtration and concentration. The final product is brown syrup using the pH-stat method. The processing of this product follows different steps including extracting, solubilizing and hydrolyzing the initial insoluble proteins. The fat mixture of phytosteryl ferulates contained in RBEE [15].

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 ω-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 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 (8.5) 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 hydrolizing 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), ω-oryzanol (1260 mg/kg), tocopherols (59 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 Zucker 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 University of Seville. 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 (EEC 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, NaHCO3 25, MgSO4 1.2, CaCl2 1.8, KH2PO4 1.18 and glucose 11). Aortic rings to be used for detection of superoxide anion (O2•−) 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 phenylephrine-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 for each gene (NOX-1, p47phox and p22phox) were quantified by real-time PCR system (Pierce Chemical Company, Rockford, IL, USA) and was approved by the Ethical Committee for Animal Research of the University of Seville.
2.7. In situ detection of superoxide anion

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate production of arterial \( \cdot O_2^\bullet^- \) in situ as previously described [19]. Fourteen-micrometer-thick aortic sections were placed on gelatin-coated slides and equilibrated for 30 min at 37°C in Krebs-HEPES buffer (in mmol/L: NaCl 130, KCl 5.6, CaCl_2 2, MgCl_2 0.24, HEPES 8.3, glucose 11) (pH 7.4). Tissue sections were incubated in HEPES-buffered solution containing DHE (2 μmol/L) (546-nm excitation and 610-nm emission) in a light-protected humidified chamber (37°C, 30 min). Preparations were viewed by laser scanning confocal microscope (TCS SP2; Leica, Heidelberg, Germany; ×16 objective) using the same imaging settings in each case. In preliminary experiments, DHE fluorescence was almost abolished by the \( \cdot O_2^\bullet^- \) scavenger PEG-superoxide dismutase, indicating the specificity of this reaction.

For quantification, integrated optical densities were calculated from four sampled areas per ring for each experimental condition using MetaMorph Image Analysis Software (Molecular Devices, Sunnyvale, CA, USA).

2.8. Immunofluorescence

Transverse sections (14 μm) of aortic rings were placed on gelatin-coated slides and air-dried. After blockade, sections were incubated with a polyclonal antibody against iNOS (1:100; Thermo Scientific) in 2% bovine serum albumin and air-dried. After blockade, sections were incubated with a polyclonal antibody followed by the same protocol as above. Under these conditions, no staining was observed in the vessel wall in any experimental situation.

Optical density of immunofluorescence was assayed with MetaMorph Image Analysis Software (Molecular Devices). Four areas per ring were sampled for each experimental condition. The integrated optical densities in the target region were calculated.

2.9. Drugs and chemicals

All chemicals were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). 1400 W was supplied by Tocris Bioscience (Bristol, UK). All drugs were prepared in distilled water.

2.10. Expression of results and statistical analysis

Data represented are means±S.E.M. of \( n=7 \) rats. Vasodilator responses were expressed in g/mg of dry tissue. Vasodilatation was expressed as a percentage of the previous tone generated by Phe. Two-way analysis of variance followed by Bonferroni’s comparison test was used to compare the results. Differences were considered significant when \( P<.05 \). A Prism GraphPad Software 5.0 (San Diego, CA, USA) was used for statistical analysis.

3. Results

3.1. Effect of RBEE on vascular reactivity

3.1.1. Vasodilatation

To evaluate endothelial function, endothelium-dependent vasodilatation to ACh was examined in aortas from the experimental groups. ACh induced concentration-dependent relaxation that was significantly reduced in aortic rings from OC rats compared to lean rats (Fig. 1A). The endothelial dysfunction in OC was also revealed by a lower value of pEC50 (−logEC50) when comparing curves to ACh (pEC50 OC: 7.25±0.06, LC: 7.86±0.24; \( P<.05 \)), without altering maximal relaxing responses. RBEE significantly restored this impairment in endothelial-dependent vasodilatation to ACh in aorta from obese Zucker rats (Fig. 1A). This restoration was particularly evident in rats treated with the lowest concentration of RBEE (01%), reaching similar levels of vasodilatation to those observed in lean rats (Fig. 1A) (pEC50: 7.58±0.06, \( P<.05 \)). Endothelium-independent vasodilation induced by the NO donor SNP in endothelium-denuded aortic rings did not differ significantly between the experimental groups (Fig. 1B).

Aortas from lean Zucker rats treated with RBEE-enriched diet did not show significant changes in endothelium-dependent and -independent dilatation compared to nontreated lean rats (Supplementary Figure 1 (A–B)).

The contribution of NO in the vasodilatation to ACh was also analyzed. Inhibition of NOS with L-NAMe evoked a marked reduction in endothelium-dependent vasodilatation for all the experimental groups, and this attenuation was even more evident in aorta from obese animals (Fig. 2A). To evaluate the contribution of vascular iNOS in this effect, we used the selective iNOS inhibitor 1400W. Vasodilatation curves to ACh in the presence of 1400W (Fig. 2B) revealed a marked implication of iNOS in the vasodilator response to ACh in aortic rings from OC compared to LC. This contribution was significantly attenuated by treatment with 1% RBEE. Representation of the differences between the area under the curves in the presence of L-NAMe (nonselective inhibition of NOS isoforms) and in the presence of 1400W (selective inhibition of iNOS) revealed the participation of eNOS in vasodilatation in each experimental group (Fig. 2C). In this sense, aortas from OC exhibited a decreased contribution of eNOS — and therefore an enhanced iNOS impliciation — that was significantly restored by RBEE in a concentration-dependent manner (Fig. 2C).

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Fig. 1. Concentration–response curves to ACh (A) or SNP (B) to evaluate endothelium dependence or independence of the vasodilatation, respectively. Data are mean±S.E.M. (\( n=7 \)). **\( P<.01 \), ***\( P<.001 \) vs LC.
3.1.2. Vasoconstriction

Endothelium-intact aortic rings from OC rats exhibited an increased contractile response to Phe in comparison to LC (Fig. 3A). Treatment with RBEE significantly attenuated this hyperreactivity to Phe in obese animals (Fig. 3A). This attenuation was especially evident in the O1% group, showing a similar pattern of contraction to Phe than that reached in LC (Fig. 3A). Removal of endothelium enhanced vasoconstriction in all experimental groups when comparing to contractile curves in aortas with functional endothelium (Fig. 3B, P < 0.05). The attenuation of the hyperreactivity to Phe induced by 1% RBEE in intact aortic rings was significantly reversed by endothelium removal (Fig. 3B). RBEE treatment did not modify Phe responses in aortas from lean Zucker rats (Supplementary Figure 1 (C–D)).

The presence of either L-NAME or 1400W enhanced contractile responses to Phe in all experimental groups compared to intact aortic rings in the absence of the inhibitor (Fig. 3C and D, P < 0.05). Under these conditions, OC rats still showed hyperreactivity to Phe in comparison to LC that remained unaltered by the presence of either L-NAME or 1400W (Fig. 3C and D). On the other hand, O1% group exhibited a marked attenuation of the concentration–response curve to Phe after NOS inhibition (Fig. 3C and D), especially when iNOS was selectively inhibited. Regarding O5%, vasoconstriction was not altered by L-NAME, whereas selective inhibition of iNOS with 1400W induced a significant attenuation Phe response when compared to intact aortic rings (Fig. 3D, P < 0.05).

3.2. Effects of RBEE on vascular eNOS

Aortas from lean and nontreated obese Zucker rats exhibited similar levels of eNOS protein expression (Fig. 4A). However, aortas from RBEE-treated animals exhibited a significant up-regulation of eNOS expression, especially in the 5% RBEE-treated group (Fig. 4A). Regarding aortic mRNA levels of eNOS, no differences were appreciated between the experimental groups, indicating a posttranscriptional regulation of eNOS (Fig. 4B).

3.3. Effects of RBEE on vascular iNOS and TNF-α

Aortic tissue from OC animals showed an increased protein expression of iNOS, particularly in the smooth muscle layer, whereas no immunostaining was found in arterial preparations from lean rats (Fig. 5A). Treatment with RBEE induced a marked attenuation of aortic iNOS immunofluorescence in the vascular wall (Fig. 5A). Quantification of the fluorescence signals (Fig. 5B) evidenced the significant inhibition of iNOS aortic immunofluorescence by RBEE in obese animals. According to these results, analysis of mRNA levels of vascular iNOS revealed a substantial increase in OC compared to LC and a significant attenuation in obese groups treated with RBEE (Fig. 5C). Therefore, RBEE decreased vascular induction of iNOS in obese Zucker rats at both mRNA and protein level.

Fig. 5D shows vascular expression of TNF-α mRNA in the experimental groups. Expression levels of the inflammatory cytokine were significantly increased in nontreated obese rats compared to LC, whereas the O1% and O5% groups revealed a marked reduction of aortic mRNA levels of TNF-α (Fig. 5D).

3.4. Effects of RBEE on vascular superoxide anion production and NADPH oxidase

To characterize and localize vascular O$_2^\cdot$– production, aortic sections were exposed to DHE, which is oxidized by O$_2^\cdot$– to yield
the red fluorescent DNA stain ethidium. Aortic sections from OC rats showed marked increase staining throughout the vessel wall when compared to LC (Fig. 6A and B). Red fluorescence from all vascular cell layers was significantly reduced in aortic preparations from obese rats treated with RBEE (Fig. 6A and B).

Analysis of mRNA levels of NADPH oxidase subunits, which are the major source of vascular \( \text{O}_2^- \), showed augmented mRNA levels of NOX-1 and p22phox subunits in OC aortic rings compared with LC, whereas arterial segments from 1% and 5% RBEE-treated animals evidenced a significant reduction of these values (Fig. 6C and D). However, no significant differences were observed when comparing mRNA levels of p47phox subunit (Fig. 6E).

4. Discussion

Several studies support the beneficial properties of rice bran extracts in cardiometabolic risk factors such as dyslipidemia, hypertension and glucose metabolism [22,23]. Recent investigations of our group have evidenced the beneficial effects of a novel water-soluble RBEE in vivo on animal models of hypercholesterolemia [24] and metabolic syndrome [16]. Particularly, obese animals fed an RBEE diet showed an important improvement on lipid profile, blood pressure levels, insulin resistance and plasmatic levels of inflammatory biomarkers compared to nontreated obese rats [16]. The present investigation has found the beneficial effect of RBEE on vascular and endothelial dysfunction associated with obesity and the main mechanisms involved in this activity.

Vascular abnormalities related to obesity, in particular the impaired vasodilatation in various vascular beds and in response to different stimuli, might affect vascular homeostasis, peripheral vascular resistance and the delivery of substrates to metabolically active tissues, thereby contributing to hypertension, atherosclerosis and metabolic disorders [5]. One of the most characteristic vascular alterations in metabolic syndrome is the endothelial dysfunction, which seems to involve a reduction in the amount of the available endothelium-derived NO within the vasculature. Indeed, patients with metabolic syndrome secondary to central obesity show impaired forearm vasodilator response to ACh [25]. Regarding our results, a moderate grade of endothelial dysfunction was developed by obese Zucker rats that was restored by RBEE-supplemented diet, especially by 1% RBEE. The fact that aortas from RBEE-treated obese rats showed up-regulation of eNOS protein expression, but not at mRNA levels, compared to nontreated animals suggests that RBEE could induce a posttranscriptional activation of eNOS and hence increase endothelial NO production, which is the main mediator of vasodilatation in conductance arteries such as aorta. However, it is important to take into account that eNOS expression does not reflect enzyme activity. In line with these results, the use of nonselective and selective inhibitors of iNOS revealed the potential participation of eNOS to vasodilatation in aortas from RBEE-treated obese rats.

Another vascular alteration present in obese Zucker rats is vascular hyperreactivity, evidenced by an increased vascular tone. This hyperresponse has been associated with an increased myogenic activation [26], an elevated expression and activity of serotonin [27] and endothelin receptors [28], as well as an increased sympathetic relaxation.
and pancreatic results. The excessive NO production from iNOS has been shown to increased adrenergic activity is frequently observed [30], which can nervous system activity [29]. In the development of obesity, an increased adrenergic activity is frequently observed [30], which can have a profound impact on the perfusion of tissues which are sensitive to adrenergic modulation and subsequently on blood pressure levels [31]. In the present study, RBEE treatment ameliorated hypercontractility to Phe, reaching a vasoconstriction profile similar to that found in lean rats. This response had an important endothelium-dependent component and revealed a different grade of participation of NOS isoforms. The up-regulation of eNOS evidenced in RBEE-treated groups compared to nontreated rats might be involved in the attenuation of vascular vasoconstriction to Phe by RBEE in intact aortic segments. In addition, the use of nonselective and selective inhibitors of iNOS revealed an important contribution of this isoform in aortic hyperreactivity of obese Zucker rats. Treatment with RBEE seemed to attenuate iNOS involvement, reaching similar levels of participation as observed in lean rats. This finding suggests the potential action of RBEE on vascular inflammation related to obesity, which will be discussed later. We could also suggest that the effect of RBEE on vasoconstriction, joined with its direct effects on vascular endothelium, might be involved in the attenuation of blood pressure levels previously evidenced in obese Zucker rats treated with this rice bran extract [16].

On the other hand, it is known that obesity is characterized by iNOS overexpression in the vasculature [32], as confirmed by our results. The excessive NO production from iNOS has been shown to reduce insulin-mediated glucose uptake and induce cellular stress and pancreatic β-cell death in obese animals [32]. For this reason, the implication of this enzyme as an obesity-associated inflammation biomarker was evaluated. Selective inhibition of iNOS by 1400W confirmed the important involvement of this isoform in the vascular reactivity of obese Zucker rats, which was modified by the RBEE-supplemented diet. Indeed, while obese animals showed augmented values of iNOS aortic protein expression and mRNA levels compared to LC, these parameters were significantly restored in aortas from RBEE-treated animals. Therefore, the key role developed by NO from vascular iNOS was significantly ameliorated by treatment with a diet supplemented in RBEE. These results are in accordance with our previous investigation in which obese rats treated with RBEE showed an important attenuation of the proinflammatory plasmatic levels of nitrates and nitrites when compared to nontreated obese animals [16].

Accordingly, it has been demonstrated that the best characterized adipocytokine causing insulin resistance and endothelial dysfunction in metabolic syndrome is TNF-α, a proinflammatory molecule which under normal circumstances circulates in low concentrations [33]. Increased expression levels of iNOS are strongly related to an up-regulation of TNF-α [34], as we have found in aorta from obese Zucker rats. In this regard, vascular expression of TNF-α was reduced with both concentrations of RBEE in accordance to iNOS down-regulation and plasmatic adiponectin restoration (Supplementary Table I and Ref. [16]). The attenuation of the vascular proinflammatory state associated with obesity agrees with previous studies where a diet supplemented in γ-oryzanol and other bioactive components of rice bran improved levels of different inflammatory biomarkers in obese subjects [35].

Vascular oxidative stress plays a key role in the vascular impairment associated with metabolic syndrome. In general, treatment of obese Zucker rats with antioxidants has improved vasodilator responses to NO-dependent stimuli in isolated microvessels and using in situ preparations [36], suggesting that vascular reactivity can be improved by acute treatment with oxidative radical scavengers in this animal model. In this regard, an elevated antioxidant capacity has been attributed to rice bran because of its high content in phenolic compounds [37,38]. Besides, it has been demonstrated that γ-oryzanol and related compounds have antioxidant and ROS scavenging potency, inhibiting proinflammatory cytokines activity and increasing blood adiponectin levels in vitro and in vivo [13]. Antioxidant activity of γ-oryzanol has been attributed to its ferulic acid moiety [39] as well as to the sterol moiety in living cells [40]. In our study, the content of RBEE in these bioactive components could play a critical role in the antioxidant action induced by RBEE in the vascular wall of obese Zucker rats.

The NADPH oxidase system is a major source of vascular ROS [41]. It has been suggested that rice bran constituents may interfere with the NADPH oxidase system, which in turn inhibits ROS production [13]. Several studies support the important role of NADPH oxidase-derived ROS in hypertension and atherosclerosis using animal models with genetic disruption of subunits of the enzyme [42,43]. When evaluating the antioxidant effects of RBEE in the vascular tissue, another target in our investigation was to determine aortic expression levels of NADPH subunits. It was found a reduction in RBEE-treated aortas in the expression of the subunits NOX-1 and p22phox, which were importantly increased in obese animals, contributing to vascular dysfunction and partially to hypertension. Vascular ROS generation may be the upstream target of RBEE followed by the improvement of vascular reactivity and inflammatory biomarkers.

In summary, this investigation demonstrates that chronic administration of this novel water-soluble enzymatic extract of rice bran could be a suitable treatment for improving or alleviating vascular alterations associated with metabolic syndrome. RBEE-supplemented diet ameliorates vascular impairment related to obesity by restoring endothelial dysfunction and
vascular hyperreactivity via a counteraction of iNOS and TNF-α involvement probably in association with a notable reduction of vascular oxidative stress (Fig. 7). According to the present study and our previous investigations [16], the beneficial effect of RBEE on vascular alterations linked to metabolic syndrome seems to be a consequence of a reduction in vascular, adipose and plasmatic

Fig. 5. iNOS protein expression by immunofluorescence (A,B) and mRNA levels of iNOS (C) and TNF-α (D) in aorta. Data are mean±S.E.M. (n=5–7). *P<.05, ***P<.001 vs LC; #P<.05, ##P<.01 vs OC.

Fig. 6. Aortic production of superoxide anion by DHE staining (A, B) and mRNA levels of the NADPH oxidase subunits NOX-1 (C), p22phox (D) and p47phox (E). Data are mean±S.E.M. (n=5–7). *P<.05, ***P<.001 vs LC; #P<.05, ##P<.01 vs OC.
proinflammatory and pro-oxidants biomarkers joined with an improvement in lipid profile and insulin resistance. These findings support the idea that RBEE could be considered an exemplary candidate to be used as functional food in the treatment of vascular complications associated with obesity.

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Appendix A


References


Fig. 7. Schematic representation of mechanisms involved in the beneficial effects of RBEE in obesity-related vascular impairment. RBEE treatment attenuates vascular ROS production. This effect is related to a restoration of vascular reactivity by reducing endothelial dysfunction and hypercontractility as well as up-regulating eNOS expression. Also, RBEE attenuates vascular inflammation by reducing iNOS and TNF-α involvement, thus contributing to the improvement of vascular disorders associated with obesity. NADPHox, nicotinamide adenine dinucleotide phosphate-oxidase.


