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ARTICLE

Wild blueberry consumption affects aortic vascular function in the obese Zucker rat

Stefano Vendrame, Aleksandra S. Kristo, Dale A. Schuschke, and Dorothy Klimis-Zacas

Abstract: This study evaluates the effect of wild blueberry (WB) consumption on the biomechanical properties of the aorta in the obese Zucker rat (OZR), a model of the metabolic syndrome. Thirty-six OZRs and 36 lean controls (lean Zucker rats) were placed either on a WB-enriched or a control (C) diet for 8 weeks. Phenylephrine (Phe)-mediated vasoconstriction and acetylcholine (Ach)-mediated vasorelaxation in the aortic vessel were investigated, as well as the contribution of the nitric oxide synthase and cyclooxygenase (COX) pathways in each of the above responses by using specific inhibitors. Obese Zucker rats exhibited a reduced vasocontstrictor response to Phe and an exaggerated vasorelaxant response to Ach. The WB diet partially restored Phe-induced constrictor responses and attenuated Ach-induced relaxant responses in OZR. Plasma nitric oxide was significantly attenuated (22.1 ± 1.1 μmol·L^{-1}, WB vs 25.6 ± 1.4 μmol·L^{-1}, C, $p \leq 0.05$) with the WB diet. Thromboxane A2 levels in the aortic effluent were not significantly affected in the WB diet group, while PGI_2 concentration significantly increased (766.5 ± 92.2 pg·mg^{-1} aorta in the WB vs 571.7 ± 37.8 pg·g^{-1} aorta in the C group, $p \leq 0.05$). Downregulation of inducible nitric oxide synthase and COX_2 expression in the OZR aorta was observed in the WB diet group. In conclusion, WB consumption altered the biomechanical properties of the OZR aorta by partially restoring the impaired Phe-induced constrictor responses and attenuating the exaggerated response to Ach-induced vasorelaxation.

Key words: anthocyanins, endothelial dysfunction, metabolic syndrome, nitric oxide, vasoconstriction, vasorelaxation.

Résumé : Cette étude évalue l'effet de la consommation de bleuets sauvages (« WB ») sur les propriétés biomécaniques de l'aorte du rat Zucker obèse (« OZR »), un modèle de syndrome métabolique. On répartit 36 OZR et 36 rats de contrôle maigres (« lean Zucker rats ») dans deux groupes : apport alimentaire enrichi en WB ou apport de contrôle (« C ») durant 8 semaines. On examine dans l'aorte la vasoconstriction médiée par la phényléphrine (« Phe ») et la vasorelaxation médiée par l'acétylcholine (« Ach ») ainsi que la contribution des voies de l'oxyde nitrique synthase (« NOS ») et de la cyclooxygénase (« COX ») au moyen d'inhibiteurs spécifiques. Les rats Zucker obèses présentent une vasoconstriction réduite en réponse à la Phe et une vasorelaxation disproportionnée en réponse à l'Ach. Le régime enrichi en WB chez les OZR corrige en partie la réponse de vasoconstriction et atténue la réponse de vasorelaxation. Dans la condition enrichie en WB, on observe une diminution plasmatique significative de l'oxyde nitrique (C, 25,6 ± 1,4 μmol·L^{-1} comparativement à WB, 22,1 ± 1,1 μmol·L^{-1}, $p \leq 0,05$). Dans la condition WB, on n'observe pas de modification significative du niveau de thromboxane A2 dans l'effluent aortique, mais on observe une augmentation significative de la concentration de PGI_2 (C, 571,7 ± 37,8 pg·mg^{-1} d'aorte comparativement à WB, 766,5 ± 92,2 pg·mg^{-1} d'aorte, $p \leq 0,05$). Dans la condition WB, on observe chez les rats OZR une régulation à la baisse de l'expression synthase inductible de l'oxyde nitrique et de COX_2. En conclusion, la consommation de WB modifie les propriétés biomécaniques de l'aorte de rats OZR en corrigeant partiellement l'altération de la vasoconstriction médiée par la Phe et en atténuant l'excès de vasodilatation médiée par l'Ach.

Mots-clés : anthocyanines, dysfonction endothéliale, syndrome métabolique, oxyde nitrique, vasoconstriction, vasorelaxation.

Introduction

The metabolic syndrome (MetS) is a cluster of strictly interrelated risk factors dramatically increasing the risk of developing type 2 diabetes and cardiovascular disease, which are leading causes of death in the United States and worldwide (Roger et al. 2012). While the diagnostic criteria for MetS involve blood lipid profile, fasting blood glucose, blood pressure, and waist circumference measurements, the development of endothelial dysfunction and a chronic pro-inflammatory, pro-oxidative, and pro-thrombotic environment are landmark characteristics of this condition (Prasad et al. 2012).

Endothelial dysfunction can be created by an imbalance between vasoconstrictor and vasodilator responses leading to impaired vascular tone, peripheral vascular resistance and organ perfusion, and is one of the earliest events in the development of atherosclerotic lesions (Marti et al. 2012).

Recent evidence indicates that dietary bioactive compounds can play a fundamental role in preventing and reversing endothelial dysfunction (Landberg et al. 2012).

Wild blueberries (WBs) are one of the richest fruit sources of anthocyanins (ACN) and other polyphenols (Häkkinen and Törrönen 1999). The ability of WB consumption to affect the biomechanical properties of rat aortas has been repeatedly shown ex vivo. Consumption of an 8% WB diet resulted in attenuated phenylephrine (Phe)-induced vasoconstriction in weanling Sprague–Dawley rats after 7 weeks (Kalea et al. 2009) or 13 weeks (Norton et al. 2005), and in adult spontaneously hypertensive rats (SHR) after 8 weeks (Kristo et al. 2010). Enhanced acetylcholine (Ach)-induced vasorelaxation was observed in young SHRs fed an 8% WB

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diet for 7 weeks (Kalea et al. 2010). Reduced Phe-induced vasoconstriction and enhanced Ach-induced vasorelaxation has also been reported with a 2% highbush blueberry diet for 10 weeks in Wistar rats fed a high fat and cholesterol diet to induce endothelial dysfunction (Rodriguez-Mateos et al. 2013).

Furthermore, WB diets have been shown to remodel the structure of the aortic extracellular matrix by altering the concentration and sulfation patterns of glycosaminoglycans, both in Sprague–Dawley (Kalea et al. 2006) and SHR (Kristo et al. 2012), which may favorably affect signal transduction pathways involved in endothelial function.

However, no specific data are available on the effects of WB on endothelial function as related to the metabolic syndrome. Thus, in this study, we evaluate for the first time the effect of WB consumption on the biomechanical arterial properties of the aorta in the obese Zucker rat (OZR), which is a valid experimental model of MetS (De Artiñano and Castro 2009).

As a result of a spontaneous mutation, OZRs (fa/fa) develop obesity, insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypercholesterolemia already in their first weeks of life (De Artiñano and Castro 2009). Their lean littermate controls (Fa/Fa) do not exhibit any of the above-mentioned abnormalities.

In this study, both Phe-mediated vasoconstriction and Ach-mediated vasorelaxation in the aortic vessel are investigated, as well as the contribution of the nitric oxide synthase (NOS) and cyclooxygenase (COX) pathways in each of the above responses. We also evaluate the expression of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and COX_2 in the aortic tissue, plasma concentrations of nitric oxide, and concentrations of prostacyclin I_2 (PGI_2) and thromboxane A_2 (TXA_2) in the aortic effluent.

Materials and methods

Zucker rats

Thirty-six male OZR (fa/fa) and 36 male lean Zucker rats (LZR) (Fa/Fa) were purchased from Charles River Laboratories (Raleigh, N.C., USA). Animals from each group were randomly assigned to either a control (C) or a WB-enriched diet for 8 weeks, from ages 8 to 16 weeks. Rats were caged individually in a room maintained at 22 °C and with a 12-h light/12-h light cycle. Food consumption was recorded daily and rat weight was measured weekly. The experimental protocol was approved by the University of Maine Institutional Animal Care and Use Committee.

Diets

Wild blueberries were provided as a composite by Wyman's (Cherryfield, Maine, USA) and processed following standard procedures to obtain a freeze-dried powder (FutureCeuticals, Momence, Ill., USA). The WB powder was vacuum-packed in plastic bags and stored at –20 °C until use. Total ACN content of the WB powder was 1.5% *w*/*w*, with malvidin-3-galactoside and peonidin-3-glucoside being the most abundant forms, as previously reported (Del Bo' et al. 2012).

Diets were prepared weekly from purified ingredients and stored at 4 °C. The C diet was prepared using dextrose, egg white solids, vitamin mix, mineral mix, DL-methionine, biotin, and corn oil as previously described (Norton et al. 2005). The WB diet was prepared using the same ingredients, but replacing 8% *w*/*w* of dextrose with an equivalent amount of WB powder.

Sample collection

At the end of the experimental period, rats were anesthetized with 95% CO_2/5% O_2 for approximately 2 min and exsanguinated by cardiac puncture. Blood was collected in a tube with 200 μL of a 5% EDTA solution to prevent clotting, and centrifuged for 15 min at 2300*g* for plasma separation and storage at –80 °C until further analysis.

Four aortic rings that were each 3 mm in length were prepared from the middle of the aorta for vasorelaxation and vasoconstriction experiments, while a 1-cm-long section from the abdominal end was used for assaying prostanoid release as described below. The remaining portion of aorta was quickly snap-frozen in liquid nitrogen and stored at –80 °C for subsequent mRNA isolation as described below.

Phe-induced vasoconstriction with or without NO and COX pathway inhibition

From a subset of 40 rats, a Phe dose–response curve was generated using 4 aortic rings from each animal to evaluate vasoconstriction.

Each aortic ring was suspended by 2 stainless steel wire triangles and mounted in a Radnoti tissue bath that contained physiologic saline solution (PSS) at 37 °C and aerated with a 95%O_2/5%CO_2 gas mix. Rings were connected to tissue force analyzers (MicroMed, Louisville, Ky., USA) to record the force developed during the experiment.

After a preload to adjust resting tension to 1.5 g and a preconditioning with 1 dose of each agonist (Ach10^{-8} mol·L^{-1} and Phe 10^{-8} mol·L^{-1}) to saturate the nonselective receptors, each aortic ring was randomly assigned to either a treatment with N^G-monomethyl-L-arginine (L-NMMA, 10^{-4} mol·L^{-1}), which inhibits NO synthases ((eNOS, iNOS, and neuronal nitric oxide synthase (nNOS)); a treatment with mefenamic acid (MFA, 10^{-5} mol·L^{-1}), which inhibits cyclooxigenases (COX_1 and COX_2); or a treatment with no inhibitor.

The aortic rings were subsequently contracted with 6 cumulative Phe doses (from 10^{-8} to 3×10^{-6}), and allowed to reach maximum contraction force for 6 min after each dose. After the last dose, a single dose of Ach (3×10^{-6}) was applied to confirm the integrity of the endothelium.

The maximum force of contraction (F_{max}) was determined for each ring as the highest value of each Phe curve and used to evaluate the effect of treatments on aorta contractility. The effective concentration of the agonist to obtain 50% of maximum response (EC_{50}) and vessel sensitivity to the α_1-adrenergic receptor response (pD_2, $-\log_{10}$ EC_{50}) were also determined for each ring (Norton et al. 2005).

Ach-induced vasorelaxation with or without NO pathway and COX pathway inhibition

From the other subset of 32 rats, an Ach dose–response curve was generated using 4 aortic rings from each animal to evaluate vasorelaxation. Each aortic ring was mounted and preloaded as described above, and subsequently randomly assigned to either a treatment with L-NMMA (10^{-4} mol·L^{-1}), a treatment with MFA (10^{-5} mol·L^{-1}), or a treatment with no inhibitor.

Rings were precontracted with 1 maximal Phe dose (10^{-6} mol·L^{-1}) for 10 min, until the contraction curve reached a plateau. The aortic rings were subsequently exposed to 6 cumulative Ach doses (from 10^{-8} to 3×10^{-6} mol·L^{-1}), and allowed to reach maximum vasorelaxation for 6 min after each dose. The relaxant effect to each dose of Ach was expressed as a percentage of vasorelaxation of the maximum Phe-induced precontraction force. The effective concentration of agonist at which 50% vasorelaxation is obtained (EC_{50}) was determined for each ring, as well as vessel sensitivity to Ach (pD2, $-\log_{10}$ EC_{50}).

Plasma NO

Total NO in plasma was estimated using the Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit (R&D Systems), a nitrate/nitrite colorimetric assay based on spectrophotometric detection of a Griess Reaction product at 540 nm, following the instructions provided by the manufacturer.

PGI_2 and TXA_2 in the aortic effluent

A segment of aorta (~1 cm long) from each animal was incubated in a 2-mL Radnoti tissue bath containing PSS at 37 °C and aerated with 95% O_2/5% CO_2. Tissue was allowed to equilibrate for 20 min before adding Phe (10^{-6} mol·L^{-1} for 10 min) followed by Ach (10^{-5} mol·L^{-1} for 10 min) to stimulate prostanoids release to the medium. Aortic tissue was then dried under a stream of nitrogen and weighed. The effluent was collected and PGI_2 levels were estimated using the enzyme immunoassay 6-keto-PGF1α EIA Kit (Cayman). 6-keto-PGF1α is a metabolite of non-enzymatic hydrolysis of PGI_2. TXA_2 levels in the aortic effluent were estimated by using the enzyme immunoassay Thromboxane B_2 EIA Kit (Cayman). TXB_2 is a metabolite of non-enzymatic hydrolysis of TXA_2. Results were normalized to the dry weight of the aorta section stimulated with the agonists for prostanoid generation.

mRNA expression of iNOS, eNOS, and COX_2 in the aorta

mRNA was isolated from frozen aorta samples using the RNeasy Fibrous Tissue Mini Kit (Qiagen no. 74704). cDNA was subsequently obtained from mRNA using the QuantiTect Reverse Transcription Kit (Qiagen no. 205313). The reverse transcription product was subjected to 2-step, real time, reverse transcription polymerase chain reaction (PCR) amplification on a quantitative PCR System (Bio-Rad CFX96) by using a Sybr Green master mix (SSoFast EvaGreen, Bio Rad no. 172-5202) and the following rat-specific primers: *Nos2* (RefSeq NM_012611, Qiagen no. QT00178325), *Nos3* (RefSeq NM_021838, Qiagen no. QT01570618), *Ptgs2* (RefSeq NM_017232, Qiagen no. QT00192934), *Actb* (RefSeq NM_031144, Qiagen no. QT00193473).

For each primer, the analysis was performed in triplicate with a reaction volume of 20 μL per well (1.5 μL reverse transcription product, 10 μL Sybr Green Mix, 2 μL primers, and 6.5 μL RNase free water) and the following protocol: enzyme activation at 95 °C for 30 s; 45 amplification cycles (denaturation at 95 °C for 2 s, annealing/extension at 60 °C for 5 s); melting curve 75–95 °C to ensure specificity of amplification. The ΔΔCt method (Livak and Schmittgen 2001) was used to calculate relative expression of the target genes normalized to the housekeeping gene β-actin. Results were reported as fold-variation compared with LZR on the C diet.

Statistical analysis

Results from vasoconstriction and vasorelaxation experiments were evaluated using 2-way ANOVA with Student–Newman–Keuls comparisons on equal numbers of rank-ordered observations, in the absence or presence of inhibitors, in OZR and LZR. Gene expression data, plasma nitrites plus nitrates, and aortic prostanoid concentrations were analyzed using 2-way ANOVA, with diet (WB vs. C diets) and animal model (OZR and LZR) as independent factors. Significant main effects and interactions were further evaluated by means of the Tukey HSD post hoc test. Statistical analysis was performed using R statistical software version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria). Results were expressed as means ± SE and considered significant at $p < 0.05$.

Results

Food intake and animals weight

Average food intake was significantly higher in the OZR group (29.7 ± 2.60 g·day^{-1}) compared with the LZR group (22.6 ± 1.89 g·day^{-1}), but it was homogeneous within each group between WB and C animals. Average weight (439.5 ± 25.3 g OZR and 302.7 ± 22.1 g LZR) and average weight gain (281.1 ± 27.3 g OZR and 159.7 ± 23.5 g LZR) during the experimental period were both significantly higher in the OZR group compared with the LZR group, with no significant differences within each group between WB and C animals.

Phe-induced vasoconstriction

As shown in Fig. 1, Phe-induced vasoconstriction was significantly lower in OZR-C (F_{max} 0.43 ± 0.02 g) compared with LZR-C (F_{max} 0.74 ± 0.02 g). WB feeding partially restored Phe-induced constrictor responses in OZR, with significant increase in the maximal force that was developed (F_{max} 0.55 ± 0.02 g, $p < 0.05$). Constrictor responses in LZR were unaffected by WB in response to all Phe doses.

When rings were pretreated with NOS inhibitor L-NMMA or with COX inhibitor MFA, the constrictor responses to Phe significantly increased in both OZR (F_{max} 1.54 ± 0.04 g and 0.71 ± 0.04 g, respectively) and LZR (F_{max} 1.79 ± 0.04 g and 1.11 ± 0.04 g, respectively). In OZR assigned to WB diet, pretreatment with L-NMMA caused a further increase in vasoconstrictor response (F_{max} 1.79 ± 0.04 g, $p < 0.05$), elevating the maximal force developed to the same level of LZR. With MFA pretreatment, the increased vasoconstrictor response induced by WB in OZR was maintained, still resulting in lower vasoconstrictor response in OZR compared with LZR.

Vessel sensitivity to the α1 adrenergic agonist Phe (Table 1) was similar in OZR and LZR, and was not affected by the WB diet. With both L-NMMA–induced NOS inhibition and MFA-induced COX inhibition, vessel sensitivity significantly increased in both OZR and LZR ($p < 0.05$). Furthermore, when L-NMMA was added, WB diet resulted in increased vessel sensitivity in OZR.

Ach-induced vasorelaxation

The cumulative dose–response curves to Ach-induced endothelium-dependent vasorelaxation are shown in Fig. 2.

The maximal relaxation in response to Ach was significantly higher in OZR (97.62% ± 1.78%) compared with LZR (80.26% ± 1.66%). The WB diet did not have any effect in OZR, but resulted in enhanced relaxation in LZR (maximal relaxation 90.73% ± 1.80%).

Following pretreatment with L-NMMA, vasorelaxant responses were significantly lowered in OZR and LZR on the WB diet but were not significantly affected in OZR and LZR on the C diet. As a result, the difference in maximal relaxation in response to Ach between LZR-C and LZR-WB was lost, while a significantly lower relaxant response was observed in OZR-WB compared with OZR-C.

Following pretreatment with MFA, a trend toward lower vasorelaxation responses could be observed in OZR, although this effect was statistically significant in OZR-WB only, and vasorelaxation responses were unaffected in OZR fed a WB diet. Vasorelaxation responses following MFA pretreatment were unaffected in LZR, and the higher maximal relaxation in LZR-WB was maintained.

Vessel sensitivity to Ach (Table 2) was significantly higher in OZR compared with LZR, independent of diet, both in the absence of the inhibitors and following L-NMMA or MFA pretreament. WB diet significantly attenuated vessel sensitivity in OZR, and significantly increased it in LZR.

Pretreatment with L-NMMA decreased vessel sensitivity to Ach in both OZR and LZR. The differences between C and WB diet were maintained, but appeared to be enhanced within OZR, and reduced within LZR, as indicated by the difference between pD_2 values.

Following pretreatment with MFA, the difference in vessel sensitivity to Ach between OZR-C and OZR-WB was eliminated, while it was still significantly higher in LZR-WB compared with LZR-C.

Prostanoids and NO

Prostanoid concentrations in the aortic effluent and plasma NO levels are reported in Table 3. A significant effect of the model was only observed for NO, with OZR having higher NO circulating levels. Nitrites plus nitrates concentration increased in LZR and decreased in OZR with the WB diet. In the aortic effluent, 6-keto-$PGF_{1\alpha}$ concentration increased while thromboxane B_2 levels were unaffected in OZR fed the WB diet.

Fig. 1. Phenylephrine (Phe) concentration–response curves in lean (LZR) and obese Zucker rats (OZR) following wild blueberry (WB) or control (C) diet in the absence or presence of N^G-monomethyl-L-arginine (L-NMMA, 10^{-5} mol·L^{-1}) or mefenamic acid (MFA, 10^{-5} mol·L^{-1}). PSS, physiologic saline solution. *, Significant effect of diet, OZR-C vs. OZR-WB ($p < 0.05$).

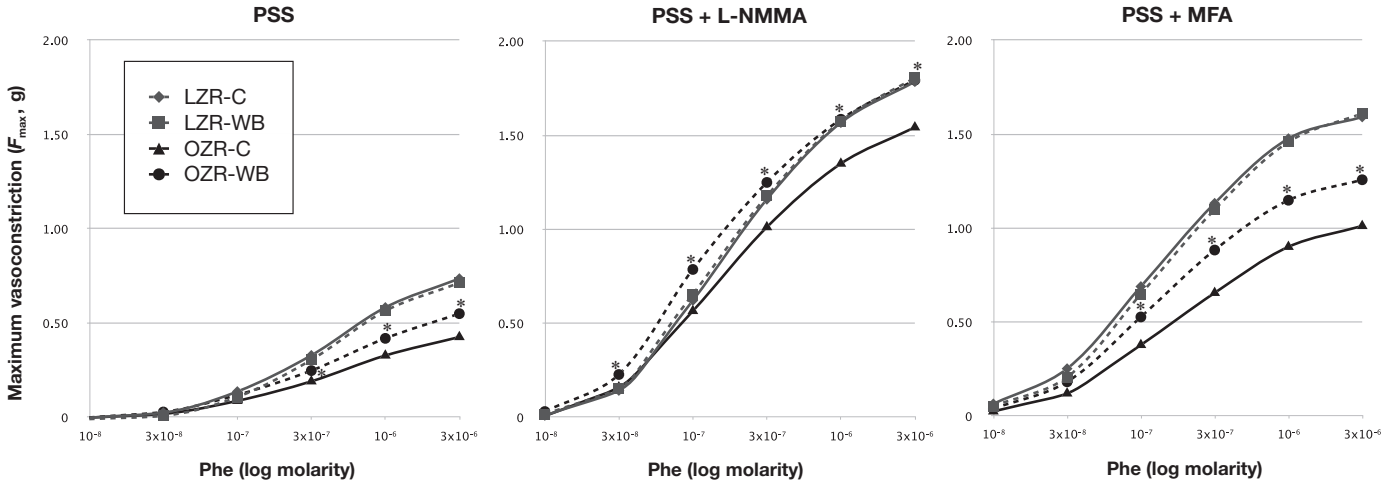


Table 1. Vessel sensitivity (pD_2) to the α1 adrenergic agonist phenylephrine in lean (LZR) and obese (OZR) Zucker rats, with or without inhibitors, following wild blueberry (WB) or control (C) diets.

	LZR-C (n = 10)	LZR-WB (n = 10)	OZR-C (n = 10)	OZR-WB (n = 10)
PSS	6.42±0.02	6.42±0.02	6.43±0.01	6.44±0.01
PSS+L-NMMA	6.75±0.01[a]	6.77±0.01[a]	6.74±0.02[a]	6.89±0.02[a,*]
PSS+MFA	6.86±0.02[b]	6.83±0.02[b]	6.81±0.02[b]	6.88±0.02[b]

Note: L-NMMA, N^G-monomethyl-L-arginine; MFA, mefenamic acid; PSS, physiologic saline solution.

[a]Significant effect of treatment, PSS vs L-NMMA ($p < 0.05$).

[b]Significant effect of treatment, PSS vs MFA ($p < 0.05$).

*Significant effect of diet, OZR-C vs OZR-WB ($p < 0.05$).

Aorta expression of iNOS, eNOS, and COX2

As shown in Fig. 3, a significant effect of the animal model was observed for mRNA expression of eNOS, iNOS, and COX-2, which were all higher in the aortic tissues of OZR compared with LZR. While no interaction with diet was observed for eNOS, the WB diet resulted in downregulation of iNOS expression both in the OZR and LZR aorta. In OZR, COX-2 expression was also significantly attenuated with the WB diet.

Discussion

The present study investigates the effects of a WB-enriched diet on vasoconstricting and vasodilating responses in the OZR model of the metabolic syndrome.

This animal model shows dramatically different and often opposite responses depending on the type of vessels studied and the different age stages, and results are often inconsistent between studies (Auguet et al. 1989; Institoris et al. 2011; Moral-Sanz et al. 2011; Oltman et al. 2006; Picchi et al. 2006; Romanko and Stepp 2005; Sánchez et al. 2010; Subramanian and MacLeod 2003; Vessières et al. 2010; Villalba et al. 2009). At least in the aortic vessel, most data point to exaggerated endothelium-dependent vasodilator responses and impaired vasoconstrictor responses, although the contribution of endothelium-derived relaxing and constricting factors, including NO and prostanoids, has not been fully elucidated (Auguet et al. 1989; Oltman et al. 2006; Subramanian and MacLeod 2003).

In our study, the OZR exhibited impaired vasoconstrictor responses to the α1 adrenergic agonist Phe, developing a lower maximal force of contraction compared with LZR, with no difference in vessel sensitivity.

Vasoconstrictor responses in OZR arteries have been consistently found to be lower compared with LZR (Auguet et al. 1989; Moral-Sanz et al. 2011; Oltman et al. 2006; Romanko and Stepp 2005). Constrictor reactivity to norepinephrine was reduced in 15- to 18-week-old OZR mesenteric arteries (Romanko and Stepp 2005), maximal Phe-induced vasoconstriction was reduced in the thoracic aorta of 16-week-old OZR (Oltman et al. 2006), the thoracic aorta of 14- to 15-week-old OZR (Auguet et al. 1989), and the resistance pulmonary arteries of 17- to 18-week-old OZR (Moral-Sanz et al. 2011).

This impaired vasoconstriction is endothelium-dependent, as demonstrated by Auguet et al. (1989), who observed that removing the endothelium abolished any difference between LZR and OZR in constrictor responses to Phe. With intact endothelium, Phe-induced contraction was diminished in both LZR and OZR, suggesting that endothelium exerts an inhibitory effect on contraction. Such effect was more pronounced in OZR (Auguet et al. 1989).

In the present study, the WB diet did not have any significant effect on LZR vasoconstrictor responses, but significantly increased the maximal force of contraction in response to Phe in OZR.

When rings were pretreated with L-NMMA to inhibit NOS, the maximal tension to Phe increased as expected in all groups, but the increase in OZR was accentuated with WB. Indeed, the maximal force of contraction in OZR on the WB diet reached the same level of LZR with NOS inhibition. This, together with the significantly higher vessel sensitivity in the OZR that were fed WB with NOS inhibition, points to an involvement of the NO pathway in the impaired constrictor responses observed in OZR.

In contrast, pretreatment with MFA to inhibit COX increased the force of contraction and vessel sensitivity in all groups, but did not alter the effect of WB on constrictor responses, suggesting that the COX pathway does not play a significant role in this regard.

In the current study, vasorelaxant responses to Ach were found to be significantly higher in the OZR compared with LZR. Endothelium-dependent relaxation responses to Ach have been previously found to be reduced in mesenteric arteries of 15- to 18-week-old OZR (Romanko and Stepp 2005; Vessières et al. 2010), in brain arteries of 11- to 13-week-old OZR (Institoris et al. 2011), and in penile arteries of 17- to 18-week-old OZR (Villalba et al. 2009). Responses to Ach were also slightly impaired in coronary arteries from 12- to 18-week-old OZR (Picchi et al. 2006; Sánchez et al. 2010), but were not affected in coronary

Fig. 2. Acetylcholine (Ach) concentration–response curves in lean (LZR) and obese Zucker rats (OZR) following wild blueberry (WB) or control (C) diet, in the absence or presence of N^G-monomethyl-L-arginine (L-NMMA, 10^{-5} mol·L^{-1}) or mefenamic acid (MFA, 10^{-5} mol·L^{-1}). PSS, physiologic saline solution. *, Significant effect of diet, OZR-C vs. OZR-WB ($p < 0.05$); †, significant effect of diet, LZR-C vs. LZR-WB ($p < 0.05$).

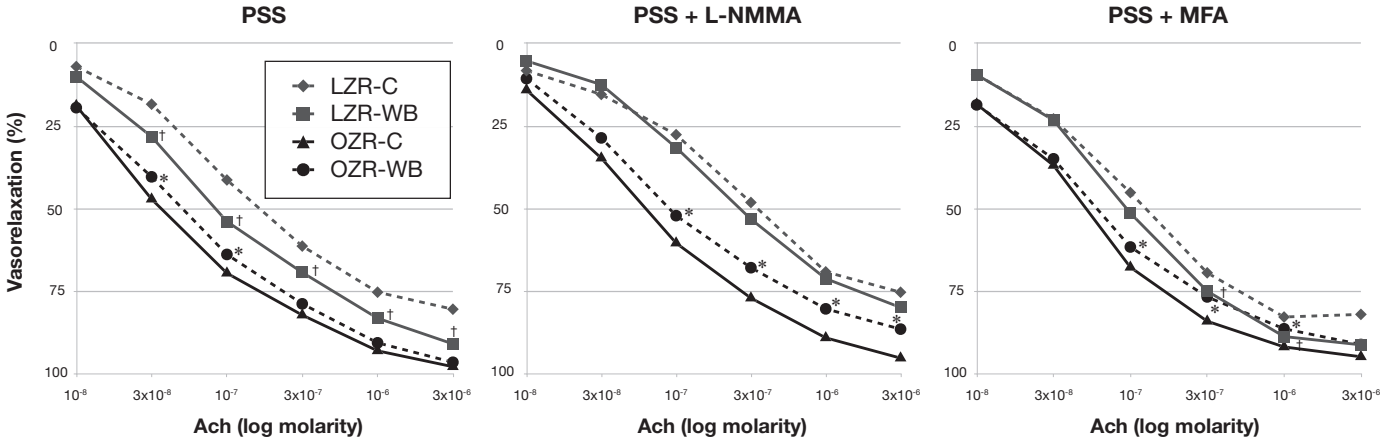


Table 2. Vessel sensitivity (pD_2) to acetylcholine in lean (LZR) and obese (OZR) Zucker rats, with or without inhibitors, following wild blueberry (WB) or control (C) diets.

	LZR-C (n = 10)	LZR-WB (n = 10)	OZR-C (n = 10)	OZR-WB (n = 10)
PSS	6.71±0.03	6.95±0.02†	7.35±0.03‡	7.22±0.03*
PSS+L-NMMA	6.45±0.03[a]	6.57±0.03[a,†]	7.15±0.04[a,‡]	6.92±0.04[a,*]
PSS+MFA	6.89±0.04[b]	6.99±0.03†	7.28±0.02[b,‡]	7.25±0.04

Note: Values are means ± SE. L-NMMA, N^G-monomethyl-L-arginine; MFA, mefenamic acid; PSS, physiologic saline solution.
[a]Significant effect of treatment, PSS vs L-NMMA ($p < 0.05$).
[b]Significant effect of treatment, PSS vs MFA ($p < 0.05$).
*Significant effect of diet, OZR-C vs OZR-WB ($p < 0.05$).
†Significant effect of diet, LZR-C vs LZR-WB ($p < 0.05$).
‡Significant effect of model, LZR-C vs OZR-C ($p < 0.05$).

arteries of 17- to 18-week-old OZR (Villalba et al. 2009). Conversely, Ach response has been found to be increased in the thoracic aorta of 32-week-old OZR (Subramanian and MacLeod 2003), and endothelium-dependent relaxation induced by the cholinergic agonist carbachol was higher in thoracic aorta of 14- to 15-week-old OZR (Auguet et al. 1989).

In our study, the WB diet in the LZR resulted in enhanced Ach-mediated vasorelaxation and vessel sensitivity to the agonist. The effect on vessel sensitivity was diminished but not abolished with both NOS inhibition and COX inhibition, suggesting the partial involvement of both pathways. However, the effect of WB on maximal relaxation was abolished with NOS inhibition and unaffected by COX inhibition, suggesting that the effect is independent from the COX pathway and mainly dependent on the NO pathway.

The effect of WB on vasorelaxation in the OZR tended to be in opposite direction compared with the effect observed in LZR. The WB diet did not affect maximal relaxation but decreased vessel sensitivity to Ach. Cyclooxygenase inhibition partially attenuated this effect on vessel sensitivity with no effect on maximal relaxation, while NOS inhibition resulted in decreased maximal relaxation and accentuated the difference in vessel sensitivity to Ach following WB, suggesting a partial involvement of both pathways in WB induced responses.

A similar response has been previously reported in hindquarter vessel preparations of 13-week-old OZR that showed higher Ach-induced vasorelaxation compared with LZR (Andrews et al. 2000). Following 4 weeks of supplementation with the antioxidant vitamin E, vasodilator responses were enhanced in LZR, and reduced in OZR (Andrews et al. 2000), similarly to what we observed in the aortas with WB diet. This suggests that the overcompensated endothelial function observed in OZR may be the result of a protective adaptation to the increased endogenous levels of oxidative stress.

One of the possible explanations for the vascular hyporesponsiveness to Phe in OZR and the exaggerated vasodilator reactivity to Ach is the pathologic release of NO by the inducible, calcium-independent isoform of NOS. This enzyme can be induced both in the endothelium and the smooth muscle by pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and by endotoxin lipopolysaccharides, and has been shown in vitro to lead to vascular relaxation that is resistant to vasoconstrictors, similar to the situation characteristic of septic shock (Moncada and Higgs 1993). NO overproduction has been observed in many inflammatory conditions (Mollace et al. 2005), possibly as a compensatory adaptation to preserve blood flow in a pro-oxidant, pro-inflammatory, and dyslipidemic environment. However, under such conditions, iNOS-derived NO is likely not converted to nitrite as it happens physiologically, and instead reacts with superoxide anion to generate the highly reactive peroxynitrite radical, further contributing to oxidative stress and endothelial dysfunction.

Indeed, in this study we detected significantly higher circulating levels of NOx in OZR, and a 2.5-fold higher expression of iNOS in the aorta of OZR-C compared with LZR-C. Endothelial NOS expression was also slightly higher in OZR compared with lean controls.

The variations of plasma NOx mirrored the variations in vasodilator reactivity. Following the WB diet, NO plasma concentrations increased in LZR and decreased in OZR. Endothelial NOS expression in the aorta was unaffected by the WB diet, while iNOS expression was significantly down-regulated in both OZR and LZR. This effect is mediated by the transcription factor nuclear factor kappa-B (Nf-kB), as it has been observed for flavonoids in vitro (Liang et al. 1999; Mollace et al. 2005). We have previously reported that WB consumption is able to attenuate the augmented pro-inflammatory environment that is characteristic of OZR. Eight weeks of WB consumption significantly reduced plasma concentrations of pro-inflammatory cytokines interleukin-6 (IL-6) and TNF-α as well as C-reactive protein, and significantly downregulated expression of Nf-kB, IL-6, and TNF-α both in the liver and the abdominal adipose tissue of OZR (Vendrame et al. 2013).

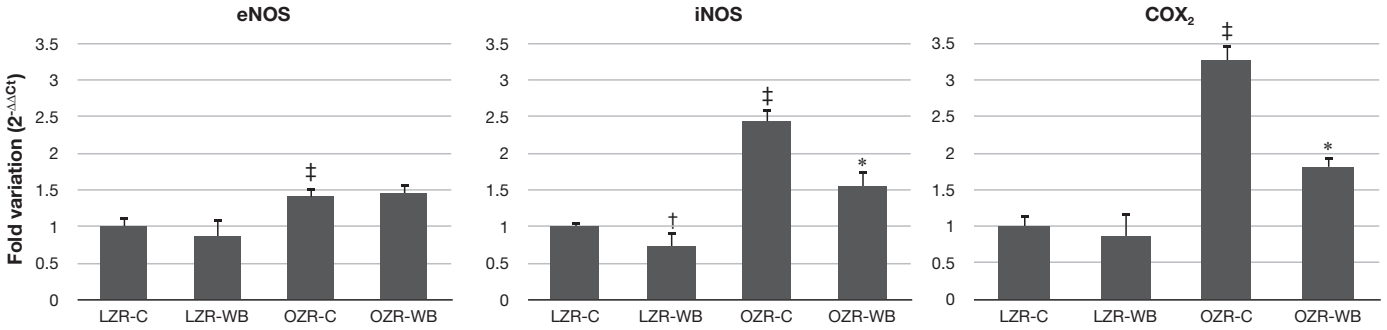
The involvement of the COX pathway is more difficult to elucidate. In our study, inducible COX-2 expression in the aortic tissue was more than 3-fold higher in OZR-C compared with LZR-C, similarly to what has been previously reported in OZR coronary arteries (Sánchez et al. 2010) and mesenteric arteries (Vessières et al. 2010) and consistent with the above-mentioned upregulation

Table 3. Concentrations of plasma nitric oxide and aortic-released prostanoids in lean (LZR) and obese (OZR) Zucker rats following wild blueberry (WB) or control (C) diets.

	LZR-C (n = 10)	LZR-WB (n = 10)	OZR-C (n = 10)	OZR-WB (n = 10)
Plasma NO (μmol·L^{-1})	17.58±0.57	20.52±0.75†	25.63±1.36‡	22.07±1.08*
6kPGF$_{1\alpha}$ (pg·mg^{-1} of aorta)	517.66±60.42	601.49±65.76	571.67±37.85	766.49±92.21*
TXB$_2$ (pg·mg^{-1} of aorta)	4.24±0.40	4.35±0.44	4.42±0.89	4.16±0.63

Note: 6kPGF$_{1\alpha}$, 6-keto-prostaglandin F$_{1\alpha}$; NO, nitric oxyde; TXB$_2$, thromboxane B$_2$.
*Significant effect of diet, OZR-C vs. OZR-WB ($p < 0.05$).
†Significant effect of diet, LZR-C vs. LZR-WB ($p < 0.05$).
‡Significant effect of model, LZR-C vs. OZR-C ($p < 0.05$).

Fig. 3. Relative gene expression (ddCt) of endothelial NOS (eNOS), inducible NOS (iNOS), and cyclooxygenase 2 (COX$_2$) in the aorta of lean (LZR) and obese Zucker rats (OZR), following wild blueberry (WB) or control (C) diet. Values are means ± SE, expressed as $2^{-\Delta\Delta Ct}$ normalized to β-actin and relative to LZR-C. *, Significant effect of diet, OZR-C vs. OZR-WB ($p < 0.05$); †, significant effect of diet, LZR-C vs. LZR-WB ($p < 0.05$); ‡, significant effect of model, LZR-C vs. OZR-C ($p < 0.05$).



of Nf-kB activity, which reflects the pro-inflammatory environment typical of OZR (De Artiñano and Castro 2009). However, concentrations of PGI$_2$ and TXA$_2$ in the aortic effluent were not significantly different between LZR and OZR.

Following WB consumption, COX-2 expression in the aorta was significantly downregulated in OZR, reflecting the anti-oxidant and anti-inflammatory effect of WB on Nf-kB activation. Interestingly, WB consumption did not have any effect on TXA$_2$ concentrations in the aortic effluent, and increased prostacyclin concentrations in OZR. The lack of changes in TXA$_2$ concentrations following WB could be attributed to the primary contribution of the constitutive COX$_1$ isoform to the basal production of this prostanoid in the arterial wall. The increase in PGI$_2$ in spite of COX$_2$ downregulation could be attributed to a shift in prostanoid production. The contribution of PGI$_2$ to endothelium-dependent relaxation is generally limited, while its effects on inhibition of platelet aggregation are more relevant, synergistically with NO (Vanhoutte 2003). The increased levels of PGI$_2$ following WB may therefore reflect a compensatory mechanism to preserve antithrombotic function, in response to the decreased NO (Mollace et al. 2005).

In conclusion, 8 weeks of WB consumption altered the aortic biomechanical function of the OZR by partially restoring the impaired Phe-induced constrictor responses, and attenuating the exaggerated response to Ach-induced vasorelaxation.

Our results suggest that both COX$_2$ and the NOS pathways account for the observed responses, but the contribution of NOS appears to be greater. We speculate that the main biological effect of WB consumption on endothelial function of OZR is related to its antioxidant and anti-inflammatory action, resulting in down-regulation of iNOS expression. This attenuates the chronic overproduction of NO, which causes overcompensated endothelial-mediated responses and generation of peroxynitrite radical under pro-oxidative and pro-inflammatory conditions.

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