Vascular Reactivity Is Affected by Dietary Consumption of Wild Blueberries in the Sprague-Dawley Rat

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ABSTRACT We have previously reported that consumption of blueberry-enriched (BB) diets attenuates the arterial contractile response to α1-adrenergic stimuli and affects vasomotor tone via endothelium-related pathways. The present study was designed to evaluate vascular function and responsiveness in aortas of weanling male Sprague-Dawley rats fed a control (C) or a BB diet for 7 weeks. Vascular ring studies were conducted in 3-mm isolated rat aortic ring preparations to investigate vasoconstriction induced by L-phenylephrine (Phe) (10^{-8}–3 × 10^{-6} M) and vasorelaxation induced by acetylcholine (ACh) (10^{-8}–3 × 10^{-6} M). Agonists were used alone and in the presence of nitric oxide (NO) synthase and cyclooxygenase (COX) inhibitors. We observed a significantly diminished vasoconstrictor response to Phe in aortic rings from rats fed the BB diet. Inhibition of NO synthase but not COX caused a significant increase in the constrictor response in both dietary groups, with the BB group having the greater response. Similarly, the participation of the NO pathway in endothelium-dependent vasorelaxation induced by ACh was greater in the rats fed a BB diet, while COX inhibition showed no effect on maximum ACh-induced vasorelaxation in any diet group. The vessel sensitivity of BB aortic rings to the vasoconstrictor and vasodilator was significantly reduced when compared to controls. We have concluded that diets enriched with blueberries, fed for 7 weeks in Sprague-Dawley rats, seem to affect NO metabolic pathways in the aorta at basal and stimulated levels.

KEY WORDS: • blueberries • endothelium • nitric oxide • vasoconstriction • vasorelaxation

INTRODUCTION

Epidemiological studies indicate that consumption of foods rich in bioactive components is associated with lower incidence of cardiovascular events such as myocardial infarction, stroke, and cardiac death.1–3 Blueberries (Vaccinium angustifolium) contain polyphenols and other antioxidants and have the highest antioxidant capacity among fruits and vegetables, which has been correlated with their anthocyanin and total phenolics content.4 Numerous studies have demonstrated the protective in vitro effect of bioactive compounds on vascular function. Experimental evidence has suggested that isolated polyphenols and flavonoids elicit endothelium-dependent vasorelaxation in experimental animals, as well as human subjects,5–12 and that this effect is mediated in part by the nitric oxide (NO)/cyclic GMP pathway.11,13–15 The antioxidant capacity of berry phenolics has been extensively studied in the past, supporting their vasoprotective effect.16–20 Plant pigment anthocyanin extracts appear to relax the vascular contractile machinery of porcine coronary arteries in vitro,21 while recently it has been reported that the dietary consumption of a synthesized anthocyanidin-derivative (HK-008) augments the acetylcholine (ACh)-induced endothelium-mediated vasorelaxation in the perfused rat mesenteric arterial bed.22 However, there have been only a few studies in the literature describing the role of polyphenolic compounds administered intragastrically10 or of dietary consumption of foods naturally rich in polyphenols and flavonoids (blueberries, grape juice, tea, cocoa, etc.)23–27 on the vascular contractile machinery.

Endothelium-released vasoactive mediators, which affect vascular functional properties may be either endothelium-derived relaxing factors such as NO, prostacyclin (prostaglandin [PG] I2), and endothelium-derived hyperpolarizing factor or endothelium-derived contracting factors, such as endothelin-1, thromboxane A2, PGH2, etc.28,29 Basal vascular tone is the net result of the balance between contractors and dilators.29,30 Shear stress and activation of cell membrane receptors stimulate the release of NO from the endothelium,31 which diffuses to the vascular smooth muscle and induces vasorelaxation via a cyclic GMP-activated pathway.32 Prostacyclin, on the other hand, a product of cyclooxygenase (COX), leads to vasorelaxation via a cyclic...
AMP-mediated pathway. The NO signaling pathway also interferes with endothelial cell–arachidonic acid pathways by stimulating the activity of COX enzymes and hence the release of 6-keto-PGF1α. This leads to the augmented production of COX metabolites and alters not only the production of vasoconstrictor or vasodilator prostanooids but also the antiplatelet and anti-inflammatory properties of NO. Alterations in the production and effects of these endothelial-derived factors lead to vascular dysfunction and disruption of vasomotor tone, which are observed in many cardiovascular disease states such as atherosclerosis, hypertension, heart failure, coronary heart disease, and stroke.

Despite the increasing interest in the effects of polyphenolic compounds on arterial functional properties as related to the cardiovascular system, there is some discrepancy in the mechanisms involved for their effect. This is attributed to the fact that most of the previous studies focused on the in vitro effect of different plant and fruit extracts or of isolated compounds on the vascular contractile machinery and compared it to the effect of vasodilator agonists. Our hypothesis was that blueberry (as a whole fruit) consumption alters the responses of the contractile machinery to vasoconstrictors and vasodilators in a manner that involves NO signaling. Therefore, the purpose of this study was to determine whether blueberry-enriched (BB) diets consumed by Sprague-Dawley rats affect changes in vasoconstrictor responses induced by L-phenylephrine (Phe) and/or (2) endothelium-dependent vasodilator responses induced by ACh in rat aortas, in the presence or absence of NO synthase (NOS) and COX inhibitors.

**MATERIALS AND METHODS**

**Animal model**

Weanling male Sprague-Dawley rats (Charles River Laboratories Inc., Wilmington, MA) were randomly assigned to a control AIN-93 (C) diet (n = 10) or a control diet in which wild blueberries were added (BB) (n = 10) for 7 weeks. All rats were individually housed in metal mesh-bottomed cages in an environmentally controlled room maintained at 22°C with a 12:12-hour light:dark cycle. Body weights were measured weekly. The Animal Care and Use Committee of the University of Maine approved all animal care and experimental procedures.

**Diets**

Diets were mixed in our laboratory from purified ingredients, as described before, and were composed of dextrose, egg white solids, vitamin mix, DL-methionine, biotin, mineral mix, and corn oil. Vitamin mix (A.O.A.C. Special Vitamin Mixture, Harlan Teklad, Indianapolis, IN) and mineral mix (MMP Biochemicals, Cleveland, OH) were commercially prepared. Whole wild blueberries were purchased as a composite of blueberries from Wyman’s (Cherryfield, ME), freeze-dried with standard procedures by Oregon Freeze Dry (Albany, OR), powdered, and incorporated into the diet at 8% (wt/wt), followed by an 8% decrease of carbohydrates (dextrose) in the BB diet. Diets were prepared fresh and were stored at 4°C for a maximum of 3–6 days following preparation. Tap water and food was provided for the animals ad libitum.

**Drugs and chemicals**

ACh chloride, Phe, L-NG-monomethyl-arginine (L-NMMA), mefenamic acid (MFA), and salts for the stock solutions of the physiologic salt solution (PSS) (NaCl, KCl, NaHCO₃, KH₂PO₄, MgSO₄, dextrose, and CaCl₂) were purchased in pure forms from Sigma-Aldrich Chemical Co. (St. Louis, MO).

**Tissue sampling**

At the end of each feeding period (7 weeks), food was withheld for 12–14 hours. Rats were anesthetized in a chamber with 95% CO₂/5% O₂ for <2 minutes, and they were exsanguinated by withdrawal of blood from the heart. Thoracic aortas were removed quickly and washed with PSS (composed of 118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 1.17 mM MgSO₄, 11 mM dextrose, and 1.25 mM CaCl₂).

**General preparation of rings**

After harvest, vessels were immediately placed in cold PSS, carefully cleaned of fat and adherent periadventitial tissue, and cut into four rings ~3 mm long. Rings were mounted on two triangular stainless steel wire specimen holders as previously described and transferred to 20-mL Radnotti tissue baths, filled with PSS aerated with 95% O₂/5% CO₂ (37°C, pH 7.4). Each ring was attached to a fixed glass hook in the tissue bath and through a weightless wire hook to a force transducer connected to a digital tissue force analyzer (model 410, MicroMed, Louisville, KY) for the measurement of isometric force. Changes in isometric force were recorded on a personal computer through the use of a system integrator software program (DMSI-410 version 1.01, MicroMed). Rings were placed on passive tension to yield a preload of 1.5 g and allowed to equilibrate at this tension for 45 minutes. During this period, tissues were washed with fresh aerated PSS twice, and the resting force on the rings was adjusted until the set preload of 1.5 g was maintained.

After equilibration, rings used to study receptor-mediated constriction or relaxation were exposed to a maximal dose of either agonist (Phe and ACh) for 10 minutes in order to alleviate nonspecific tissue binding. At the completion of this preconditioning dose, rings were washed for 25 minutes with fresh aerated PSS (minimum of four rinses). This was followed by a 5-minute equilibration period to allow the tension on the rings to return to baseline.

In some aortic preparations inhibitors were added in the tissue baths during the preconditioning period and remained...
in the tissue bath throughout the experiment. For each inhibitor ring there was a ring acting as “no inhibitor” control, which was otherwise treated identically to the ring with the “added inhibitor.” The ring treatments before the application of cumulative doses of Phe and ACh were the following: (1) one or two rings were washed with PSS without the addition of an inhibitor (no inhibitor, control ring), (2) one ring where t-NMMA (10^{-4} M) was added to inhibit NOS I, II, and III, and (3) one ring, where MFA (10^{-5} M) was added, as a COX 1 and 2 inhibitor. In some experiments we used one ring treatment with both t-NMMA (10^{-4} M) and MFA (10^{-5} M) to inhibit simultaneously NOS and COX. All inhibitors were incubated in the tissue baths in PSS for at least 25 minutes before the initiation of the agonist (Phe or ACh) dose–response curves.

**Phe-induced constrictor studies**

Each ring was contracted with cumulative applications of six concentrations of Phe (in threefold steps) over the range (10^{-8}–3 × 10^{-6} M) described before. A drug–tissue contact time of 6 minutes was allowed for each Phe concentration to achieve the maximum contraction. The presence of viable endothelium was assessed in all preparations by determining the ability of ACh (3 × 10^{-6} M) to induce more than 70% of relaxation of rings in the presence of Phe. After each agonist treatment the rings were washed four times over a 25-minute period with PSS (37°C, pH 7.4) to bring aortic tension down to or slightly below the original preload level; rings were allowed to equilibrate to baseline as described previously, and the dose–response curve was repeated.

**ACh-induced relaxation studies**

All four aortic ring preparations from each animal were precontracted with one maximal dose of the α1-adrenergic agonist Phe (10^{-6} M) for 10 minutes, which was the duration necessary for the contraction curve to reach a plateau and achieve submaximum contraction. Following the Phe precontraction, cumulative applications of six concentrations of ACh (10^{-8}–3 × 10^{-6} M) in threefold steps were applied, allowing a drug–tissue contact time of 6 minutes during which maximum vasorelaxation was achieved. Rings were then washed and allowed to equilibrate to baseline as described previously, and the dose–response curve was repeated. The maximum Phe-induced precontraction force was considered to be the point of 0% vasorelaxation (percentage of the level of precontraction), and the baseline point (preload, 0 g of force developed) was considered to be the 100% relaxation. The relaxant effect to each agonist dose was expressed as a percentage vasorelaxation of the initial Phe precontraction.

EC_{50} (the effective concentration of agonist in which 50% of maximum response was obtained) values were determined for each ring. The negative log (base 10) of the EC_{50} value was calculated. Concentration–response curves were fitted by nonlinear regression. The pD_{2} (–log_{10} EC_{50}) were calculated to evaluate the vessel sensitivity to the agonists (Phe and ACh).

**Statistics**

The relaxant effect to each ACh dose was expressed as a percentage vasorelaxation to the initial Phe precontraction. Concentration–response curves were fitted by nonlinear regression, and EC_{50} and pD_{2} (–log_{10} EC_{50}) were calculated, to evaluate the vessel sensitivity to the agonists. All results were expressed as mean ± standard error of the mean (SEM) values. Vasoocontriction and vasorelaxation in each dose were compared in different two-way analysis of variance tests using Student-Newman-Keuls comparisons in order to determine the effect of different diets and inhibitors on vasoreactivity. Vessel sensitivity to the agonists as described by pD_{2} values were compared in paired t tests. A value of P ≤ .05 was considered statistically significant. The statistical program used was the Sigmastat Statistical Program Package version 2.0 (SPSS Inc., Chicago, IL).

**RESULTS**

**Animal growth**

As previously described all animals fed a C or a BB diet gained weight, and the difference in growth rate was not statistically significant. The mean daily food intake was measured in previous experiments in which we observed...
Assessment of vasomotor function

**Phe-induced vasoconstriction.** The maximum contractile force developed in response to Phe was observed at concentrations of $10^{-6}$ M and higher (Figs. 1 and 2). Washout of agonist over a 25-minute period reduced contraction force at least to the preload value. The mean maximum force of contraction ($F_{\text{max}}$) developed in response to each concentration of Phe is presented in Figures 1 and 2. The contractile response to Phe at concentrations of $3 \times 10^{-7}$ M and higher was significantly less in BB than in C vessels ($P < .05$), including a significant decrease in maximal developed tension from 0.76 to 0.51 g with $3 \times 10^{-6}$ M Phe (SEM $= 0.033$, $P < .001$). Decreased sensitivity to Phe in BB (Table 1) compared to control aortas was defined by a shift in $EC_{50}$ (decrease in $pD_2$ value) (Fig. 1).

The Phe concentration–response curves in the presence of NO inhibition with L-NMMA show a significantly greater contraction in both dietary groups compared to the respective control curves. Differences were seen with Phe concentrations of $10^{-7}$ M and higher (Fig. 1). Statistical analysis further demonstrated that the contractile force developed in the BB group during NO blockade was greater than the C group at Phe concentrations of $3 \times 10^{-7}$–$3 \times 10^{-6}$ M (Fig. 1). These results demonstrate that a diet enriched in blueberries attenuates $\alpha_1$-adrenergic-induced vasoconstriction by promoting the NO signaling pathway during basal/nonstimulated conditions.

When the COX inhibitor MFA was present, we did not observe any significant differences in responses to Phe-induced vasoconstriction (Fig. 2). This suggests that BB diets do not reduce Phe-induced vasoconstriction by influencing the primary end product of the endothelial COX-mediated arachidonic acid metabolism. The vessel sensitivity to Phe was reduced in the BB group only in the absence of inhibitors (Table 1).

**ACh-induced vasorelaxation.** ACh-induced vasorelaxation was reduced in the BB-fed group when compared to the control group in doses $3 \times 10^{-9}$–$3 \times 10^{-7}$ M ($P < .05$) (Figs. 3 and 4). However, relaxation at ACh concentrations $>3 \times 10^{-7}$ M and maximum percentage of vasorelaxation were not significantly different between groups. These results show a signaling effect of the BB diet without an effect on relaxation capacity. Decreased sensitivity to ACh in BB compared to control aortas was defined by a shift in $EC_{50}$ (decrease in $pD_2$ value) (Table 1).

Addition of L-NMMA reduced the relaxation response to ACh in both dietary groups (Fig. 3). However, the L-NMMA effect was significantly greater in BB-fed animals when compared to the respective concentration–response curves without L-NMMA ($-41\%$ vs. $-27\%$) ($P = .045$). In addition to the significantly different effect of L-NMMA on maximal relaxation within a dietary group, the NO blocker had

![FIG. 2. Contractile responses of thoracic aortic rings from control (C) (triangles) and blueberry-fed (BB) (circles) animals before (black lines) and after COX (gray lines). The graphs represent the developed tension in response to Phe ($n = 10$ rats per group) in the absence of an inhibitor and in the presence and absence of MFA ($10^{-5}$ M) in aortic rings of rats fed a C or a BB diet. When the COX inhibitor MFA was present, we did not observe any significant differences in responses to Phe-induced vasoconstriction between the two diet groups.](image)

### TABLE 1. VESSEL SENSITIVITY ($pD_2 = -\log_{10} EC_{50}$) TO PHE AND ACH BEFORE AND AFTER ADDITION OF INHIBITORS OF NOS (L-NMMA) AND COX (MFA)

<table>
<thead>
<tr>
<th>Diet group</th>
<th>$pD_2$ to Phe</th>
<th>$pD_2$ to ACh</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PSS</td>
<td>LNMMMA</td>
</tr>
<tr>
<td>Control</td>
<td>6.603 ± 0.066</td>
<td>6.908 ± 0.043</td>
</tr>
<tr>
<td>BB</td>
<td>6.504 ± 0.027*</td>
<td>6.893 ± 0.044</td>
</tr>
</tbody>
</table>

Vessel sensitivity to Phe was reduced in the BB-fed group only in the absence of inhibitors. Dietary enrichment in blueberries reduced the vessel sensitivity to ACh both in the presence and absence of inhibitors. Data are mean ± SEM values.

Comparisons were conducted among diet groups in the same treatment (in each column), and statistically significant differences from the control (C) group are indicated: *$P < .05$. 
A greater inhibitory effect on the BB concentration–response curve at ACh concentrations ≥3 × 10⁻⁷ M (Fig. 3). These results demonstrate that a diet enriched in blueberries alters the NO-mediated component of ACh-induced vasorelaxation.

When the COX inhibitor MFA was present, we did not observe any significant differences in ACh-induced vasorelaxation (concentrations >3 × 10⁻⁸ M) (Fig. 4). In the presence of MFA and in lower ACh doses (10⁻⁸–10⁻⁷ M), we observed an equal magnitude enhancement in vasorelaxation in rings from both control and BB-fed animals (Fig. 4). These results suggest that blockade of COX metabolites reduces the level of a constituent vasoconstrictor but that the BB diet does not alter the concentration of that vasoconstrictor. The vessel sensitivity to ACh was significantly reduced in the BB group also in the presence of both inhibitors (Table 1).

**DISCUSSION**

Our present study, using a BB diet for a short duration (7 weeks), confirmed our hypothesis that dietary blueberries suppress the α₁-adrenergic agonist-induced vasoconstriction via an NO-mediated pathway. Blockade of basal levels of NO, but not COX metabolites, eliminates the depressed vasoconstrictor response to Phe seen in the BB-fed group (Figs. 1 and 2). Our conclusion is supported by our observations when we studied NO signaling under stimulated conditions. ACh-induced vasorelaxation was enhanced in BB-fed animals in comparison to controls, even though the differences in the maximum vasorelaxation (>10⁻⁶ M) did not appear to be significantly different (Figs. 3 and 4). L-NMMA but not MFA attenuated the relaxation response to ACh in both dietary groups, but the NOS blockade had a significantly greater effect in the BB group (Figs. 3 and 4).

One possible mechanism for the augmented NO signaling in the BB group is the antioxidant properties of blueberries.16–20 It has previously been reported that reactive oxygen species are involved in the mechanism of norepinephrine-induced contractions of rat aorta, since reactive oxygen species scavengers were shown to decrease the contractile responses to the agonist.47 Even though Ignarro et al.,48 when treating rat aortic vascular smooth muscle cells in vitro with blueberry juice, suggested that its antioxidant activity protects and maintains the functional levels of NO within the cell culture, this may not be the case with our control group, which is free of risk factors for developing vascular disease, and there is no known evidence for increased reactive oxygen species presence under these normal physiological conditions. Apparently for the same reasons, our control group has greater baseline ACh-induced vasorelaxation than that observed in aortic preparations from diseased animals models used as controls in similar experiments.8,49 These factors may explain the lack of an effect of the blueberry diet on maximum vasorelaxation in response to ACh in our BB group, which is not known to be under an oxidative stress condition. In our study we focused...
on how the BB diet alters the vasodilatory action of ACh
and the vasoconstrictor action of Phe. Our data in our ex vivo experimental design agree with the existing literature supporting the proposal that a BB diet preserves NO bioavailability in the vasculature under basal and stimulated levels.

It has been well established that there is a synergistic action of NOS-derived NO and COX-derived vasodilator PGs such as prostacyclin, with endothelium-derived NO being the major contributor of the vasodilator response to ACh in rat aorta. In conditions of acute withdrawal of NO, COX-derived vasodilator prostanooids may to some extent compensate for the reduced NO levels, and the enhancement of prostacyclin production by the constitutive isoform of COX may modulate agonist-induced vasorelaxation in certain vascular beds. Reduction in the production of vasodilator prostanooids in the face of decreased NO availability may further attenuate the vasodilator response to ACh. However, in our study, inhibition of COX with the nonselective COX inhibitor MFA did not modify the maximum relaxation in response to ACh in either treatment group, which might explain the enhanced vasorelaxation in ACh concentrations \(<10^{-7} M\). In our vasoconstriction experiments there was a trend toward a decrease of vasoconstriction to Phe in the BB group in the presence of MFA, which did not reach statistical significance. Under stimulated conditions, such as in ACh-induced vasorelaxation, we did not observe any differences in vasorelaxation in any group when compared to the baseline maximum vasorelaxation in the absence of inhibitors. However, a minor participation of vasodilator prostanooids cannot be excluded under basal conditions. The inability of the COX inhibitor to affect significantly vasoconstriction to Phe or vasorelaxation to ACh in our experiments was expected, because of the absence of a physiological condition that would justify elevated COX (1 or 2) baseline levels in our animal groups. In the presence of COX inhibitors, the response to ACh remains unaltered in healthy animals and humans, suggesting that products of COX do not play a major role in endothelium-dependent regulation of vascular tone under normal physiological conditions. The cellular effect of the BB diet could potentially influence vascular tone in vivo in a multifaceted manner, not only by improving NO signaling but also by affecting the vessel sensitivity to agonists. Similar maximal responses to ACh suggest the involvement of competitive inhibitory actions at endothelial muscarinic receptor sites because increased levels of agonist did overcome the BB effect. Herrera et al. have reported that the structure of individual flavonoids (such as the position of hydroxyl groups or the phenyl ring) affects the activity of vasoactive agonists at different concentration levels. Our previous studies established a definite link between a diet rich in blueberries and structural alterations in aortic tissue glycosaminoglycans in Sprague-Dawley rats. Glycosaminoglycans are important structural components of the arterial wall with great structural and functional diversity, implicated in the organization of the extracellular matrix and regulation of numerous vascular functions, which have been reported to be modified by procyanidolic oligomers and apigenin. Available data suggest a role of undersulfated glycosaminoglycans in reducing the frequency of ACh receptor clustering by diminishing the agrin signal transduction pathway, which might explain our observations of reduced vessel sensitivity to muscarinic and adrenergic receptors due to alterations of the glycosaminoglycan structure and sulfation pattern of the BB-fed rat aortas.

The main purpose of the current study was to assess the ex vivo effect of a diet enriched in blueberries, consumed as a whole fruit, on the vasomotor tone of isolated arteries. With our present study we confirm the conclusions of previous studies conducted in vitro (using blueberry extract or its components, directly into tissue baths or in cell culture) by conducting (1) an animal feeding study and (2) an ex vivo method to study vascular functional properties. Data on the effects of a BB diet on vascular cell signaling and homeostasis are necessary in future studies.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES
ALTERED VASCULAR TONE OF BLUEBERRY-FED RATS


