Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 14–22
Journal of Nutritional Biochemistry

RESEARCH ARTICLES

Dietary enrichment with wild blueberries (*Vaccinium angustifolium*) affects the vascular reactivity in the aorta of young spontaneously hypertensive rats☆

Anastasia Z. Kalea[a], Kateryna Clark[a], Dale A. Schuschke[b], Aleksandra S. Kristo[a], Dorothy J. Klimis-Zacas[a,*]

[a]*Department of Food Science and Human Nutrition, University of Maine, Orono, ME 04469, USA*
[b]*Department of Physiology and Biophysics, School of Medicine, University of Louisville, Louisville, KY 40292, USA*

Received 10 June 2008; received in revised form 22 September 2008; accepted 23 September 2008

Abstract

We have previously reported on the positive effects of wild blueberries on arterial contractile response to α_1 adrenergic stimuli and on endothelium-mediated vasorelaxation. Our present study was designed to evaluate the effects of the dietary enrichment with wild blueberries on aortic function and reactivity in the developmental phase of essential hypertension in spontaneously hypertensive rats (SHR). We investigated the possible influence blueberries may have on the acetylcholine (Ach)-induced endothelium-dependent vasorelaxation and phenylephrine-induced vasoconstriction in young SHRs, as well as the contribution of the nitric oxide (NO) synthase and cyclooxygenase (COX) pathways in each of the above responses in an animal model with dysfunctional endothelium. Vascular ring studies were conducted in 3-mm isolated rat aortic ring preparations to investigate vasoconstriction induced by L-Phenylephrine (Phe, 10^{-8} to 3×10^{-6}M) and vasorelaxation induced by acetylcholine (Ach, 10^{-9} to 3×10^{-6}M). The major findings of our study were that in Phe-induced vasoconstriction, SHR-BB aortas relaxed to a greater degree in comparison to controls when mefenamic acid (MFA) was present and that the incubation with this COX inhibitor failed to restore — and in fact decreased — the maximum vasodilator response to Ach, in comparison to controls. Our vessel reactivity index (pD_2) observations indicate that blueberries appear to modulate cell membrane–agonist (Ach) interactions primarily in response to Ach in the young SHR model, but not to the α_1 adrenoreceptor agonist. Incorporating wild blueberries in the diet seems to affect the endothelium-dependent vasorelaxation by modulating alternative metabolic pathway(s) (such as affecting the production/activity of COX-derived products) in the young SHR aorta.

Keywords: Hypertension; Vasoconstriction; Vasorelaxation; Blueberries

1. Introduction

Epidemiological studies have demonstrated a negative correlation between dietary intake of fruits and vegetables and vascular disease [1–3]. Aside from their vitamin and mineral content, fruits and vegetables are a rich source of chemical substances collectively referred to as phytochemicals, some of which have been shown to possess potent bioactive properties including several cardioprotective effects. Low-bush blueberries (*Vaccinium angustifolium*) have exhibited one of the highest recorded *in vitro* antioxidant capacities of vegetables and fruits tested to date. This high antioxidant capacity has been correlated with their anthocyanin and total

Abbreviations: Ach, acetylcholine; BB, blueberries; C, control; COX, cyclooxygenase; L-NMMA, L-N^G-monomethyl-arginine; MFA, mefenamic acid; NO, nitric oxide; NOS, nitric oxide synthase; Phe, L-Phenylephrine; SHR, spontaneously hypertensive rats.

☆ This work was supported in part by a grant from the Wild Blueberry Commission of Maine, the Wild blueberry Association of North America, the USDA/CSREES, and the Regular Research Faculty Fund of the University of Maine to Dr. Dorothy Klimis-Zacas (Maine Agriculture and Forestry Experiment Station, Scientific Contribution, #2980).

* Corresponding author. Department of Food Science and Human Nutrition, College of Natural Sciences, Forestry and Agriculture, University of Maine, Orono, ME 04469, USA. Tel.: +1 207 581 3124; fax: +1 207 581 1636.

E-mail address: dorothy.klimis.zacas@umit.maine.edu (D.J. Klimis-Zacas).

0955-2863/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.09.005

phenolic content [4]. There is substantial experimental evidence that some flavonoid-rich foods and isolated flavonoids positively affect endothelium-dependent vasorelaxation in animal models as well as humans, thus contributing to maintenance of the overall vascular homeostasis [5–11]. This effect is mediated, at least in part, by the nitric oxide (NO)/cGMP pathway [7,8,11]. Previous studies conducted in our laboratory demonstrated positive effects of wild blueberries on endothelial function. In a study conducted in young male normotensive Sprague-Dawley (SD) rats, wild blueberries incorporated into the diet affected the vascular smooth muscle contractile machinery by suppressing the α_1 adrenergic receptor–agonist-mediated contraction through an endothelium-dependent pathway [12]. In another study, wild blueberry consumption altered the structure of the extracellular matrix of male SD rat aortas, by increasing the concentration of glycosaminoglycans and decreasing the sulfation of all glycosaminoglycan-type molecules, suggesting a possible effect of wild blueberries on endothelial and vascular smooth muscle signaling pathways [13].

Accumulating scientific data suggests that the endothelium plays a central role in the regulation of vascular homeostasis via the secretion of vasoactive factors [14], defined as endothelium-derived relaxing factors such as NO prostacyclin (PGI_2); endothelium-derived hyperpolarizing factor; and endothelium-derived contracting factors such as endothelin-1, thromboxane A_2 and prostaglandin H_2. Endothelial dysfunction is characterized by a shift of endothelial actions toward reduced vasorelaxation and enhanced vasoconstriction. To date, the two major mechanisms responsible for impaired endothelial function have been identified as (1) alteration in the NO pathway (decreased production and/or availability of NO and (2) cyclooxygenase (COX)-dependent overproduction of endothelium-derived contracting factors, including vasoconstrictor prostanoids and reactive oxygen species (ROS) [15–17].

Spontaneously hypertensive rats (SHR) are extensively used as an animal model of endothelial dysfunction in various experimental designs, including pharmacological and dietary studies. In the SHR, endothelial dysfunction is attributed to the concomitant reduction in the availability of endothelium-derived vasodilator factors and increased production of vasoconstrictors [18,19]. Mounting evidence suggests that there is exaggerated production of superoxide anion and subsequent increase in oxidative stress in the vascular wall of the SHR that may cause endothelial dysfunction in this model of essential hypertension [20–24].

Numerous studies have demonstrated marked improvement in endothelial function in SHRs treated with antioxidant-rich dietary ingredients [9,25]. However, the above studies used primarily aged animals or salt-sensitive strains (stroke-prone SHRs) on a high-salt (8%) diet, where essential hypertension was already established. In our study, we hypothesized that dietary wild blueberry enrichment affects the vasomotor tone in the developmental phase of essential hypertension in the aorta of SHRs. Therefore, we investigated the possible influence wild blueberries may have on the acetylcholine (Ach)-induced endothelium-dependent vasorelaxation and phenylephrine (Phe)-induced vasoconstriction in the aorta of young SHRs, as well as the possible mechanism(s) by which blueberries may exert their action(s) on endothelial function in an animal model with dysfunctional endothelium.

2. Materials and methods

2.1. Experimental animals

Weanling male SHR (Charles River Laboratories) were randomly assigned to a control diet (SHR-C) (modified AIN-76) [12] or a diet in which freeze-dried wild blueberry powder was added (SHR-BB) 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-h 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.

2.2. Diets

Diets were mixed in our laboratory from purified ingredients, as described before [13], and were composed of dextrose, egg white solids, vitamin mix, DL-Methionine, biotin, mineral mix and corn oil. Vitamin (AOAC Special Vitamin Mixture, Harlan Teklad) and mineral mix (MMP Biochemicals, Cleveland, OH, USA) were commercially prepared. Wild blueberries were purchased from Wyman's (Cherryfield, ME, USA) as a blueberry composite; they were then freeze-dried with standard procedures by Oregon Freeze Dry (Albany, OR, USA), powdered and incorporated into the diet at 8% (w/w). Diets were prepared fresh and were stored at 4°C for a maximum of 3 to 6 days following preparation. Animals had free access to tap water and food.

2.3. Drugs and chemicals

Acetylcholine chloride, L-Phenylephrine, L-N^G-monomethyl-arginine (L-NMMA), mefenamic acid (MFA) and salts for the stock solutions of the physiologic salt solution (PSS: NaCl, KCl, $NaHCO_3$, KH_2PO_4, $MgSO_4$, dextrose, $CaCl_2$) were purchased in pure forms from Sigma-Aldrich Chemical Co.

2.4. Experimental design

At the end of each feeding period (7 weeks) and after a 12- to 14-h fasting, the rats were anesthetized in a chamber with 95% CO_2/5% O_2, for a maximum of 2 min. Anesthesia was confirmed with loss of toe pinch reflex. Thoracic aortas and livers were removed and washed PSS (with composition in millimolar: NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; KH_2PO_4, 1.18; $MgSO_4$, 1.17; dextrose, 11; $CaCl_2$, 1.25).

Two separate series of experimental animals were used in this study, and each series contained the control diet

group (SHR-C, n=10) and the blueberry-enriched diet group (SHR-BB, n=10) each time. In the first series of experiments, we examined the vasoconstrictor responses of the thoracic aortic ring preparations and in the second series we examined the effect of vasorelaxation. In each of the two series, we examined responses in the presence and absence of agonist inhibitors. For each inhibitor ring, there was a ring acting as "no inhibitor" control which was otherwise treated identically to the rings where inhibitors were added. The ring treatments before the application of cumulative doses of Phe (vasoconstrictor) and Ach (vasodilator) were the following: (a) one or two rings were washed with PSS without the addition of an inhibitor (no inhibitor, control ring); (b) one ring where L-NMMA (L-N^G-monomethyl-arginine, 10^{-4} M) was added to inhibit NOS I, II and III; and (c) one ring, where MFA (mefenamic acid; 10^{-5} M) was added, as a COX I and II inhibitor. All inhibitors were incubated in the tissue baths in PSS for at least 25 min before the initiation of the agonist (Phe or Ach) dose–response curves.

2.4.1. Aortic ring preparation

After harvest, vessels were immediately placed in cold PSS, carefully cleaned of fat and adherent periadventitial tissue and cut into rings of ~3 mm length. Rings were mounted on two triangular stainless-steel wire specimen holders as previously described [12,26,27] and transferred to 20-ml Radnoti tissue baths, filled with PSS and aerated with 95% O_2/5% CO_2 (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) for the measurement of isometric force, and which were recorded by an integrator software program (DMSI-410 version 1.01, Micro-Med, Kentucky, USA). Rings were placed on passive tension to yield a preload of 1.5 g and were allowed to equilibrate at this tension for 45 min. 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 that were used to study receptor-mediated constriction or relaxation were incubated with a maximal dose of either agonist (Phe and Ach) for 10 min in order to alleviate nonspecific tissue binding. At the completion of this preconditioning dose, rings were washed four times and allowed to equilibrate for 25 min with fresh aerated PSS (minimum of four rinses). At this point, the relevant inhibitors were added in some of the rings (depending on the protocol designed for each study) and remained in the tissue bath until the end of the protocol; in one of the four rings of each animal no inhibitors were added and it was used as a control.

2.4.2. Phenylephrine-induced contractile responsiveness

All four rings from each animal were exposed to six cumulative concentrations of Phe (in threefold steps) over the range 10^{-8} to 3×10^{-6} M, as it has been described before [26,28]. A drug–tissue contact time of 6 min was allowed for each Phe concentration to achieve the maximum contraction. Next, all rings were relaxed with one dose of Ach (3×10^{-6} M) for 10 min to demonstrate Ach-induced, endothelium-dependent relaxation in order to verify endothelial viability. At the end of each protocol, all rings were washed four times over 25 min with PSS (37°C, pH 7.4) to bring again aortic tension down to or slightly below the original preload level; rings were allowed to equilibrate to baseline as described previously [27,28] and the dose–response curve was repeated.

2.4.3. Acetylcholine-induced relaxation responses

In preliminary experiments, the dose–response relations to Phe were determined and the dose of Phe causing a 60–70% of maximal contractile response was estimated to be 10^{-6} M. This was used as a precontraction dose for our Ach cumulative dose–response curves. All rings were precontracted with this maximal dose of the α_1 adrenergic agonist Phe (10^{-6} M) for 10 min, which was the duration necessary for the contraction curve to reach a plateau and achieve submaximum contraction. Following the Phe precontraction, cumulative applications of eight concentrations of Ach (10^{-9} to 3×10^{-6} M) in threefold steps were applied allowing a drug–tissue contact time of 6 min during which maximum vasorelaxation was achieved. Rings were then washed and allowed to equilibrate to baseline as previously described and the dose–response curve was repeated.

2.5. Data analysis

In the vasoconstriction experiments, the maximum force of contraction (Fmax in g) with exposure to each Phe dose in the presence or absence of different inhibitors was determined for each ring from computer stored digitized raw data. In the vasorelaxation experiments, the Fmax for Phe (precontraction) and the maximal relaxation to Ach in each ring were used to calculate Ach relaxation as a percentage of the initial precontraction. In both experimental series and in intact rings without any inhibitor present, presence of viable endothelium was accepted at Ach-induced endothelium-dependent relaxation >70%. EC_{50} values were determined for each ring (the median effective concentration of agonist). Concentration–response curves were fitted by nonlinear regression. The pD_2 ($-\log_{10}$ EC_{50}) values were calculated as an estimate of vessel sensitivity to the agonists (Phe and Ach).

2.6. Statistical analysis

Animal body weights were compared using a one-way analysis of variance (ANOVA). Vasoconstriction and vasorelaxation in each dose were compared in different two-way ANOVA tests using Student–Newman–Keuls comparisons in order to determine the effect of different diets on vasoreactivity. The maximum force developed in each Phe dose and the relaxant effect to each Ach dose expressed as a percentage of vasorelaxation to the initial Phe

precontraction were compared. Individual ring values within each agonist (i.e., Phe), each treatment (i.e., MFA or L-NMMA) and each rat were averaged and used to calculate group means. Concentration–response curves were fitted by nonlinear regression, and EC_{50} and pD_2 ($-\log_{10} EC_{50}$) were estimated, to evaluate the vessel sensitivity to the agonists. All results were expressed as mean values±S.E.M.; *n* represents the number of rats in each experimental group in each protocol. Vessel sensitivity to the agonists as described by pD_2 values was compared in paired *t* tests. A *P*-value level of .05 or less was considered statistically significant. The statistical program used was the Sigmastat Statistical Program Package version 2.0 (SPSS Inc.).

3. Results

3.1. Animal growth

All animals gained weight during the diet study. No significant differences were detected in mean body weight between diet groups (SHR-C: 253±16.2 g *vs.* SHR-B: 237±27.7 g, *P*=.139). Mean daily food intake was measured in previous experiments in which dietary blueberries were added to a modified AIN-76 diet and no significant differences in daily food intake were observed [29].

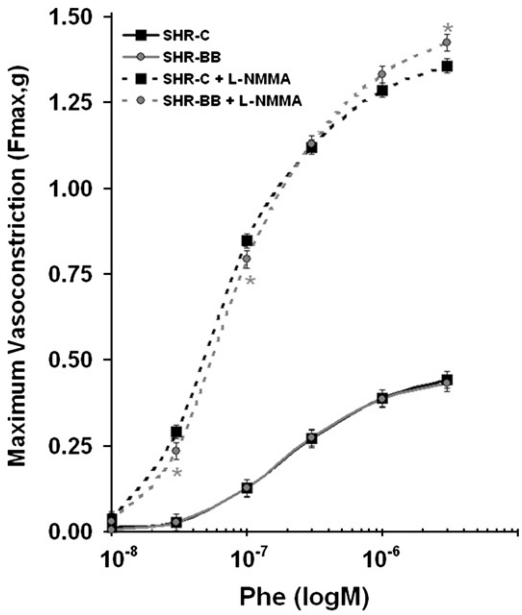

Fig. 1. Contractile responses of thoracic aortic rings from SHRs fed a control (SHR-C, black squares) and blueberry-enriched (SHR-BB, gray circles) diet, before (continuous lines) and after NOS inhibition (dashed lines). The graphs represent the developed tension to L-Phenylephrine [Phe (g), *n*=10 rats per group] in the absence of an inhibitor and in the presence of L-NMMA (10^{-4} M) in the aortic rings of SHRs fed a control or a BB diet. In the presence of Phe, the addition of L-NMMA in SHR-BB rings induced a lower vasoconstriction at Phe doses $\leq 10^{-7}$ M and a greater maximum vasoconstriction when compared to the control group. Phe-induced vasoconstriction when NOS was inhibited was more pronounced in aortas from BB-fed animals (*P<.001).

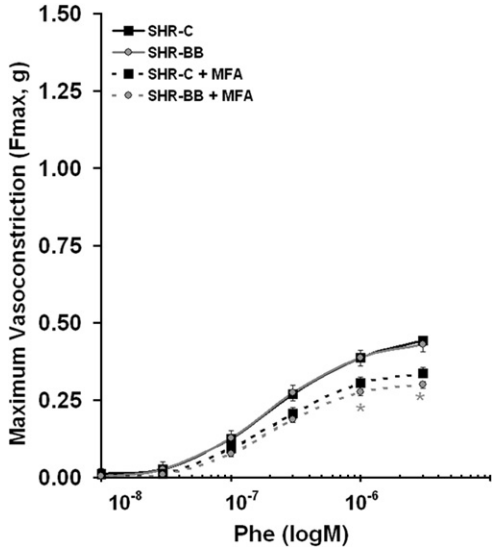

Fig. 2. Contractile responses of thoracic aortic rings from SHRs fed a control (SHR-C, black squares) and blueberry-enriched (SHR-BB, gray circles) diet, before (continuous lines) and after COX inhibition (dashed lines). The graphs represent the developed tension to L-Phenylephrine [Phe, *n*=10 rats per group] in the presence and absence of MFA (10^{-5} M) in the aortic rings of SHRs fed a control or BB diet. When the COX inhibitor MFA was present, we observed a lower Phe-induced vasoconstriction in SHR-BB compared to controls (*P<.001).

3.2. Endothelium-dependent vasoconstriction

The constrictor responses of SHR aortic rings in response to Phe were comparable between SHR-C and SHR-BB diet groups (0.442 *vs.* 0.432 g, S.E.M.=0.018) (Fig. 1 and 2). In isolated aortic rings where aortas were pretreated with a NOS inhibitor (L-NMMA) to inhibit basal (nonstimulated) NO synthesis, the maximal tension to Phe was increased as expected in both groups, but the increase was comparable between the two diet groups (increase of maximum force of

Table 1
Vessel sensitivity (pD_2) of SHR-C and SHR-BB aortic rings to Phe (A) and Ach (B) in the presence and absence of NOS (L-NMMA) and COX (MFA) inhibitors

(A)			
Diet groups	pD_2		
	Phe	Phe+L-NMMA	Phe+MFA
SHR	6.679±0.012	7.162±0.019	6.678±0.006
SHR-BB	6.698±0.012	7.058±0.019*	6.648±0.006*
(B)			
Diet groups	pD_2		
	Ach	Ach+L-NMMA	Ach+MFA
SHR	8.25±0.01	6.79±0.04	8.69±0.05
SHR-BB	8.34±0.01*	7.05±0.04*	8.49±0.05*

[a]Values are expressed as means±S.E.M.

* Statistically significant differences at $P \leq .05$ compared to SHR-C (control) group.

contraction in SHR-C: 0.92 g *vs.* SHR-BB: 0.99 g) (Fig. 1). The constrictor response was lower in the SHR-BB group in lower Phe concentrations ($10^{-8} \leq Phe \leq 10^{-7}$ M), while the highest dose of Phe yielded a significantly greater constrictor response in the SHR-BB group (Fig. 1). Similarly, in aortas isolated from blueberry-fed SHRs, the concentration–response curve to Phe after pretreatment with the non-selective COX inhibitor (MFA) showed a decreased constriction in Phe doses of 10^{-7} M and higher (Fig. 2). Greater constriction in the presence of NO blockade and less constriction in the presence of COX blockade suggests that the blueberry diet promotes both basal NO-mediated dilation and COX-mediated constriction, in response to Phe. Vessel sensitivity to the α_1 adrenergic agonist Phe did not appear to be influenced by the presence of blueberries in the SHR (Table 1A). However, when inhibitors were added, we observed, as expected, a significant decrease in the vessel sensitivity to the agonist in SHR-BB aortas compared to controls in both NOS and COX inhibitors (Table 1A) (L-NMMA: 7.162 *vs.* 7.058, S.E.M.=0.019 and MFA: 6.678 *vs.* 6.648, S.E.M.=0.006, respectively).

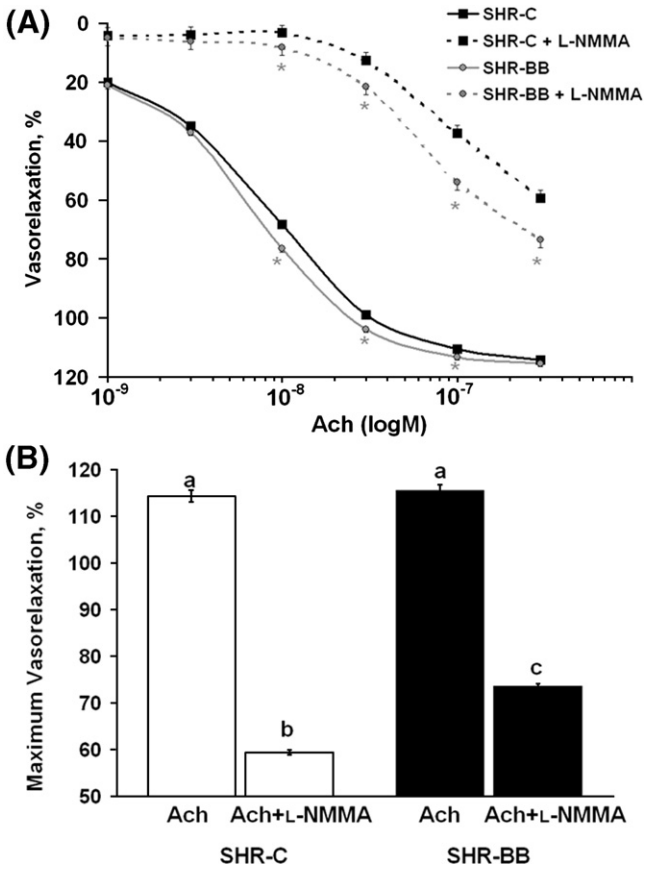

Fig. 3. Acetylcholine (Ach) responses of thoracic aortic rings from SHRs fed a control (SHR-C, black squares) and blueberry-enriched (SHR-BB, gray circles) diet after precontraction with L-Phenylephrine (Phe, 10^{-6} M), before (continuous lines) and after NOS inhibition (dashed lines). (A) The graphs represent relative relaxation to Ach (Ach, n=10 rats per group) in the absence and in the presence of L-NMMA (10^{-4} M) in aortic rings of SHRs fed a control or BB diet. Ach-induced vasorelaxation was significantly increased in SHR-BB animals for doses $10^{-8} \leq Ach \leq 10^{-7}$ M, without affecting the maximum vasorelaxation force to Ach (*P<.001). (B) Blockade of NOS decreased maximum vasorelaxation in both diet groups, with the maximum vasorelaxation in SHR-BB aortas to be greater in the presence of a NOS inhibitor. Significant differences are noted with different letters ($^{a,b,c}P$<.05).

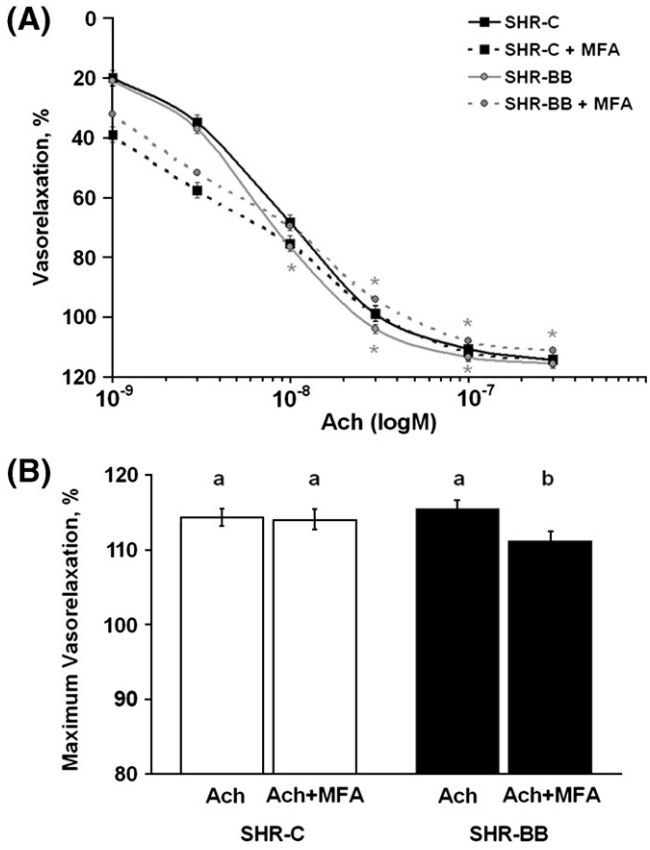

Fig. 4. Acetylcholine (Ach) responses of thoracic aortic rings from SHRs fed a control (SHR-C, black squares) and a blueberry-enriched (SHR-BB, gray circles) diet after precontraction with L-Phenylephrine (Phe, 10^{-6} M), before (continuous lines) and after COX inhibition (dashed lines). (A) The graphs represent relative relaxation to Ach (Ach, n=10 rats per group) in the absence and in the presence of MFA (10^{-5} M) in aortic rings of SHRs fed a control or BB diet. In the absence of MFA, we did not observe any significant differences in Ach-induced vasorelaxation, while when MFA was present, there was a reduced vasorelaxation (4%) of the SHR-BB rings in response to Ach, when compared to controls (*P<.01). (B) Blockade of COX significantly decreased the maximum vasorelaxation in SHR-BB group. Significant differences are noted with different letters ($^{a,b}P$<.05).

3.3. Endothelium-dependent vasorelaxation

Fig. 3 depicts the cumulative dose–response curves to Ach, an endothelium-dependent vasodilator, in aortas obtained from SHR animals fed a control or a blueberry-enriched diet. The maximal relaxation in response to Ach was significantly increased in SHR-BB animals for doses $10^{-8} \leq Ach \leq 10^{-7}$ M (Fig. 3A). This increase in relaxation agrees with the significant increase in vessel sensitivity to Ach in the SHR-BB group as described by the pD_2 values (Table 1B). The maximum vasorelaxation force to the highest concentration of Ach in response to

Ach was not significantly different between our diet groups, suggesting that the BB diet does not alter dilator capacity (Fig. 3A and B). The presence of the NOS inhibitor (L-NMMA) significantly decreased maximum vasorelaxation in both diet groups but resulted in a differential effect in the two diet groups (48% *vs.* 34% inhibition of vasorelaxation, in SHR-C and SHR-BB, respectively) (Fig. 3B). The L-NMMA pretreatment decreased the sensitivity to Ach in both groups but did not eliminate the greater sensitivity of the SHR-BB group relative to controls (Table 1B). The addition of MFA in the tissue baths (Fig. 4A and B) affected only the SHR-BB aortas leading to a small but significant inhibition of vasorelaxation (4%) (Fig. 4B). Therefore, in the presence of MFA, we observed a decreased vasorelaxation of SHR-BB aortas in comparison to the controls. This attenuated dilation in the SHR-BB group correlates with a significant decrease in the sensitivity to Ach in the same group (Table 1B).

4. Discussion

In the present study, we investigated the *in vivo* effect of wild blueberries on the vascular biomechanical properties using aortic ring preparations from young SHRs. Previous studies conducted in our laboratory reported that wild blueberry-enriched diets affect the contractile machinery of normotensive (Sprague-Dawley) rats through an endothelium-mediated pathway [12]. Our data suggest that the vasoconstrictor pathway in response to Phe does not seem to be the primary pathway affected by our blueberry diet in the SHR, even though there are small but significant differences in vasoconstriction when NOS and COX production are inhibited. We observed that L-NMMA, a NO synthase inhibitor, significantly increased the basal tone in both diet groups and potentiated the contractile responses to Phe; this effect being more pronounced in SHR-BB aortas at a Phe dose of 3×10^{-6} M. The above findings indicate the existence of a basal (not stimulated) NO release, which negatively modulates the Phe-induced vasoconstriction in both diet groups. While incubation with a COX inhibitor failed to restore — and in fact decreased — the vasodilator response to Ach of aortas from blueberry-fed SHR, in Phe-induced vasoconstriction, the suppression of vasoconstriction in SHR-BB aortas was greater, in comparison to SHR-C, when MFA was present. Even though there is functional evidence of α-adrenergic receptors in the vasculature of young SHRs, their effect is limited compared to aged SHRs [30,31]. In the current study, we support that there is no significant effect of our blueberry-enriched diet on the vessel sensitivity to the adrenoreceptor, unless NOS and COX enzyme synthesis is inhibited.

On the contrary, wild blueberries incorporated at 8% in the diet and fed for 7 weeks significantly increased the induced Ach-mediated vascular smooth muscle relaxation (Fig. 3A and 4A). This vasorelaxation response was not completely abolished in the presence of L-NMMA (SHR-C 48% *vs.* SHR-BB 34% inhibition) (Fig. 3B), suggesting the concomitant augmentation of alternative metabolic pathway (s) in SHR (such as COX pathway), which seem to be affected by our dietary treatment. This conclusion is supported by our finding that maximum vasorelaxation is significantly attenuated in the presence of MFA, even when NO signaling is not blocked (Fig. 4B). Additionally, our vessel reactivity index (pD_2) data document that wild blueberries appear to modulate cell membrane–agonist (Ach) interactions primarily in response to Ach in the young SHR model, since NOS inhibition significantly increased pD_2 in the SHR-BB group leading to an increased Ach-mediated vasorelaxation, compared to the SHR-C rings (Table 1B). The effect of MFA addition on pD_2 to Ach was different in SHR-BB aortas; we observed a decrease in vessel sensitivity to the agonist, which explains the reduced vasorelaxation (4%) of the SHR-BB rings, when compared to controls, where the presence of MFA had no significant effect.

The major novel finding of our study was that incorporating wild blueberries in the diet affects the endothelium-dependent vasorelaxation by modulating the production/activity of COX-derived products in the young SHR aorta. The nonselective COX inhibitor (MFA) did not have any effect on the Ach-mediated endothelium-dependent response of control SHR aortas, but inhibited the vasorelaxation of SHR-BB aortas in response to the agonist. This small attenuated dilation in the SHR-BB group is probably attributed to the significant decrease in the sensitivity to Ach when MFA is present and is a membrane-related event, irrelevant of events at the contractile machinery level. This observation is not in agreement with a previous study by Akpaffiong and Taylor [32], where the presence of MFA restored either partly or completely the impaired endothelium-dependent response of SHR arteries to Ach. However, even though the above observations were made in SHRs, the administration of antioxidants was intraperitoneal and the animals were 12–14 weeks old. It has been previously reported that in young SHRs the endothelium-mediated vasorelaxation seems to be similar to that of normotensives of the same age [33]. Even though the COX system does not seem to be compromised in our young SHRs, the favorable effect of blueberries towards the production of vasodilators or suppression of vasoconstrictors in response to Ach is apparent in our findings.

The effect of wild blueberries on vasorelaxation and vasoconstriction may be strain dependent and/or depend on the physiological state of the aorta (functional *vs.* dysfunctional endothelium). Even though we have previously shown an effect of blueberries in decreasing vasoconstriction in normotensive SD rats, this effect is not the same in vascular beds from hypertensive rats. On the contrary, blueberries seem to affect the vasorelaxation pathways in SHR vascular beds. We do know that different vascular beds from hypertensive rat strains possess an increased affinity and/or

number of vascular adrenergic receptors, leading to enhanced vascular contraction in response to adrenergic stimuli [34]. This and other similar differences in vascular responsiveness among strains may explain the differential effects of our dietary treatments on the studied pathways.

Young SHR are considered normotensive because hypertension in this strain develops after the 14th week. In young SHRs, the endothelium-mediated vasorelaxation seems to be similar to normotensives of the same age [33]. Koga et al. [35] did not report any significant differences in Ach-mediated vasorelaxation among normotensive and young SHR even after treatment with a nonselective COX inhibitor, while the release of COX-dependent vasoconstrictors was present only in vessels of aged normotensive or SHR animals and in response to higher concentrations of Ach. The significant differences in vessel sensitivity observed in the presence of the inhibitors, which in that case eliminate the basal presence of NO and COX products, show that the blueberry diet decreases the α_1-adrenergic reactivity. Since chronic exposure to free radicals sensitizes and potentiates the α_1-adrenergic pathway in low concentrations in mesenteric beds of SHRs (as previously reported on 15-week-old SHRs) [36], our blueberry-enriched diet may reverse the above effect. In our studies, the absence of an effect of the diet on maximal vasoconstriction may be attributed to the fact that the mesenteric vessel is more sensitive to oxidative stress than the SHR aorta and therefore even if a higher antioxidant reserve exists in our SHR-BB group, this effect will not be pronounced in certain vascular beds and at this stage of hypertension.

Under normal physiological conditions, there is constant, simultaneous and specifically regulated release of constitutive isoforms of NOS and COX products (with predominant COX products being vasodilator prostanoids), while under inflammatory states NO and PGs are released in larger micromolar concentrations, presumably due, at least in part, to the activation of inducible enzymes [37]. We should also note here that the concentration of wild blueberries in the diet (8% of powder) is significantly below the concentration that would compromise the health of the animals, and, moreover, the SHR-BB did not display any differences in body weight compared to the control group. It has been extensively reported in the past that the SHR animal model is characterized by an imbalance between endothelium-derived vasoconstrictors and vasodilators, where COX-dependent vasoconstrictors become more predominant [37,38]. In the SHR aorta, selective inhibition of COX-1 (but not COX-2) up-regulates the endothelium-dependent vasorelaxation in response to Ach [22]. Modulation of endothelium-dependent vasorelaxation in the SHR aorta has been associated with increased release of COX-dependent vasoconstrictor prostanoids, rather than with impaired release of NO in the SHR [39,40], accompanied with a greater expression of COX [40]. Moreover, numerous *in vivo* studies have demonstrated that in young SHR, NOS activity is not impaired [41]; in fact, it might be even enhanced in aged SHR compared to normotensive controls. Therefore, in our experimental animal model (SHR, during the developmental phase of hypertension) and, similarly, to the early established hypertensive stage, the effect of dietary blueberries on NO synthesis and thus NO-mediated vasorelaxation may be of limited contribution to the endothelial function affecting the onset of hypertension [41]. However, since in genetic hypertension there is a greater concentration of superoxide anion [21,42,43], which affects the later stages of endothelial function and disease onset, the ability of blueberries to preserve NO availability becomes important.

The exact mechanism involved in the modulation of COX pathway by blueberries is unknown and may engage multiple modes of action. Cyclooxygenase enzyme overexpression, along with the exaggerated production of vasoconstrictor prostanoids in different vascular beds of the SHR, has been widely reported [19,21,44,45]. The catalytic activity of COX consists of a series of reactions that use molecular oxygen and generate intermediate oxygen-derived free radicals [46,47], leading to subsequent oxidative stress associated with the pathophysiology of essential hypertension. Increased superoxide and hydrogen peroxide release are generated in aortic preparations from SHR [45,48] and they potentiate endothelium-dependent contractions to Ach. Reactive oxygen species such as superoxide play a dual role in the development and maintenance of endothelial dysfunction in SHR by decreasing the availability of NO and, furthermore, by stimulating COX activity [19].

Antioxidant therapy has been shown to restore the endothelium-dependent vasodilator response to Ach and overall endothelial function in both animal models of endothelial dysfunction such as SHR, as well as in human subjects with established endothelial dysfunction [49]. In our study, we observed significant differences in Ach-mediated vasorelaxation in SHR-BB animals at doses $10^{-8} \leq \text{Ach} \leq 10^{-7}$ M, when the dilator capacity was similar (similar to the maximum vasorelaxation in the final Ach dose of the dose–response curve). This effect was unmasked when COX was inhibited, in which case we observed a greater vasoconstriction in response to Ach (Fig. 4B), suggesting that blueberries may suppress a COX-dependent vasoconstrictor triggered by Ach. However, blueberries are known to possess strong antioxidant properties and it is conceivable that blueberries may, to some extent, preserve endothelial function by scavenging ROS. A previous study by Serraino et al. [50] revealed the protective effects of cyanidin-3-*O*-glucoside (a typical, although not predominant, anthocyanin present in blueberries) from blackberry extract against peroxinitrite-induced endothelial dysfunction in which COX is up-regulated. The mechanisms by which antioxidant compounds augment Ach-induced vasorelaxation remain speculative, since the majority of studies have either studied the pharmacologic effects of antioxidants or been conducted *in vitro*. Scavenging of peroxinitrite by anthocyanins may, in our case and to some extent, down-

regulate COX activity, resulting in reduced production of COX-derived products, including reduced production of COX-derived vasodilators. However, the small attenuation of vasorelaxation we observed in our BB group occurs only in the presence of MFA and is attributed to a great degree in the lower vessel sensitivity to Ach when MFA is present.

Dietary enrichment with wild blueberries for 7 weeks significantly increased the induced Ach-mediated vascular smooth muscle relaxation. This effect was augmented when the NO pathway was abolished and attenuated when the COX-derived metabolites were eliminated. The above effects are attributed partly to the changes in vessel sensitivity to the agonist in the above conditions. The effect of wild blueberries on aortic vasoconstrictor properties in response to Phe was less pronounced. Our data suggest that blueberries affect both NO and COX signaling pathways stimulated by Ach. A different experimental approach with a metabolically less complex animal model of hypertension may allow the dissection of the above events observed in the two pathways, which seem to be affected by our dietary treatment. Our observations and previous studies from other research groups suggest that mechanisms other than antioxidant activity of wild blueberry components may be responsible for modulating the endothelial function in SHRs. Understanding the role of wild blueberry-enriched diets in aged SHRs with established hypertension, as well as their effect(s) on the signaling pathways that lead to hypertensive complications in several disease conditions, may help us use this dietary treatment as an alterative therapeutic tool for the prevention of vascular complications.

Acknowledgments

The authors would like to express their gratitude to Ms. Julie Benoit for assisting with animal care.

References

[1] Rissanen TH, Voutilainen S, Virtanen JK, Venho B, Vanharanta M, Mursu J, et al. Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. J Nutr 2003;133:199–204.

[2] Liu S, Manson JE, Lee IM, Cole SR, Hennekens CH, Willett WC, et al. Fruit and vegetable intake and risk of cardiovascular disease: the Women's Health Study. Am J Clin Nutr 2000;72:922–8.

[3] Visioli F, Borsani L, Galli C. Diet and prevention of coronary heart disease: the potential role of phytochemicals. Cardiovasc Res 2000;47: 419–25.

[4] Prior R, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, et al. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity and variety of *Vaccinium* species. J Agric Food Chem 1998;46:2686–93.

[5] Duarte J, Perez-Palencia R, Vargas F, Angeles Ocete M, Perez-Vizcaino F, Zarzuelo A, et al. Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. Br J Pharmacol 2001; 133:117–24.

[6] Nakamura Y, Matsumoto H, Todoki K. Endothelium-dependent vasorelaxation induced by black currant concentrate in rat thoracic aorta. Jpn J Pharmacol 2002;89:29–35.

[7] Andriambeloson E, Magnier C, Haan-Archipoff G, Lobstein A, Anton R, Beretz A, et al. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. J Nutr 1998;128:2324–33.

[8] Fitzpatrick D, Hirschfield S, Ricci T, Jantzen P, Coffey R. Endothelium-dependent vasorelaxation caused by various plant extracts. J Cardiovasc Pharmacol 1995;26:90–5.

[9] Mizutani K, Ikeda K, Kawai Y, Yamori Y. Extract of wine phenolics improves aortic biomechanical properties in stroke-prone spontaneously hypertensive rats (SHRSP). J Nutr Sci Vitaminol 1999;45: 95–106.

[10] Diebolt M, Bucher B, Andriantsitohaina R. Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression. Hypertension 2001;38:159–65.

[11] Bernátová I, Pechánová O, Babál P, Kyselá S, Stvrtina S, Andriantsitohaina R. Wine polyphenols improve cardiovascular remodeling and vascular function in NO-deficient hypertension. Am J Physiol Heart Circ Physiol 2002;282:H942–8.

[12] Norton C, Kalea AZ, Harris PD, Klimis-Zacas D. Wild blueberry-rich diets affect the contractile machinery of the vascular smooth muscle in the Sprague–Dawley rat. J Med Food 2005;8:8–13.

[13] Kalea AZ, Lamari FN, Theocharis AD, Cordopatis P, Schuschke DA, Karamanos NK, et al. Wild blueberry (*Vaccinium angustifolium*) consumption affects the composition and structure of glycosaminoglycans in Sprague-Dawley rat aorta. J Nutr Biochem 2006;17: 109–16.

[14] Verma S, Buchanan MR, Anderson TJ. Endothelial function testing as a biomarker of vascular disease. Circulation 2003;108:2054–9.

[15] Arnal JF, Dinh-Xuan AT, Pueyo M, Darblade B, Rami J. Endothelium-derived nitric oxide and vascular physiology and pathology. Cellular and Molecular Life Sciences (CMLS) 1999;55:1078–87.

[16] Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87:840–4.

[17] Salvemini D, Currie M, Mollace V. Nitric oxide-mediated cyclooxygenase activation. J Clin Invest 1996;97:2562–8.

[18] Lüscher T, Vanhoutte P. The endothelium modulator of cardiovascular function. Boca Raton (Fla): CRC Press; 1990.

[19] Vanhoutte PM, Feletou M, Taddei S. Endothelium-dependent contractions in hypertension. Br J Pharmacol 2005;144:449–58.

[20] Kerr S, Brosnan MJ, McIntyre M, Reid JL, Dominiczak AF, Hamilton CA. Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium. Hypertension 1999;33:1353–8.

[21] Zalba G, Beaumont FJ, San Jose G, Fortuno A, Fortuno MA, Diez J. Is the balance between nitric oxide and superoxide altered in spontaneously hypertensive rats with endothelial dysfunction? Nephrol Dial Transplant 2001;16:2–5.

[22] Yang D, Feletou M, Boulanger CM, Wu HF, Levens N, Zhang JN, et al. Oxygen-derived free radicals mediate endothelium-dependent contractions to acetylcholine in aortas from spontaneously hypertensive rats. Br J Pharmacol 2002;136:104–10.

[23] Cuzzocrea S, Mazzon E, Dugo L, Di Paola R, Caputi AP, Salvemini D. Superoxide: a key player in hypertension. FASEB J 2004;18:94–101.

[24] Luscher TF, Vanhoutte PM. Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. Hypertension 1986;8:344–8.

[25] Machha A, MR M. Chronic treatment with flavonoids prevents endothelial dysfunction in spontaneously hypertensive rat aorta. J Cardiovasc Pharmacol 2005;46:36–40.

[26] Kalea AZ, Harris PD, Klimis-Zacas DJ. Dietary manganese suppresses α_1 adrenergic receptor-mediated vascular contraction. J Nutr Biochem 2005;16:44–9.

[27] Kalea AZ, Schuschke DA, Harris PD, Klimis-Zacas DJ. Cyclooxygenase inhibition restores the attenuated vasodilation in manganese-deficient rat aorta. J Nutr 2006;136:2302–7.

[28] Kawabe T, Harris PD, Zakaria REL, Garrison NR. Sepsis alters vessel contraction by adrenoceptor-induced nitric oxide and prostanoid. J Surg Res 2003;110:352–9.

[29] Kalea A, Norton C, Harris P, Klimis-Zacas D. Whole wild blueberries suppress α_1 adrenergic agonist induced contraction in rat aorta. FASEB J 2003.
[30] Nyborg NC, Bevan JA. Increased alpha-adrenergic receptor affinity in resistance vessels from hypertensive rats. Hypertension 1988;11: 635–8.
[31] Villalobos-Molina R, Lopez-Guerrero JJ, Ibarra M. Functional evidence of [alpha]1D-adrenoceptors in the vasculature of young and adult spontaneously hypertensive rats. Br J Pharmacol 1999;126: 1534–6.
[32] Akpaffiong M, Taylor A. Antihypertensive and vasodilator actions of antioxidants in spontaneously hypertensive rats. Am J Hypertens 1998; 11:1450–60.
[33] Cappelli-Bigazzi M, Rubattu S, Battaglia C, Russo R, Enea I, Ambrosio G, et al. Effects of high-cholesterol and atherogenic diets on vascular relaxation in spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 1997;273:H647–54.
[34] White RM, Rivera CO, Davison CB. Differential contribution of endothelial function to vascular reactivity in conduit and resistance arteries from deoxycorticosterone-salt hypertensive rats. Hypertension 1996;27:1245–53.
[35] Koga T, Takata Y, Kobayashi K, Takishita S, Yamashita Y, Fujishima M. Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat. Hypertension 1989;14:542–8.
[36] Girouard H, de Champlain J. Acute and chronic effects of free radicals on [alpha]1-adrenergic-induced vasoconstriction in mesenteric beds of spontaneously hypertensive rats. J Hypertens 2005;23:807–14.
[37] Davidge ST, Baker PN, McLaughlin MK, Roberts JM. Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. Circ Res 1995;77:274–83.
[38] Mombouli J, Nakashima M, Hamra M, Vanhoutte P. Endothelium-dependent relaxation and hyperpolarization evoked by bradykinin in canine coronary arteries. Br J Pharmacol 1999;117:413–8.
[39] Diederich D, Yang ZH, Buhler FR, Luscher TF. Impaired endothelium-dependent relaxations in hypertensive resistance arteries involve cyclooxygenase pathway. Am J Physiol Heart Circ Physiol 1990; 258:H445–51.
[40] Ge T, Hughes H, Junquero DC, Wu KK, Vanhoutte PM, Boulanger CM. Endothelium-dependent contractions are associated with both augmented expression of prostaglandin H synthase-1 and hypersensitivity to prostaglandin H2 in the SHR aorta. Circ Res 1995;76:1003–10.
[41] Radaelli A, Mircoli L, Mori I, Mancia G, Ferrari AU. Nitric oxide–dependent vasodilation in young spontaneously hypertensive rats. Hypertension 1998;32:735–9.
[42] Sawada Y, Sakamaki T, Nakamura T, Sato K, Ono Z, Murata K. Release of nitric oxide in response to acetylcholine is unaltered in spontaneously hypertensive rats. J Hypertens 1994;12:745–50.
[43] Chou TC, Yen MH, Li CY, Ding YA. Alterations of nitric oxide synthase expression with aging and hypertension in rats. Hypertension 1998;31:643–8.
[44] Carneado J, Alvarez de Sotomayor M, Perez-Guerrero C, Jimenez L, Herrera M, Pamies E, et al. Simvastatin improves endothelial function in spontaneously hypertensive rats through a superoxide dismutase mediated antioxidant effect. J Hypertens 2002;20:429–37.
[45] Grunfeld S, Hamilton CA, Mesaros S, McClain SW, Dominiczak AF, Bohr DF, et al. Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. Hypertension 1995;26:854–7.
[46] Katusic Z. Superoxide anion and endothelial regulation of arterial tone. Free Radic Biol Med 1996;20:443–8.
[47] Marnett LJ. Cyclooxygenase mechanisms. Curr Opin Chem Biol 2000; 4:545–52.
[48] Cosentino, Patton S, d'Uscio L, Werner E, Werner-Felmayer G, Moreau P, et al. Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. J Clin Invest 1998;101:1530–7.
[49] Newaz M, Yousefipour Z, Nawal N, Adeeb N. Nitric oxide synthase activity in blood vessels of spontaneously hypertensive rats: antioxidant protection by gamma-tocotrienol. J Physiol Pharmacol 2003;54:319–27.
[50] Serraino I, Dugo L, Dugo P, Mondello L, Mazzon E, Dugo C, et al. Protective effects of cyaniding-3-*O*-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure. Life Sci 2003;73:1097–114.