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Intake and time dependence of blueberry flavonoid–induced improvements in vascular function: a randomized, controlled, double-blind, crossover intervention study with mechanistic insights into biological activity[1–3]

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ABSTRACT

Background: There are very limited data regarding the effects of blueberry flavonoid intake on vascular function in healthy humans.

Objectives: We investigated the impact of blueberry flavonoid intake on endothelial function in healthy men and assessed potential mechanisms of action by the assessment of circulating metabolites and neutrophil reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity.

Design: Two randomized, controlled, double-blind, crossover human-intervention trials were conducted with 21 healthy men. Initially, the impact of blueberry flavonoid intake on flow-mediated dilation (FMD) and polyphenol absorption and metabolism was assessed at baseline and 1, 2, 4, and 6 h after consumption of blueberry containing 766, 1278, and 1791 mg total blueberry polyphenols or a macronutrient- and micronutrient-matched control drink (0 mg total blueberry polyphenols). Second, an intake-dependence study was conducted (from baseline to 1 h) with 319, 637, 766, 1278, and 1791 mg total blueberry polyphenols and a control.

Results: We observed a biphasic time-dependent increase in FMD, with significant increases at 1–2 and 6 h after consumption of blueberry polyphenols. No significant intake-dependence was observed between 766–1791 mg. However, at 1 h after consumption, FMD increased dose dependently to ≤766 mg total blueberry polyphenol intake, after which FMD plateaued. Increases in FMD were closely linked to increases in circulating metabolites and by decreases in neutrophil NADPH oxidase activity at 1–2 and 6 h.

Conclusions: Blueberry intake acutely improves vascular function in healthy men in a time- and intake-dependent manner. These benefits may be mechanistically linked to the actions of circulating phenolic metabolites on neutrophil NADPH oxidase activity. This trial was registered at clinicaltrials.gov as NCT01292954 and NCT01829542. *Am J Clin Nutr* doi: 10.3945/ajcn.113.066639.

INTRODUCTION

Evidence has suggested that polyphenol-rich foods may exert cardiovascular health benefits, with randomized, controlled human-intervention trials indicating a positive effect of such foods on several well-characterized cardiovascular disease (CVD)[4] risk factors, including endothelial dysfunction, hypertension, lipid metabolism, and platelet activity (1). Such beneficial vascular effects may be mediated by the ability of absorbed polyphenols and their circulating metabolites to improve nitric oxide bioavailability (2–5), although the precise mechanism is unclear. Notably, flavanol-rich cocoa has been shown to acutely improve endothelium-dependent vasodilation, as assessed by flow-mediated dilation (FMD), in healthy individuals and patients with coronary artery disease, hypertension, or diabetes (4, 6–11). These vascular improvements were correlated in time with changes in plasma flavanol metabolites and were also observed after pure (−)-epicatechin intake, suggesting a cause-and-effect relation between flavanols and vascular improvements (2).

With respect to anthocyanin-rich foods, data from human trials have been less clear, in part because of a lack of well-conducted randomized controlled trials (RCTs). Indeed, although improvements in FMD have been observed in hypercholesterolemic individuals after pure anthocyanin (320 mg) ingestion (3) and in healthy individuals and individuals with the metabolic syndrome or coronary heart disease after the intake of grape polyphenols (12–14), other studies with grape or grape extracts showed no significant vascular impact (15, 16). Datasets on blood pressure have also been mixed, with blueberry intake decreasing blood pressure in individuals with the metabolic syndrome (17) but not in chronic smokers (18) or obese insulin-resistant individuals

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[4] Abbreviations used: CVD, cardiovascular disease; DVP, digital volume pulse; DVP-RI, digital volume pulse reflection index; DVP-SI, digital volume pulse stiffness index; FMD, flow-mediated dilation; PWA, pulse wave analysis; PWV, pulse wave velocity; RCT, randomized controlled trial.

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(19). There have also been a number of uncontrolled studies that indicated that cranberry juice, pomegranate, and purple-grape juice may improve FMD acutely after ingestion (20–22), whereas blueberry intake by animals has been shown to decrease blood pressure and improve endothelium-dependent vasodilation ex vivo (23–28).

Such discrepancies in the impact of blueberry intake on vascular outcomes have likely been due to differences in the anthocyanin, flavanol (monomers and oligomers), and hydroxycinnamic acid amounts contained within intervention diets in the various studies (29, 30). Studies have largely based interventions on the amount of blueberry consumed rather than the delivery of polyphenol amounts, meaning that such studies are difficult to compare and difficult to interpret. To address this problem, in current RCTs, we have designed our interventions around the amount of total blueberry polyphenols (flavonoids and hydroxycinnamates) to investigate both the time-dependent and intake-dependent changes in endothelial function by brachial artery FMD and assess potential mechanisms of vascular action. Because specific polyphenols and their metabolites have been suggested to act as inhibitors of NADPH oxidase activity in vitro (31), thereby reducing superoxide generation and sparing nitric oxide, we also investigated the potential for blueberry polyphenol intake to affect the activity of this enzyme in vivo.

SUBJECTS AND METHODS

Materials

All individual flavonoid and phenolic acid standards were obtained from Sigma-Aldrich Co Ltd or Extrasynthese. Water, methanol, acetic acid, and acetonitrile (HPLC grade) were purchased from Fisher Scientific. HPLC columns were obtained from Hichrom. β-glucuronidase and sulfatase (*Helix pomatia*, type H1) was purchased from Sigma-Aldrich Co Ltd. Oasis HLB solid-phase extraction cartridges were purchased from Waters. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich Co Ltd or Fisher Scientific.

Intervention study subjects

Twenty-one healthy men were recruited from the University of Reading and surrounding area. Volunteers were assessed before the start of the trial for good health and were selected according to the following after-inclusion criteria: a signed consent form, inclusive age of 18–40 y, good general health assessed by their responses to a standard medical questionnaire, blood results (normal liver enzymes, hemoglobin, hematocrit, and leukocyte counts), and the absence of diabetes, hypertension (blood pressure $>$150/90 mm Hg) and anemia. Exclusion criteria were as follows: individuals who were taking antiinflammatory, antibiotics, or blood pressure–lowering medication within a 2-mo period before the study. Volunteers were instructed not to alter their usual dietary or fluid intake. Individuals selected for the study were asked to refrain from the following for 24 h before and during the study: the consumption of polyphenol-rich foods including fruit, vegetables, cocoa, chocolate, coffee, tea, and wine; the intake of nitrate rich foods including leafy green vegetables and beetroot; participation in vigorous exercise ($>3 \times 20$ min/wk); and the consumption of $>$168 g alcohol (any form)/wk. Written informed consent was obtained from all subjects before their participation in the study.

Study design

The first RCT was designed to investigate time-course effects and was a randomized, double-blind, crossover, controlled intervention trial in which volunteers were asked to consume a blueberry drink that contained 766, 1278, and 1791 mg total blueberry polyphenols (equivalent to 240, 400, and 560 g fresh blueberries, respectively) or a macronutrient and micronutrient control drink matched for the macronutrient and micronutrient of the 1278 mg blueberry dose (**Figure 1**A). On arrival at the Nutrition Unit, subjects rested for 30 min in a quiet, temperature-controlled room before they were cannulated, and blood samples were collected in the fasted state and at 0, 1, 2, 4, 6, and 24 h after consumption of each intervention drink. FMD of the brachial artery was the primary outcome, and secondary outcomes included the pulse wave velocity (PWV), pulse wave analysis (PWA), peripheral and central blood pressure, and digital volume pulse (DVP). Vascular measurements were conducted before the consumption of each intervention drink and at 2, 4, and 6 h after consumption. In addition, FMD was also measured at 1 h after consumption. The study was conducted with 10 volunteers from September to December 2010.

The second intervention study (dose-dependency study) was also a double-blind, randomized, 6-arm study in which volunteers (different individuals from the first RCT) were asked to consume either a blueberry drink that contained 319, 639, 766, 1278, or 1791 mg of total polyphenols (equivalent to 100, 200, 240, 400, and 560 g fresh blueberries, respectively) or a macronutrient and micronutrient control drink (Figure 1B). FMD (main outcome), blood pressure measurements (secondary outcome), and blood samples were collected before the consumption of the drink and at 1 h after consumption. Systolic and diastolic blood pressures were measured by using an Omron MX2 automatic digital upper-arm blood pressure monitor (Omron Healthcare UK Ltd). All subjects completed a 2-d diet diary of their habitual dietary intake during the day before the study and on the study day arm to make sure they complied with the low flavonoid diet. Plasma samples were drawn into EDTA-containing vials that were supplemented with 2% formic acid and immediately stored at -80°C until analysis. The study was conducted with 11 volunteers from March to May 2012.

A qualified nurse enrolled participants on the study. Study nurses, care providers and researchers involved with the assessment of study outcomes were all blinded to the interventions. An independent researcher generated the random allocation to the treatment sequence (by using a Williams design) and implemented the allocation sequence. All studies were conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the University of Reading Research Ethics Committee (reference: 06/37 and 12/14). The studies were registered at clinicaltrials.gov as NCT01292954 and NCT01829542.

Anthocyanin- and flavanol-containing test materials

Freeze-dried wild-blueberry powder was kindly supplied by the Wild Blueberry Association of North America and was stored at -20°C until use. Blueberry drinks were analyzed for

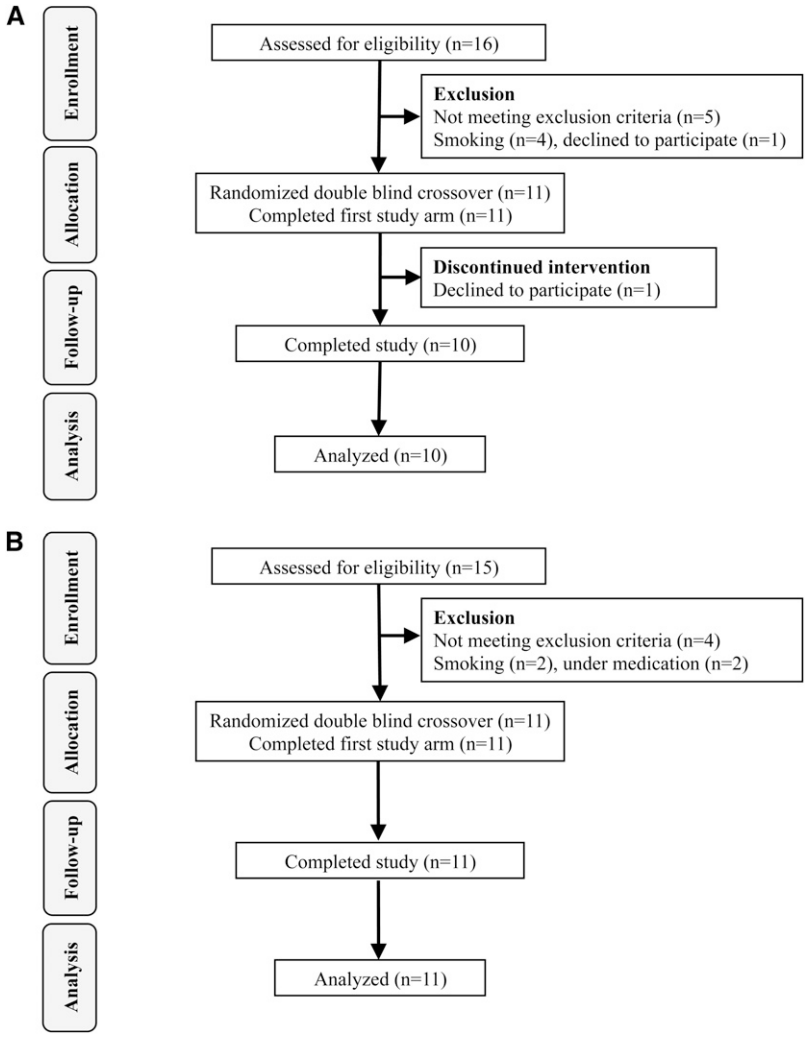

FIGURE 1. CONSORT flow diagrams for the time-course study (A) and dose-dependency study (B).

flavonoid and phenolic acid content as previously described (29) with interventions that contained 319, 639, 766, 1278, and 1791 mg total major blueberry polyphenols (**Table 1**). The total anthocyanin content of drinks ranged between 129 and 727 mg; the total procyanidin content was between 57 and 320 mg, whereas the chlorogenic acid content was between 114 and 637 mg. Small amounts of the flavonol quercetin and other phenolic acids (caffeic, *p*-coumaric, and ferulic acid) were also quantified (Table 1). An independent researcher prepared the drinks immediately before consumption by dissolving the adequate amount of freeze-dried blueberry powder (Table 1) in 500 mL low-nitrate water. The control drink was matched for sugars (glucose and fructose) and vitamin C to the blueberry drink that contained 1278 mg total polyphenols. Drinks were provided in identical opaque containers with a black straw to ensure no differences in color could be detected.

FMD

FMD of the brachial artery was the primary endpoint measure of the study and was measured according to standard guidelines (32) by using an ALT Ultrasound HDI5000 system (ATL Ultrasound) in combination with a semiautomated computerized analysis system (Brachial Analyzer; Medical Imaging Applications-llc). Briefly, after a 15-min supine rest in a quiet, temperature-controlled room, the brachial artery was imaged longitudinally at 2–10 cm proximal to the antecubital fossa.

TABLE 1
Compositional analysis of the freeze-dried wild blueberry and control drinks used in human studies

	Freeze-dried wild blueberry (g)					
Compounds	14	28	34	57	80	Control
Anthocyanins (mg)	129	258	310	517	724	0
Procyanidins (mg)	57	114	137	228	320	0
Flavanol monomers (mg)	10	20	24	40	56	0
Flavanol oligomers (mg)	47	94	113	188	264	0
Chlorogenic acid (mg)	114	228	273	455	637	0
Quercetin (mg)	11	22	26	43	61	0
Caffeic acid (mg)	7	15	17	30	42	0
p-Coumaric acid (mg)	0.6	1.2	1.4	2.4	3.4	0
Ferulic acid (mg)	0.6	1.2	1.4	2.4	3.4	0
Vitamin C (mg)	1.7	3.4	4	6.8	9.5	6.8
Fructose (g)	4	7.4	9	15	21	15
Glucose (g)	3	7	8	13	19	13
Total polyphenols (mg)	319	639	766	1278	1791	0

After baseline images were recorded for 60 s, a blood pressure cuff placed around the forearm was inflated to 220 mm Hg. After 5 min of occlusion, the pressure was rapidly released to allow reactive hyperemia, with image collection that continued for 5 min after release. A single researcher, who was blinded to the measurement details, analyzed all image files, and the peak diameter was defined as the largest diameter obtained after the occlusion was released. The FMD response was calculated as the relative diastolic diameter change from baseline compared with the peak diastolic diameter.

PWV and PWA

PWV and PWA were determined by using a SphygmoCor CPV Arterial Tonometry system (ScanMed Medical). The aortic pulse waveform and augmentation index were derived at the radial artery; PWV was determined between carotid and femoral sites. A pencil-type probe was used for all measurements and was held at the base of neck for the carotid artery and over the right femoral artery. Recordings were taken when a reproducible signal was obtained with high-amplitude excursion. The distance between carotid and femoral sites was measured with a tape measure, and electrocardiogram gating permitted the time lapse between pulse waves at the carotid and femoral sites to be calculated. PWV and PWA are gold-standard techniques for the measurement of arterial stiffness, which is an assessment of both the structure and function of the arteries (33), and the augmentation index is a measure of central blood pressure. Both techniques have been shown to by strongly correlated with cardiovascular risk (33–35).

DVP

A PulseTrace PCA 2 with a photoplethysmograph transducer transmitting infrared light at 940 nm (MicroMedical, Kent) was placed on the index finger of the dominant hand and used to calculate the DVP stiffness index (DVP-SI) and DVP reflection index (DVP-RI). The DVP records the systolic and diastolic waveforms of the pulse by measuring infrared-light transmission through the finger. The DVP-SI (in m/s) is defined as the height of the subject divided by the time between the first and the second wave peaks, and it is usually correlated with the stiffness of large arteries. The DVP-RI is the relative height of the second peak compared with the first and is associated with smaller artery stiffness (36). Both of these variables provide an indication of the stiffness of the arterial system for an individual.

Plasma flavonoid and metabolite analysis

A plasma flavanol (37) and anthocyanin (38) analysis was performed as previously described. A plasma phenolic acid analysis was performed according to a method that was based on Ottaviani et al (39) with some modifications. Briefly, plasma (0.5-mL) samples were prepared by using enzymatic hydrolysis with β-glucuronidase and sulfatase (10,000 IU β-glucuronidase, 300 IU sulfatase; 40 min at 37°C) to produce nonglucuronidated and nonsulfated metabolites for analysis. The recovery standard used was 2-(4-hydroxy-phenoxy)-propionic acid (50 μL, 100 μmol/L). Samples were mixed with 1 mL 0.5% acetic acid in water (vol:vol), and centrifuged at 17,000 $\times$ *g* for 15 min at 4°C. Samples were loaded onto solid-phase extraction cartridges (Oasis HLB 60 mg, 3 cc) previously conditioned with 1 mL *N*,*N*-dimethylformamide: methanol (7:3) and 0.5% (vol:vol) acetic acid in water. Washing steps consisted of 3 mL 0.5% (vol:vol) acetic acid in water and 1 mL water:methanol:acetic acid (80:20:0.5). For elution, cartridges were dried and eluted with the addition of 1 mL *N*, *N*-dimethylformamide:methanol (7:3) twice. The eluent was collected in tubes that contained 200 μL 0.5% (vol:vol) acetic acid in methanol. The combined supernatant fluid was taken to dryness by using a Speedvac system (Thermo Fisher Scientific Inc) and redissolved in 100 μL HPLC water.

Liquid Chromatography coupled to quadrupole Time-Of-Flight mass spectrometry analysis of phenolic acid metabolites

Phenolic acid metabolites were analyzed by using an HPLC Agilent 1100 series (Agilent Technologies Ltd) equipped with a quaternary pump, autosampler, column, and sample thermostat and ultraviolet/visable detector coupled to a Bruker MicroTof QII high-resolution time-of-flight mass spectrometer (Bruker Daltonics Ltd) by using the negative-mode scanning the range of *m*/*z* 50–1100 Da. Samples (10 μL) were injected onto an Ace C18 column (2.1 $\times$ 150 mm) with a 5-μm particle size (Hichrom). The mobile phase consisted of 0.1% (vol:vol) formic acid in HPLC water (A) and 0.1% (vol:vol) formic acid in acetonitrile (B), and the following gradient protocol was run: 0 min, 95% A, 5% B; 1 min, 95% A, 5% B; 20 min, 70% A, 30% B; 25 min, 20% A, 80% B; 30 min, 20% A; 80% B; 31 min, 95% A, 5% B; and 45 min, 95% A, 5% B. The flow rate was 0.2 mL/min. The detection and quantification of phenolic acids and their metabolites was performed by using authentic standards with the exception of 3′- and 4'-hydroxyhippuric acid, which were quantified by using α-hydroxyhippuric as a standard.

NADPH oxidase activity and expression

Neutrophil NADPH oxidase activity in whole blood samples, collected from individuals after the intervention with the 766-mg total polyphenol dose, was assessed by using flow cytometry as previously described (40). Whole blood aliquots (100 μL) were incubated in the absence or presence of phorbol myristate acetate (50 ng/mL) for 45 min at 37°C in a 5% CO_2 atmosphere to induce superoxide production by neutrophils. Dihydrorhodamine 123 (30 μg/mL) (Sigma) was added and incubated for 5 min at 37°C. Red blood cells were lysed with 2 mL fluorescence-activated cell sorting lysing solution (Becton Dickinson) for 15 min at room temperature, and cells were centrifuged at 400 $\times$ *g* for 5 min at 4°C. After pellet washing with 3 mL isotonic saline solution, white blood cells were resuspended in 0.5 mL phosphate-buffered saline and analyzed by using flow cytometry. Data were analyzed by using Cell Quest software version 3.2 (Becton Dickinson), and the median intensity of fluorescence was used to evaluate the fluorescence of each tube. The NADPH oxidase activity was calculated as the difference between values obtained in phorbol myristate acetate–activated and –resting cells. Serum concentrations of soluble-gp91^{phox} were assessed as previously described (41) by the addition of serum (100 μL) to plates coated with anti-gp91^{phox} monoclonal antibody (Santa Cruz Biotech). The complex was detected by using goat anti-mouse IgG1-horseradish peroxidase (Santa Cruz Biotech) and 3,3′, 5,5′-tetramethylbenzidine as substrate. After

TABLE 2
Baseline clinical characteristics of the study population (time-course study: $n = 10$; dose-dependency study: $n = 11$)[1]

Baseline characteristics	Time-course study	Dose-dependency study	*P*
Age (y)	27 ± 1.3	27 ± 1.0	NS
BMI (kg/m^2)	25 ± 0.8	22 ± 0.9	NS
Total cholesterol (mmol/L)	4.3 ± 0.2	4.2 ± 0.2	NS
HDL cholesterol (mmol/L)	1.2 ± 0.1	1.5 ± 0.1	NS
Triacylglycerol (mmol/L)	0.8 ± 0.1	0.9 ± 0.1	NS
SBP (mm Hg)	123 ± 2.3	120 ± 1.7	NS
DBP (mm Hg)	71 ± 2.1	65 ± 1.8	<0.01
Heart rate (beats/min)	56 ± 2.7	62 ± 1.9	<0.01
Brachial diameter (mm)	3.9 ± 0.1	4.2 ± 0.1	NS
FMD (%)	7.1 ± 0.1	6.3 ± 0.2	NS

[1] All values are means ± SEMs. *P* indicates significance at $P < 0.05$ (2-factor ANOVA). DBP, diastolic blood pressure; FMD, flow-mediated dilation; SBP, systolic blood pressure.

incubation for 30 min at room temperature, the reaction was stopped with 100 μL of 1 mol/L HCl, and the absorbance was read at 405/450 nm. Quantification was achieved by using a standard curve constructed by using gp91^{phox} peptide (Abcam).

Biochemical analysis

Blood samples collected in lithium and heparin tubes were centrifuged (1700 × *g*; 10 min at 4°C) immediately after collection. Samples were also collected in serum separation tubes and allowed to stand for 30 min before centrifugation (1300 × *g*; 10 min at 21°C). All samples were frozen at −80°C until analysis. Plasma concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, glucose, and triacylglycerol were assayed with an ILAB 600 chemistry analyzer (Instrumentation Laboratory) by using enzyme-based colorimetric tests supplied by Instrumentation Laboratory. The following variables were determined in all samples: total cholesterol, LDL cholesterol, HDL cholesterol, glucose, and triacylglycerol. Plasma ascorbic acid was analyzed as previously described (42).

Power calculations and statistical analyses

Power calculations were performed for the primary endpoint of the change in FMD response. Power was based on the intra-individual variability of the operator who performed the FMD analysis (5% CV and SD of 0.3 on the basis previous studies in which the same subjects were measured on 4 different occasions at the same time of day). At a power of 0.8, a 0.05 significance level, and a mean FMD of 7.2%, the number of subjects required to detect a difference of 0.3% in the response of matched pairs in a crossover study is 10. This number is consistent with that in other studies carried out with similar endpoints and study design (2, 4). Two-factor repeated-measures ANOVA was fitted to analyze the data by using the SAS version 9.1 software package (SAS Institute) and GraphPad Prism version 5 software (GraphPad Software Inc). A post hoc analysis was carried out by using the Bonferroni test. Significance was defined at $P < 0.05$, with *P* values represented in figures as follows: *P = 0.01–0.05, **P = 0.001–0.01, ***$P < 0.001$, ****$P < 0.0001$. The area under the plasma concentration compared with time curve (AUC) was calculated by using the trapezoidal method. A correlation analysis was performed by using Pearson's correlation coefficient.

RESULTS

Baseline characteristics and tolerance of intervention

Baseline characteristics of subjects were all within normal limits, including baseline FMD and blood pressure (**Table 2**). Diastolic blood pressure and heart rate were significantly different between the 2 studies (Table 2). Both drinks were well tolerated by all subjects, and no adverse events were reported.

Blueberry induced a biphasic dose-dependent increase in FMD

A significant increase in endothelium-dependent brachial artery vasodilation, measured as FMD, was observed after ingestion of a blueberry drink that contained 766 mg total polyphenols, with 2 peaks, with the first peak at 1–2 h postconsumption and a second peak at 6 h ($P < 0.05$) (**Figure 2**A; **Table 3**). The

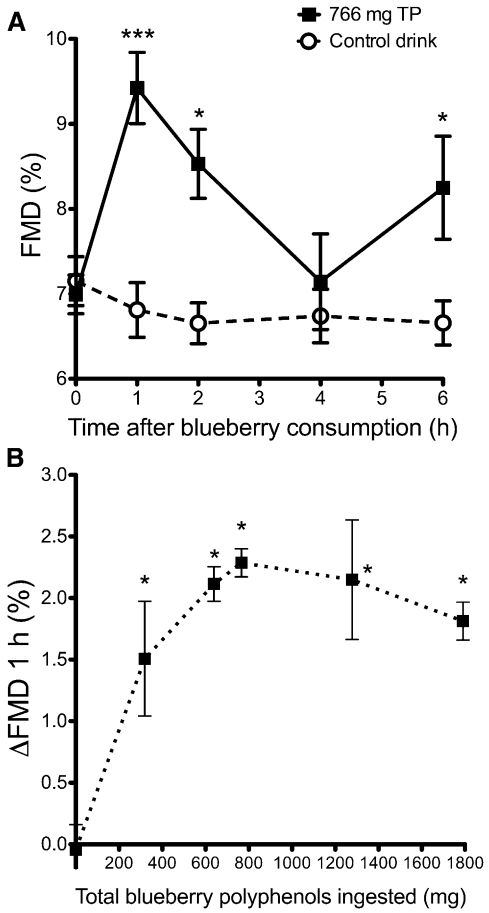

FIGURE 2. A: Time-course of FMD (mean ± SEM) after consumption of a blueberry drink containing 766 mg TP or a macronutrient- and micronutrient-matched control ($n = 10$). B: Dose-response curve of FMD (ΔFMD; mean ± SEM) after 1 h after consumption of a blueberry drink with increasing amounts of blueberry polyphenols ($n = 11$). Data were analyzed by using 2-factor repeated-measures ANOVA with time and treatment as the 2 factors [significant effects of time ($P = 0.02$), treatment ($P < 0.0001$), and the interaction between time and treatment ($P = 0.0034$)]. Post hoc analyses were conducted by using a Bonferroni multiple-comparisons test. *,****Significantly different compared with the control drink: *P = 0.01–0.05, ***$P < 0.001$. FMD, flow-mediated dilation; TP, total blueberry polyphenols.

magnitude of increase was 2.4 ± 0.5% at 1 h, 1.5 ± 0.4% at 2 h, and 1.2 ± 0.6% at 6 h (Figure 2A). No significant changes in FMD between blueberry and control drinks were observed at baseline and 4 h after consumption, and no significant changes in FMD were observed at any time after the consumption of the control drink. Changes in FMD for the 1278- and 1791-mg intakes were not significantly different to the change observed for the 766-mg intake (Table 3). No other vascular variables (blood pressure, heart rate, PWV, augmentation index, DVP-SI, and DVP-RI) were significantly altered after consumption of any of the polyphenol intakes compared with at baseline or after consumption of the control drink (Table 3 and **Table 4**).

In the dose-dependency study, FMD measurements were made at 1 h when FMD reached the maximum value according to the time-course study (Figure 2A) and indicated that endothelial function increased in a linear fashion up to the 766-mg intake, after which the vascular response plateaued and decreased slightly at higher intakes (Figure 2B). From these data, the blueberry polyphenol intake necessary to achieve half-maximal effects was calculated to be 482 mg, which corresponded to 152 g fresh blueberries.

Biphasic increase in plasma polyphenols

A total of 32 polyphenol metabolites were identified in plasma after blueberry consumption (**Table 5**). In line with increases in FMD, a biphasic increase in plasma polyphenol metabolites was observed, with a significant increase in plasma concentration of 6 metabolites at 1–2 h after consumption (**Figure 3**) and a later increase in the concentration of 8 metabolites at 6 h after consumption (Figure 3 and **Figure 4**). Metabolites that increased at 1–2 h were distinct from those that increased at 6 h, with the exception of vanillic and benzoic acids, which were significantly elevated at 2 and 6 h after consumption (Figure 3). Plasma *p*-coumaric acid metabolites also increased significantly at 2 h after consumption ($P < 0.001$), although the amounts quantified were close to the limit of detection (14 nmol/L). The AUC of the plasma concentration of total polyphenol metabolites over time was significantly higher after consumption of blueberry polyphenols that the control (16.1 ± 5.3 and 4.3 ± 1.4 μmol · h/L, respectively, $P = 0.004$). The method used for the analysis of plasma polyphenol metabolites after β-glucuronidase and sulfatase treatment resulted in limits of quantification between 6–400 nmol/L (Table 5) and an average percentage of recovery of 73% ± 2%. No flavanol or anthocyanin metabolites were detected in plasma at any time after consumption of the blueberry polyphenol intervention. No significant changes in plasma vitamin C were shown after consumption of the blueberry or control interventions, with plasma concentrations that ranged from 43 to 62 μmol/L from 0 to 6 h before and after consumption of all intervention drinks.

TABLE 3
Acute effects of blueberry polyphenols on vascular function ($n = 10$)[1]

	Baseline	1 h	2 h	4 h	6 h
FMD (%)					
766 mg TP	7.0 ± 0.2	9.4 ± 0.4[a,z]	8.5 ± 0.4[a,z]	7.1 ± 0.6	8.2 ± 0.6[a,y]
1278 mg TP	7.2 ± 0.5	9.4 ± 0.7[b,z]	8.7 ± 0.6[b,z]	7.3 ± 0.5	7.8 ± 0.8[y]
1791 mg TP	7.0 ± 0.3	8.8 ± 0.6[b,z]	8.0 ± 0.7[z]	7.1 ± 0.6	7.4 ± 0.8
Control	7.1 ± 0.3	6.8 ± 0.3	6.6 ± 0.2	6.7 ± 0.3	6.6 ± 0.3
PWV (m/s)					
766 mg TP	5.9 ± 0.2	—	5.9 ± 0.2	5.7 ± 0.2	6.2 ± 0.2
1278 mg TP	5.9 ± 0.2	—	5.9 ± 0.2	5.9 ± 0.1	6.0 ± 0.1
1791 mg TP	5.8 ± 0.2	—	6.0 ± 0.2	6.1 ± 0.2	6.3 ± 0.2
Control	5.9 ± 0.4	—	6.4 ± 0.3	6.2 ± 0.3	6.5 ± 0.3
AIx (%)					
766 mg TP	2.3 ± 3.9	—	1.8 ± 2.5	3.0 ± 3.0	1.1 ± 3.0
1278 mg TP	2.3 ± 3.4	—	5.4 ± 3.4	3.1 ± 4.1	−0.6 ± 3.2
1791 mg TP	1.4 ± 3.8	—	1.6 ± 2.8	2.6 ± 2.3	0.9 ± 2.4
Control	3.1 ± 5.9	—	5.4 ± 3.3	2.2 ± 0.9	1.1 ± 1.9
DVP-SI (m/s)					
766 mg TP	6.0 ± 0.4	—	5.8 ± 0.1	5.7 ± 0.1	5.9 ± 0.2
1278 mg TP	6.2 ± 0.4	—	6.3 ± 0.4	5.9 ± 0.1	5.8 ± 0.2
1791 mg TP	6.1 ± 0.4	—	6.1 ± 0.2	5.8 ± 0.2	5.9 ± 0.2
Control	6.4 ± 0.2		6.6 ± 0.1	6.2 ± 0.1	5.9 ± 0.1
DVP-RI (%)					
766 mg TP	67 ± 4	—	69 ± 3	67 ± 5	66 ± 4
1278 mg TP	78 ± 2	—	77 ± 2	78 ± 3	64 ± 6
1791 mg TP	70 ± 5	—	79 ± 3	70 ± 4	71 ± 4
Control	69 ± 7	—	71 ± 4	70 ± 3	65 ± 7

[1] All values are means ± SEMs ($n = 10$). [a,b]Significant difference between baseline and postintervention (1, 2, 4, or 6 h) (repeated-measures ANOVA): [a]$P < 0.05$, [b]$P < 0.01$. [y,z] Significant difference between control and blueberry treatment (2-factor repeated-measures ANOVA): [y]$P < 0.05$; [z]$P < 0.01$. AIx, augmentation index; DVP-RI, digital volume pulse reflection index; DVP-SI, digital volume pulse stiffness index; FMD, flow-mediated dilation; PWV, pulse wave velocity; TP, total blueberry polyphenols.

TABLE 4
Acute effects of blueberry on blood pressure and heart rate (n = 10)[1]

	Baseline	2 h	4 h	6 h
PSBP (mm Hg)				
766 mg TP	130 ± 3	130 ± 3	130 ± 3	132 ± 2
1278 mg TP	127 ± 3	128 ± 3	127 ± 3	127 ± 3
1791 mg TP	129 ± 3	130 ± 3	130 ± 3	130 ± 3
Control	128 ± 3	130 ± 2	132 ± 1	130 ± 1
PDBP (mm Hg)				
766 mg TP	67 ± 3	66 ± 2	67 ± 3	68 ± 3
1278 mg TP	64 ± 3	66 ± 3	65 ± 3	64 ± 3
1791 mg TP	64 ± 3	65 ± 3	66 ± 3	65 ± 3
Control	67 ± 4	68 ± 4	75 ± 1	69 ± 5
CSBP (mm Hg)				
766 mg TP	108 ± 2	107 ± 2	107 ± 2	108 ± 1
1278 mg TP	105 ± 3	107 ± 3	105 ± 3	103 ± 3
1791 mg TP	106 ± 3	106 ± 3	106 ± 3	106 ± 3
Control	106 ± 4	107 ± 4	110 ± 1	106 ± 1
CDBP (mm Hg)				
766 mg TP	67.7 ± 2.7	66.5 ± 2.5	67.8 ± 2.7	69 ± 3
1278 mg TP	64.8 ± 3.4	65.8 ± 3.5	66.3 ± 3.5	66 ± 3
1791 mg TP	64.7 ± 3.4	65.8 ± 3.5	66.3 ± 3.5	66 ± 3
Control	67.3 ± 4.0	69.1 ± 2.8	75.5 ± 0.7	69 ± 3
Heart rate (beats/min)				
766 mg TP	57 ± 2	57 ± 2	57 ± 2	58 ± 2
1278 mg TP	56 ± 3	55 ± 3	56 ± 3	57 ± 2
1791 mg TP	54 ± 3	56 ± 2	55 ± 2	57 ± 2

[1] All values are means ± SEMs (n = 10). No significant differences were shown between baseline and postintervention (2, 4, or 6 h) or between control and blueberry treatments, $P > 0.05$ (repeated-measures and 2-factor repeated-measures ANOVA). CDBP, central diastolic blood pressure; CSBP, central systolic blood pressure; PDBP, peripheral diastolic blood pressure; PSBP, peripheral systolic blood pressure; TP, total blueberry polyphenols.

Decreased NADPH oxidase activity after blueberry intervention

Highly significant reductions in neutrophil NADPH oxidase activity were measurable at 1–2 and 6 h ($P > 0.001$), although there was a smaller reduction also apparent at 4 h ($P > 0.05$) (**Figure 5**A). In contrast, there were no significant changes ($P > 0.05$) in plasma concentrations of soluble-gp91^{phox}, which is the catalytic core of phagocyte NADPH oxidase, partly reflecting NADPH oxidase expression (Figure 5B).

Plasma metabolite changes predict changes in FMD and NADPH oxidase activity

In a multivariate regression analysis including all plasma metabolites that increased over the 1–2-h time frame (Figure 3) and coincided with the first peak of FMD response, only vanillic acid (R^2 = 0.25 for the model, P = 0.02) and benzoic acid (R^2 = 0.25 for the model, P = 0.04) predicted the magnitude of FMD increase, suggesting that these metabolites may be, at least in part, involved in mediating the observed increases in endothelium-dependent vascular function between 1–2 h. A similar multivariate regression analysis conducted by using values obtained between 4 and 6 h (the second increase in FMD) with those metabolites observed to increase over this timeframe (Figure 4) indicated that hippuric acid (R^2 = 0.32, P = 0.014), hydroxyhippuric acid (R^2 = 0.30, P = 0.017), and homovanillic acid (R^2 = 0.27, P = 0.024) all predicted the magnitude of FMD increase. A multivariate analysis also indicated that changes in vanillic acid (R^2 = 0.43 for the model, P = 0.006), hippuric acid (R^2 = 0.39, P = 0.008), and homovanillic acid (R^2 = 0.49, P = 0.001) predicted declines in NADPH oxidase activity, suggesting that these may in part be responsible for the enzymes inhibition. Last, in addition to the mean time-dependent reductions in NADPH oxidase activity after the 766-mg blueberry polyphenol intervention, we also observed a significant univariate correlation between changes in FMD and neutrophil NADPH oxidase activity (r = −0.846, P = 0.0021).

DISCUSSION

In our time-course study, we observed a biphasic time-dependent increase in FMD with significantly increased brachial artery dilation observed at 1–2 and 6 h after consumption of 766–1791 mg blueberry polyphenols. Increases in FMD were accompanied by increases in plasma concentrations of phenolic acid metabolites, with some that peaked at 1–2 h and others that peaked at 6 h, and reductions in neutrophil NADPH oxidase activity. In the dose-dependency study, endothelial function (at 1 h) increased dose dependently up to the 766-mg intake and reached a plateau at higher intakes. These data are in agreement with cocoa-flavanol FMD datasets whereby FMD increased after an intake of ≤200 mg total cocoa flavanols and reached a plateau from 200 to 400 mg flavanol intake (4). Furthermore, meta-analysis data have indicated that FMD increases linearly to a consumption of ≤500 mg total cocoa polyphenols, after which a decrease in the percentage of FMD was observed at higher intakes (43), and dose-response relations were nonlinear between FMD and flavonoid dose (44). No changes in secondary markers of vascular function were observed at any of the time points or intakes used, which agreed with data that indicated that PWV (20) and blood pressure (18, 45) are not acutely altered after flavonoid interventions.

To investigate a potential cause-and effect relation between increases in FMD and the intake of blueberry polyphenols, we investigated plasma concentrations of flavonoid and phenolic acid metabolite profiles after consumption. In line with the FMD results, we also observed a biphasic time-dependent increase in plasma phenolic metabolites at 1–2 and 6 h after consumption. Ferulic acid, isoferulic acid, vanillic acid, 2-hydroxybenzoic acid, benzoic acid, and caffeic acid (sum of conjugated and nonconjugated compounds) were significantly higher at 1–2 h after blueberry polyphenol consumption and reached a plasma total concentration ∼400 nmol/L and coincided with the maximal increase in FMD at 1h (Figure 3). A correlation analysis indicated that both vanillic and benzoic acid may have been linked in part to the observed changes in FMD. Such metabolites have previously been observed in plasma after cranberry, blackcurrant, and Concord grape juice intake (46–48) and may be responsible for driving beneficial vascular responses at these time points, although we could not rule out that low amounts of anthocyanins (below the limit of detection in our study) may be partly linked to vascular improvements. Indeed, anthocyanins have previously been detected in plasma at 1–2 h after anthocyanin-rich food consumption, although only at low nanomolar concentrations (3, 49, 50). However, it was highly unlikely that flavanol metabolites mediated vascular changes in our study because we were unable to detect flavanol metabolites despite a limit of sensitivity of 2–16 nmol/L.

TABLE 5
LC-qTOF identification of phenolic acids and aromatic compounds detected in plasma after consumption of a blueberry drink containing 766 mg total polyphenols by healthy volunteers ($n = 10$)[1]

Compounds	RT (min)	$[M-H]^-$ (m/z)	Formula	LOD (μmol/L)	LOQ (μmol/L)
Gallic acid	4.6	169.0142	$C_7H_6O_5$	0.04	0.13
Protocatechuic acid	9.4	153.0193	$C_7H_6O_4$	0.10	0.33
3′/4′-Hydroxyhippuric acid	10.5	194.0459	$C_9H_9NO_4$	0.05	0.17
3,4-Dihydroxyphenylacetic acid	11.9	167.0350	$C_8H_8O_4$	0.15	0.50
p-Hydroxybenzoic acid	13.9	137.0244	$C_7H_6O_3$	0.14	0.47
2,5-Dihydroxybenzoic acid	14.4	153.0193	$C_7H_6O_4$	0.01	0.03
Chlorogenic acid	14.7	353.0878	$C_{16}H_{18}O_9$	0.01	0.03
Hippuric acid	15.4	178.0510	$C_9H_9NO_3$	0.01	0.03
4-Hydroxyphenylacetic acid	15.5	151.0401	$C_8H_8O_3$	0.10	0.33
Vanillic acid	15.8	167.0350	$C_8H_8O_4$	0.10	0.33
Caffeic acid	16.3	179.0350	$C_9H_8O_4$	0.01	0.03
2,4-Dihydroxybenzoic acid	16.3	153.0193	$C_7H_6O_4$	0.01	0.03
Isovanillic acid	16.8	167.0350	$C_8H_8O_4$	0.05	0.17
Syringic acid	16.9	197.0455	$C_9H_{10}O_5$	0.06	0.20
2,3-Dihydroxybenzoic acid	17.1	153.0193	$C_7H_6O_4$	0.02	0.07
3-Hydroxybenzoic acid	17.3	137.0244	$C_7H_6O_3$	0.04	0.13
Homovanillic acid	17.4	181.0506	$C_9H_{10}O_4$	0.03	0.10
3-Hydroxyphenylacetic acid	17.5	151.0401	$C_8H_8O_3$	0.12	0.40
3-(*p*-Hydroxyphenyl)propionic acid (RS)	19.3	165.0557	$C_9H_{10}O_3$	0.02	0.06
p-Coumaric acid	19.8	163.0401	$C_9H_8O_3$	0.02	0.08
3-(4-Hydroxy-3-methoxyphenyl)propionic acid	20.5	195.0663	$C_{10}H_{12}O_4$	0.02	0.05
3-(3-Hydroxyphenyl)propionic acid	21.0	165.0557	$C_9H_{10}O_3$	0.03	0.10
Sinapic acid	21.0	223.0612	$C_{11}H_{12}O_5$	0.02	0.07
Ferulic acid	21.1	193.0506	$C_{10}H_{10}O_4$	0.04	0.13
Isoferulic acid	22.1	193.0506	$C_{10}H_{10}O_4$	0.06	0.20
trans-3-Hydroxycinnamic acid	22.3	163.0401	$C_9H_8O_3$	0.02	0.07
Benzoic acid	24.0	121.0295	$C_7H_6O_2$	0.10	0.33
Phenylacetic acid	24.4	135.0452	$C_8H_8O_2$	0.10	0.33
2-Hydroxycinnamic acid	24.6	163.0401	$C_9H_8O_3$	0.06	0.20
Rosmarinic acid	24.7	359.0722	$C_{18}H_{16}O_8$	0.07	0.23
2-Hydroxybenzoic acid	25.5	137.0244	$C_7H_6O_3$	0.01	0.03
trans-Cinnamic acid	28.7	147.0451	$C_9H_8O_2$	0.15	0.50
Kaempferol	29.4	285.0404	$C_{15}H_{10}O_6$	0.01	0.03

[1] LC-qTOF, Liquid Chromatography coupled to quadrupole Time-Of-Flight mass spectrometry; (M-H)-, negative ion mode; LOD, limit of detection; LOQ, limit of quantification; RS, recovery standard; RT, retention time.

Improvements in FMD at 6 h after consumption were correlated with the appearance of metabolites derived from anthocyanin and chlorogenic acid, notably hippuric, hydroxyhippuric acid, and homovanillic acid. Because intact anthocyanin and flavanol glucuronides and sulfates are not expected in plasma at 6 h postconsumption (49–51), this finding suggests that these smaller phenolic derivatives are more likely to have mediated the observed vascular effects. Although hippuric acid and hydroxyhippuric acid may derive from aromatic amino acid metabolism, they have been reported as major urinary polyphenol metabolites after blueberry, blackcurrant juice, tea, and other polyphenol-rich food interventions (46, 49, 52–56). Previous studies with cocoa appeared to suggest that such metabolites are not associated with increases in FMD (8), although they are present in the circulation at a significantly lower amounts after cocoa compared with blueberry intakes (57, 58), whereas homovanillic acid, vanillic acid, and benzoic acid, which are known urinary and plasma metabolites of hydroxycinnamates and flavonoids, (59) may also influence improvements in FMD.

Such phenolic metabolites may mediate improvements in endothelial function by increasing the bioavailability of nitric oxide via their potential to inhibit NAPDH oxidase. NADPH oxidase activity was observed to be significantly reduced at all time points after intervention with blueberry polyphenols, although the degree of its inhibition was greatest at 1, 2, and 6 h postintervention and was correlated well with increases in FMD and with plasma concentrations of vanillic acid, hippuric acid, and homovanillic acid, suggesting that there may be an association between such phenolics, NADPH oxidase inhibition, and FMD. Reductions in NADPH oxidase activity have previously been linked to alterations in nitric oxide concentrations via a reduction in the production of superoxide (31, 60). Indeed, hippuric, vanillic, and homovanillic acids have structural homologies to the pharmacologic NADPH oxidase inhibitor apocynin (61) and have been proposed as potent NAPDH oxidase inhibitors in endothelial cells (31), with ferulic acid capable of restoring endothelium dependent vasodilation of aortas from spontaneously hypertensive rats via a reduction in NADPH-dependent superoxide production in aortas (62). Furthermore, the ingestion of chlorogenic acid has also been shown to improve endothelium-dependent vasodilation and decrease blood pressure in spontaneously hypertensive rats via decreased NADPH-dependent superoxide anion production and a parallel increase in urinary nitric oxide metabolites (63), whereas pure chlorogenic

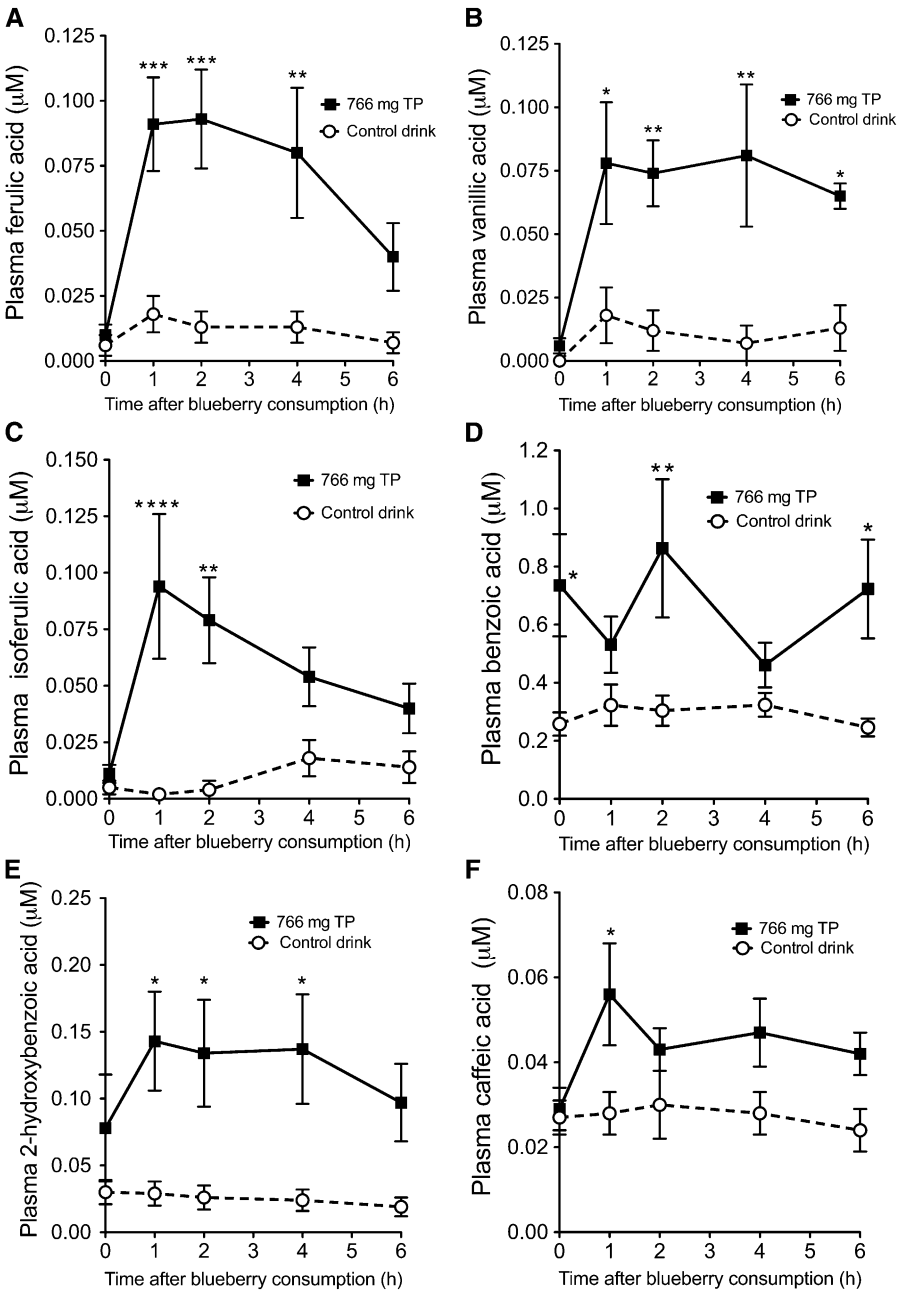

FIGURE 3. Mean (±SEM) plasma concentrations of ferulic acid (A), vanillic acid (B), isoferulic acid (C), benzoic acid (D), 2-hydroxybenzoic acid (E), and caffeic acid (F) after blueberry or control drink consumption (n = 10). Data were analyzed by using 2-factor repeated measures ANOVA with time and treatment as the 2 factors [significant effect of time ($P < 0.0008$), treatment ($P < 0.0001$), and the interaction between time and treatment ($P < 0.004$)]. Post hoc analyses were conducted by using a Bonferroni multiple-comparisons test. **********Significantly different compared with the control drink at the specified time point: *P = 0.01–0.05, **P = 0.001–0.01, ***$P < 0.001$, ****$P < 0.0001$. TP, total blueberry polyphenols.

acid has decreased blood pressure in healthy volunteers (64). Our data suggested that circulating small phenolic metabolites that derive from the ingestion of anthocyanins and other flavonoids/polyphenols present in blueberry may inhibit NADPH oxidase and, thus, lead to increased nitric oxide bioavailability and subsequent increases in endothelial-dependent dilation.

Various limitations should be considered when interpreting the trial datasets. First, although our data suggested that causality may exist between improvements in FMD and the occurrence of plasma polyphenol metabolites, we could not exclude the fact that other compounds present in blueberry and not in the macronutrient- and micronutrient-matched control drink, such as fiber and/or minerals, might also have been causally related to the biological outcomes. Second, the short time frame of the study limited the biological significance of the finding and did not predict the benefits of longer-term consumption on endothelial function. Finally, the study population, which consisted of healthy young men, cannot be extended to the general population, and additional work is required to observe whether, eg, women, the elderly, and CVD patients experience similar vascular benefits. In addition, epidemiologic studies have reported an association between a higher anthocyanin intake from the diet with

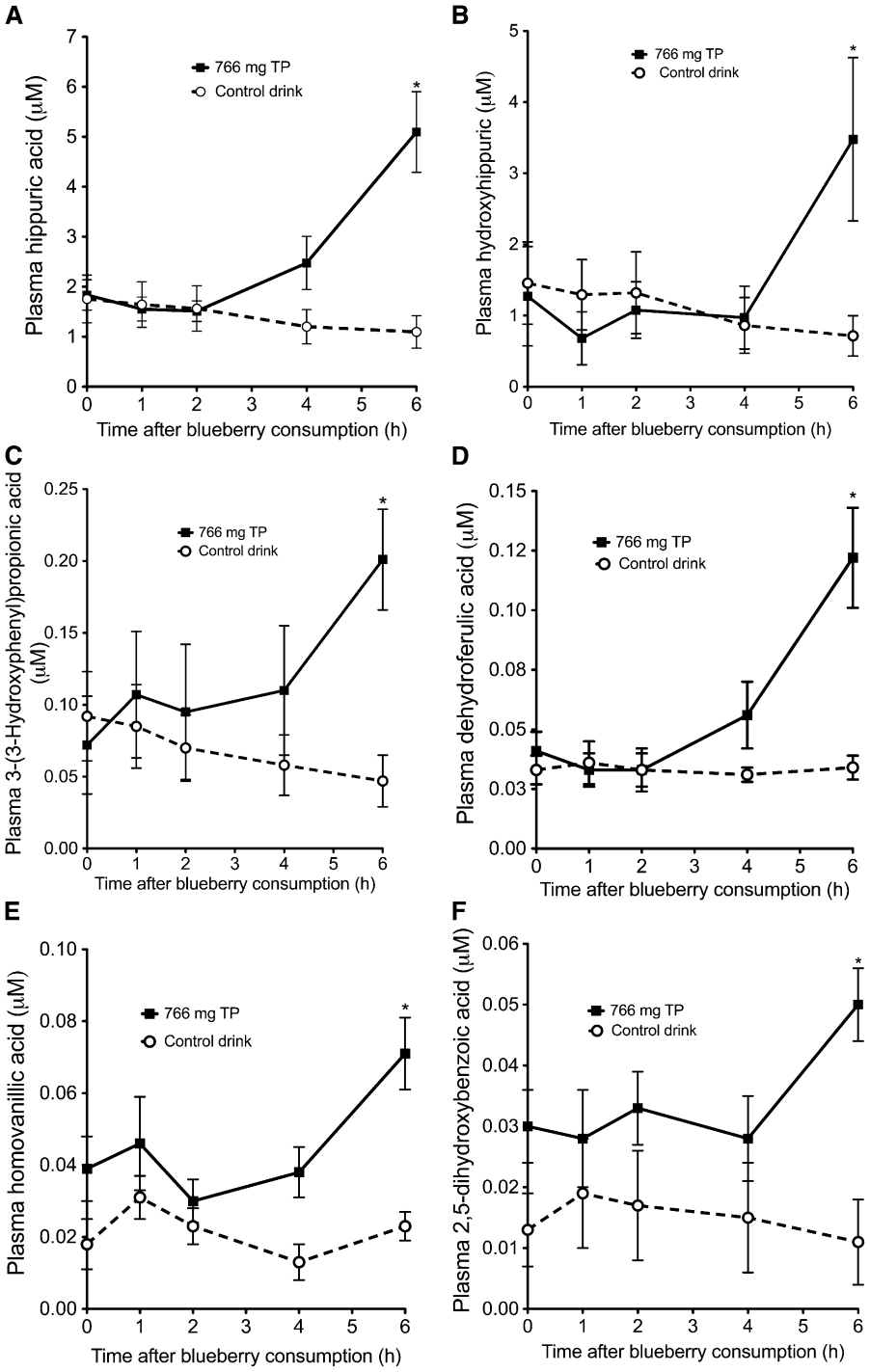

FIGURE 4. Mean (±SEM) plasma concentrations of hippuric acid (A), hydroxyhippuric acid (B), 3-(3-hydroxy)-phenylpropionic acid (C), 3-(4-hydroxy-3-methoxyphenyl)propionic acid or dehydroferulic acid (D), homovanillic acid (E), and 2,5-dihydroxybenzoic acid (F) after blueberry or control drink consumption (n = 10). Data were analyzed by using 2-factor repeated-measures ANOVA with time and treatment as the 2 factors [significant effect of time ($P < 0.036$), treatment ($P < 0.009$), and the interaction between time and treatment ($P < 0.007$)]. Post hoc analyses were conducted by using a Bonferroni multiple-comparisons test. *Significant differences compared with the control drink at the specified time point, *P = 0.01–0.05. TP, total blueberry polyphenols.

lower risk of myocardial infarction and death from CVD (65, 66); however, the range of anthocyanin intake in these studies was very low (~2–35 mg/d).

Nevertheless, to our knowledge, our data provide the first evidence that circulating small phenolic metabolites derived from blueberry polyphenols may be partly responsible for acute improvements in endothelial function in healthy individuals, and these improvements may be dependent on the potential of such metabolites to inhibit neutrophil NADPH oxidase. However, the causal relation, clinical relevance, and potential long-term health benefits of blueberry polyphenols on vascular function remain to be established.

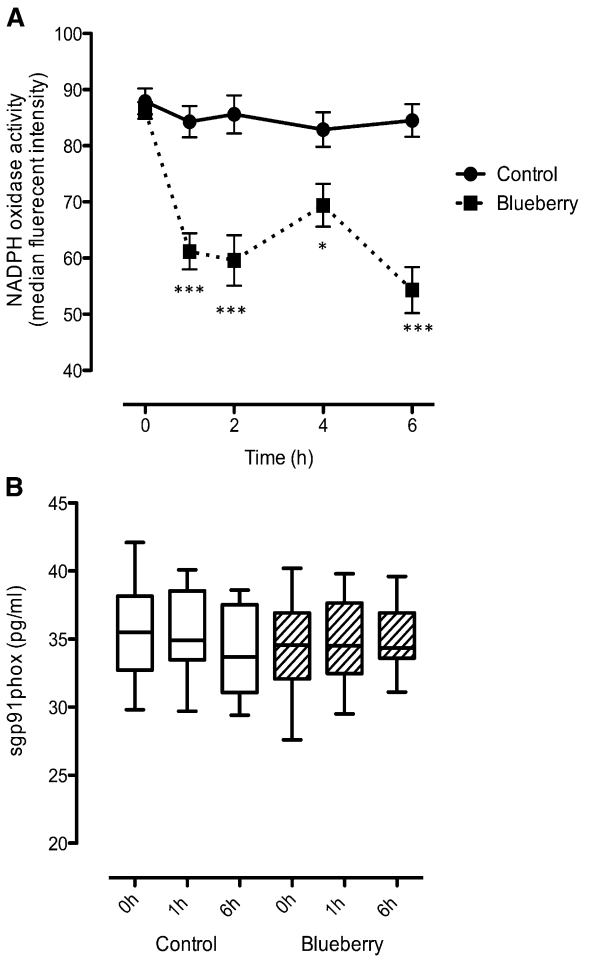

FIGURE 5. A: Mean (±SEM) neutrophil NADPH oxidase activity after a blueberry (766 mg total blueberry polyphenols) or control (0 mg total blueberry polyphenols) intervention (n = 10). Data were analyzed by using 2-factor repeated-measures ANOVA with time and treatment as the 2 factors [significant effect of time ($P < 0.001$), treatment ($P < 0.001$), and the interaction between time and treatment ($P = 0.0003$)]. Post hoc analyses were conducted by using a Bonferroni multiple-comparisons test. *·***Significant differences compared with the control drink at the specified time point: *P = 0.01–0.05, ***$P < 0.001$. B: Mean (±SEM) plasma concentrations of soluble-gp91^{phox} after a blueberry (766 mg total blueberry polyphenols) or control (0 mg total blueberry polyphenols) intervention (n = 10). No significant differences were shown between plasma concentrations of soluble-gp91^{phox} after the blueberry (766 mg total blueberry polyphenols) or control (0 mg total blueberry polyphenols) intervention ($P > 0.05$).

In conclusion, our data suggest that the consumption of blueberry at dietary intakes (100–240 g; equivalent to 319, 639, and 766 mg total polyphenols) may have public health relevance in maintaining circulatory function. Furthermore, such an intake of polyphenols need not be restricted to blueberry alone but may be achieved through the intake of other berries and anthocyanin-rich foods and beverages. With regard to the higher intakes reported in the current study, we suggest that these intakes are most likely through novel, functional foods and beverages in which polyphenols have been incorporated

The authors' responsibilities were as follows—JPES: was the principal investigator of the study; JPES, AR-M, TWG, and CH: designed study protocols; AR-M, CR, TB-M, and ST: coordinated and conducted the RCTs; AR-M: undertook all of the FMD measurements under the supervision of CH; AR-M, CR, TB-M, TWG, and ST: undertook experimental measurements, including the metabolite analysis and measurements of NADPH oxidase activity; JPES, AR-M, and CH: collaborated on the manuscript preparation; and all authors: read and approved the final manuscript. The funders of this study had no input on the design, implementation, analysis or interpretation of the data. The authors received, by way of a gift, the experimental diets from Wild Blueberry Association of North America. None of the authors declared any other conflicts of interest.

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