Free Radical Research, November 2005; 39(11): 1241–1248

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Effect of daily fruit ingestion on angiotensin converting enzyme activity, blood pressure, and oxidative stress in chronic smokers

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Accepted by Professor A. Azzi

(Received 30 June 2005; in revised form 3 August 2005)

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Abstract

Objective: This study examined whether, daily fruit (blueberries) consumption (250 g) for three weeks or acute fruit ingestion (250 g) would attenuate angiotensin converting enzyme (ACE) activity and reduce oxidative stress in chronic cigarette smokers.

Methods: Twenty subjects were recruited and randomized into fruit or control groups. Blood samples and blood pressure were obtained at baseline and then pre and one hour post when subjects returned to the lab three weeks later. To examine acute effects, the fruit group immediately ingested 250 g of blueberries after returning and at least one hour prior to the post blood draw. Plasma samples were analyzed for ACE activity, F_2- isoprostanes and lipid hydroperoxides (LH) as measures of oxidative stress, and ferric reducing ability of plasma (FRAP) as a measure of antioxidant potential. A 2 (treatment) × 3 (time) repeated measures ANOVA was used for statistical analysis. If interaction was significant, then Student's *t*-tests were used to further examine this relationship. For these comparisons, a Bonferroni adjustment was made with statistical significance set at $P < 0.025$.

Results: The pattern of change between treatments was not significant for any variable except LH ($P < 0.001$).

Conclusion: This study indicates that LH are significantly reduced by daily fruit consumption, but not affected by acute ingestion. This finding could be one way in which fruit consumption contributes to prevention of cardiovascular disease.

Keywords: *Angiotensin converting enzyme, oxidative stress, F2-isoprostanes, smokers, lipid hydroperoxides*

Introduction

Reactive oxygen species (ROS) are involved in the etiology of aging, carcinogenesis, pathophysiology of many diseases, and muscle soreness and damage during exercise [1,2]. Given the potential involvement of ROS in vascular diseases and detrimental cellular processes, much research has focused on the potential beneficial effects of antioxidant consumption and suppression of oxidative stress [3–8].

In relation to antioxidant compounds and oxidative stress, many potential health benefits of increased fruit and vegetable consumption have been suggested, such as cancer prevention and reduction of neurological degeneration [9]. Experimental and observational data strongly suggest that regular intake of fruits and vegetables reduces oxidative stress and helps prevent CVD [10]. Fruits and vegetables are known to contain varying amounts of several classes of polyphenolic antioxidant compounds [11]. Polyphenols have been

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis
DOI: 10.1080/10715760500306836

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shown to attenuate oxidative and inflammatory stress [12] and alleviate some of the detrimental effects associated with ROS production [13,14]. In particular, blueberries have been suggested to be important in preventing degeneration in neural and cerebellar function from ROS [15] and in preventing inflammation from tumor necrosis factor [12].

The renin-angiotensin system and angiotensin converting enzyme activity (ACE), has been found to be heavily involved in inflammatory vascular disease [16]. Cigarette smoking contributes to this process and is associated with elevated blood pressure and direct damage to endothelial cells [17]. Polyphenols are known to possess anti-angiogenic properties [18]. Actis-Gorretta et al. [19] found the polyphenolic compounds flavan-3-ols and procyanidins significantly reduced ACE activity *in vitro*. It was found that this inhibition of ACE activity was based on a competition for substrates rather than a direct antioxidant effect on ACE. Additionally, Zuhair et al. [20] found that pumpkin seed oil significantly enhanced the hypotensive effect of the ACE inhibitor captopril versus captopril alone. The results were attributed to antioxidant effects of the pumpkin seed oil. Aviram et al. [21] observed that 1.5 mmol of total polyphenols per day from pomegranate juice inhibited ACE activity and reduced systolic blood pressure in hypertensive subjects. Although not precisely matched to pomegranates in polyphenolic variation and content, blueberries do contain some of the same classes of polyphenols such as anthocyanins [22]. The increase in ROS and endothelial damage associated with increased ACE activity originate from increased formation of angiotensin II and not ACE itself.

Angiotensin II stimulates ROS from sources such as NADPH oxidases. As ACE is a glycoprotein peptidyldipeptide hydrolase enzyme whose main known functions are to cleave histidyl-leucine from angiotensin I to form angiotensin II, it is not known what effect ROS have on ACE directly [16]. However, antiangiogenic drugs such as captopril also exhibit antioxidant properties which interact with ROS reducing the pharmacologic potency [23]. Therefore, ROS have an indirect effect on ACE. In this regard, reducing ROS with phenolic compounds would maintain the pharmacologic activity of the ACE inhibitor.

F_2-isoprostanes are a recently described class of prostaglandin-like compounds produced by non-cylooxygenase free radical mediated lipid peroxidation of arachidonic acid. F_2-isoprostanes are recognized as a sensitive and stable marker of oxidative stress and exhibit biological activity *in vitro,* causing smooth muscle vasoconstriction and platelet aggregation [24]. Cacceta et al. [25] found that both plasma and urinary F_2- isoprostanes were significantly reduced by the polyphenols in dealcoholized red wine. We have previously observed that blueberry consumption for one week significantly reduced LH but not F_2-isoprostanes without altering the plasma antioxidant potential [7]. The reason for the difference in study effects on F_2-isoprostanes is not known. Although, we have observed no change in plasma antioxidant potential after blueberry consumption which implies the potential does not directly impact F_2-isoprostanes or LH, plasma antioxidant potential has been found to be inversely related to oxidative stress in some disease states [26].

Therefore, given the health implications of increasing polyphenols in the diet, we examined whether, blueberries given in amounts equal to the minimum suggested daily fruit recommendations would have any effect on blood pressure (BP), ACE activity, or oxidative stress. Should these benefits exist, we also sought to examine the time course and potential amounts of blueberries necessary to achieve them by examining both chronic and acute ingestion. We hypothesized that both chronic and acute blueberry consumption would attenuate ACE activity and oxidative stress markers in smokers.

Materials and methods

Participants

Twenty subjects were recruited who had smoked at least one pack of cigarettes daily for a minimum of one year. Informed written consent was obtained from each subject, and the experimental procedures were in accordance with the policy statements of the Institutional Review Board of Appalachian State University. Subjects reported to the lab for baseline testing and then again three weeks later. Height and weight measures and all blood samples and blood pressure (BP) readings for both lab sessions were taken between 7:00 a.m. and 9:00 a.m.

Blood pressure (BP)

BP readings were obtained at baseline and then again when subjects returned to the lab three weeks later. BP readings were taken, with subjects in a seated position, after subjects sat quietly for 10 min. Two BP readings were taken in the same arm ten minutes apart and the values averaged. After the initiaal three-week period, BP was taken upon arriving to the lab and again one hour later.

Supplementation

Subjects were randomized into blueberry (BB) or control (CON) groups. During the following three weeks, BB ingested 250 g of blueberries daily. Both BB and CON otherwise maintained a usual diet with the inclusion of several study restrictions. Subjects followed a diet (using a food list) that prohibited

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consumption of large amounts of fruit and vegetables, and subjects agreed to avoid all vitamin supplementation during the study. During the last week of supplementation, subjects recorded a seven-day food record to determine if both groups had ingested similar diets. The food records were analyzed using the ESHA version 7.9 food processor software program (ESHA Research, Salem, OR).

Upon arriving to the lab for the second session, the potential acute effects of BB consumption were examined. BB immediately ingested 250 g of blueberries after having initial BP taken and blood drawn and at least one hour prior to the last blood draw and BP measurement.

Sample collection

Blood samples were collected from the antecubital vein area between 7:00 a.m. and 9:00 a.m. the first day of reporting to the lab for baseline testing as well as the second lab visit three weeks later. Blood samples were collected into heparinized and serum separator (SST) vacutainer tubes. The tubes were immediately placed on ice and then spun at 1500*g* for 10 min at 4°C. The plasma 161 from the heparin tubes was aliquoted into cryotubes, snap frozen in liquid nitrogen and stored at −80°C until analysis for F_2- isoprostanes, ferric reducing ability of plasma (FRAP), and LH. The plasma from the SST tube was aliquoted into cryotubes, snap frozen in liquid nitrogen and stored at −80°C until analysis for ACE activity.

Analytical measurements

Angiotensin converting enzyme activity. ACE activity was determined using a colorimetric kit obtained from American Laboratory Products Company (Windham, NH). The assay was based upon the ability of ACE enzyme to produce hippuric acid from a synthetic substrate at a wavelength of 382 nm. One ACE activity unit was defined as 1 μmol of hippuric acid produced per minute. Values were obtained from linear regression of a standard curve ranging from 250 to 2500 μmol hippuric acid.

F_2-isoprostanes. Plasma F_2-isoprostanes were determined using gas chromatography mass spectrometry according to the methodology of Morrow [24]. Briefly, free F_2-isoprostanes were extracted from 1 ml of plasma. One to five pmol of deuterated $[^2H_4]PGF_{2\alpha}$ internal standard was added and the mixture vortexed. This mixture was then added to a C_{18} Sep Pak column, followed by silica solid phase extractions. F_2-isoprostanes were converted into pentafluorobenzyl esters, subjected to thin layer chromatography, and then converted to trimethylsilyl ether derivatives. Samples were then analyzed by a negative ion chemical ionization GC-MS using a Nermag R10-10C mass spectrometer interfaced with an Agilent computer system.

Lipid hydroperoxides. LH of duplicate samples was determined after chloroform extraction using spectrophotometric analysis and a kit (#705002) obtained from Cayman Chemical (Ann Arbor, MI). LH is highly unstable and reacts readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as chromogen. Briefly, 100 μl of plasma sample were pipetted into duplicate test tubes, and 100 μl of extract R saturated methanol (Fisher Scientific, Pittsburgh, PA) were added to each tube. All tubes were then vortexed and had 750 μl of cold chloroform (Fisher Scientific, Pittsburgh, PA) added, and the tubes were again vortexed. All tubes were then centrifuged at 1500*g* for 5 min at 4°C according to protocol instructions. Then, 500 μl of the bottom chloroform layer were extracted from each tube and immediately placed in ice. At this point, 450 μl of a 2:1 ratio deoxygenated chloroform-methanol mixture were added to each tube, and the tubes were vortexed. A standard curve was prepared using a hydroperoxide standard and varying amounts of the 2:1 ratio deoxygenated chloroform methanol mixture giving a range of zero to 5 nmol and a total volume of 950 μl. Lastly, 50 μl of chromogen were added to each sample and standard in duplicate, and the tubes were vortexed. All tubes were incubated at room temperature for 5 min, and then 300 μl of each standard and sample were removed and placed in a 96-well glass plate and read at 500 nm in a microplate reader (Biotek uQuant, Winooski, VT). LH concentration was determined from a linear regression line generated from the standard curve of cumene hydroperoxide.

Total plasma antioxidant potential. Total plasma antioxidant potential was determined by the FRAP assay according to the methodology thodology of Benzie [27]. The basis of this assay is that water-soluble reducing agents (antioxidants) in the plasma will reduce ferric ions to ferrous ions, which then react with an added chromogen. Working FRAP solution was prepared daily and consisted of 300 mmol per liter acetate buffer with the pH adjusted to 3.6 (3.1 g sodium acetate (Sigma, St. Louis, MO) and 16 ml of 1N acetic acid (Sigma, St. Louis, MO) per liter of buffer solution); 10 mmol per liter TPTZ (2,4,6-tripyridyl-s-triazine, (Sigma, St. Louis, MO) in 40 mmol HCl (Fisher Scientific, Pittsburgh, PA); 20 mmol iron trichloride hexhydrate (Sigma, St. Louis, MO) in doubly distilled deionized water water. Working FRAP reagent was prepared as required by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution,

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Table I. Descriptive characteristics.

	Blueberry	Control	*P*-value
Age (years)	25.9 ± 3.3	29.4 ± 4.2	0.592
Ht (cm)	175.2 ± 3.6	174.2 ± 4.3	0.862
Wt (kg)	91.1 ± 8.8	88.5 ± 10.2	0.863
Body fat (%)	19.9 ± 2.6	20.0 ± 2.7	0.991
BMI	29.7 ± 2.8	29.0 ± 3.2	0.854
Cigarettes per day	16.7 ± 2.4	9.2 ± 2.4	0.07
Antioxidant Intake (A, E, C)			<0.05

Values are means ± SEM.

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and 2.5 ml iron trichloride hexhydrate solution. The working FRAP solution was placed in a water bath and warmed to 37°C.
Then, 100 μl of either standard, sample, or blank (doubly distilled deionized water), respectively were added to glass test tubes containing 3.0 ml of warmed FRAP reagent and vortexed. All tubes were then incubated at 37°C for four minutes and read at 593 nm in a spectrophotometer (Genesys-5, Thermo Spectronic, Rochester, NY). Samples and standards were analyzed in duplicate, and FRAP values were expressed as vitamin C equivalents as determined by linear regression from a vitamin C standard curve (0–1000 μmol).

Statistical analysis

A 2 (treatments: BB and CON) × 3 (times: baseline, pre, and post) repeated measures ANOVA was used to test main effects. If interaction was significant, then Student's *t*-tests were used to further examine this relationship. For these comparisons, a Bonferroni adjustment was made with statistical significance set at $P < 0.025$. Pearson product-moment correlations were used to test the relationship between levels of ACE activity, plasma antioxidant potential, F_2-isoprostanes, and ROOH measures. Paired *t*-tests were used to compare differences in descriptive measures. Statistical significance was set *a priori* at the $P < 0.05$ level, and values were expressed as means ± SEM. All statistical analyses were done using Instat version1.01 (San Diego, CA) and SPSS version 11.5 (Chicago, IL). Statistical power was calculated to be at 85% or better for all variables.

Results

Subject demographics and diet

Subject descriptives are presented in Table I. These measures were not different between groups. Differences in antioxidant intake (vitamins A, E, and C) were also not significantly different (Table I). Energy and macronutrient intake also did not differ between groups (data not shown).

Biochemical measures and blood pressure

F_2-isoprostane concentration was not affected by three-weeks of blueberry supplementation or acute ingestion of blueberries ($P > 0.05$ for treatment, time and interaction effects) (Figure 1). Likewise, FRAP values (Figure 2) and ACE activity (Figure 3) were not affected by the three-week supplementation or acute ingestion ($P > 0.05$ for treatment, time, and interaction effects). Systolic and diastolic pressures were not significantly affected by any part of the study treatments (Figure 4). Interestingly, the pattern of change in LH concentration was significant and declined 50% in BB during the three-week supplementation compared to CON. The acute ingestion had no effect on LH (Figure 5).

Correlations

There were significant positive correlations between F_2-isoprostanes and systolic BP ($r = 0.474$, $P = 0.008$) and diastolic BP ($r = 0.369$, $P = 0.045$) in BB. FRAP was significantly negatively correlated with LH ($r = -0.555$, $P = 0.001$) in CON.

Discussion

We examined whether daily consumption of blueberries for three-weeks in amounts representing 50% of the servings recommended by the Food guide pyramid would have any effect on ACE activity, BP,

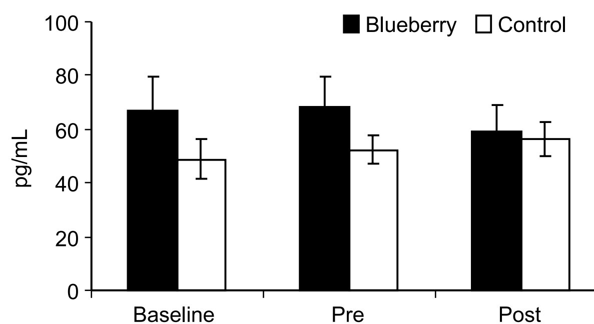

Figure 1. There was no effect of blueberry ingestion on F2-isoprostanes. Treatment: $P = 0.308$, Time: $P = 0.868$, Interaction: $P = 0.45$. Values are means ± SEM.

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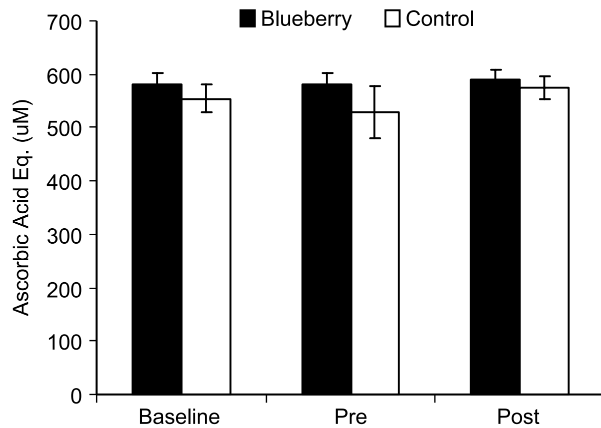

Figure 2. There was no effect of blueberry ingestion on plasma antioxidant potential. Treatment: $P = 0.291$, Time: $P = 0.533$, Interaction: $P = 0.747$. Values are means ± SEM.

and oxidative stress. We chose this amount of fruit because we thought it to be a realistic amount that might be routinely consumed for any length of time. Furthermore, we were interested as to whether fruit could be consumed in less than nutraceutical amounts and still provide immediate health benefits.

To examine changes in oxidative stress, F2-isoprostanes are recognized as a sensitive and stable marker of oxidative stress and exhibit biological activity *in vitro,* causing smooth muscle vasoconstriction and platelet aggregation [24]. F_2-isoprostanes are a recently described class of prostaglandin-like compounds produced by non-cylooxygenase free radical mediated lipid peroxidation of arachidonic acid. Whether, this occurs at physiological concentrations is not clear, but it is possible that F_2-isoprostanes may mediate some of the effects of oxidative stress. Smoking has been shown to elevate F_2- isoprostane values [24]. In support of this observation, subjects in our study had baseline F_2-isoprostane values 33% higher than those we previously observed in fit athletes [28]. We did not observe any affects of blueberry supplementation on

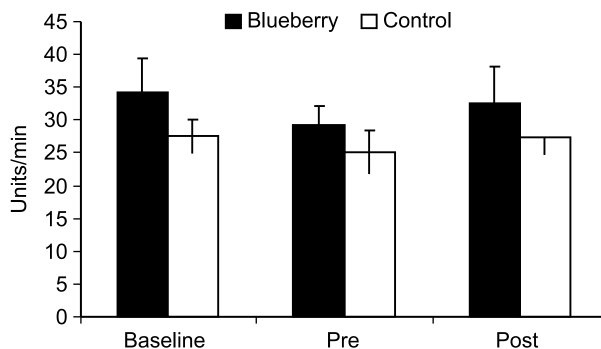

Figure 3. There was no effect of blueberry ingestion on angiotensin converting enzyme activity. Treatment: $P = 0.304$, Time: $P = 0.356$, Interaction: $P = 0.687$. Values are means ± SEM.

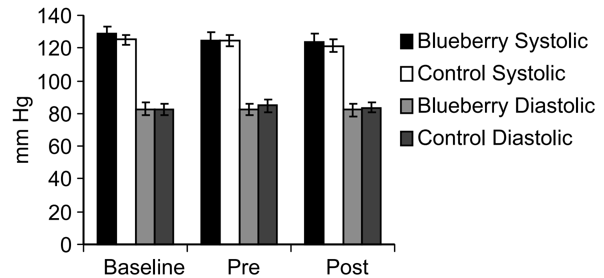

Figure 4. There was no effect of blueberry ingestion on systolic or diastolic blood pressure. (Systolic) Treatment: $P = 0.740$, Time: $P = 0.120$, Interaction: $P = 0.680$:(Diastolic) Treatment: $P = 0.856$, Time: $P = 0.606$, Interaction: $P = 0.567$. Values are means ± SEM.

F_2-isoprostanes in this study. This is in agreement with a prior study in which we supplemented young non-smoking college age males with 1.5 cups of blueberries daily for one week prior to exercising to exhaustion in the heat [7]. In contrast to the current study, and also using a different class of polyphenol, Dillon et al. [13] reported that supplementation for 14 days with aged garlic extract significantly reduced plasma and urinary F_2- -isoprostanes in groups of smoking and nonsmoking men and women [29–31]. A possible reason for the discrepancy in effects on F_2-isoprostanes is the many different types of polyphenolic compounds found in fruits and vegetables and the variation in gut bioavailability [14]. Another possibility is that a greater amount and longer period of supplementation may be necessary to achieve tissue and plasma saturation.

It was hypothesized that supplementation with blueberries would increase the antioxidant potential of the blood, but this did not occur. Plasma FRAP values were not significantly different in any group during the study. We have observed this lack of effect on FRAP

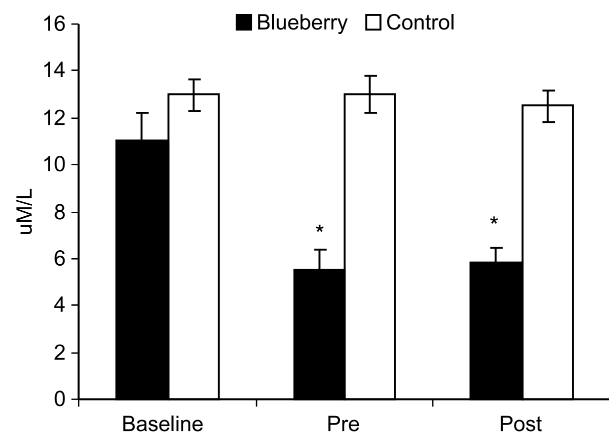

Figure 5. The pattern of change in lipid hydroperoxides was different between blueberry and control groups. Treatment: $P = 0.000$, Time: $P = 0.000$, Interaction: $P = 0.001$. *Significantly different from baseline after Bonferroni correction. Values are means ± SEM.

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values in a prior study with blueberries [7] and this is supported by Alia et al. [3] who found that grape antioxidants did not affect FRAP in rats. In contrast, although using a different antioxidant detection protocol and nutraceutical administration of tea polyphenols, Bub et al. [32] found that polyphenols were able to increase the antioxidant capacity of the blood. Whether the difference in our study is due to the difference in procedures, or type and concentration of polyphenol administered, is not known. It is of importance that total plasma antioxidant potential has been found to be inversely related to oxidative stress in some disease states [26]. In particular, FRAP has been found to be inversely correlated with values of glycated hemoglobin in type 2 diabetics [33] and significantly decreased in patients with fatty liver disease [34]. Interestingly, a high polyphenol intake might be less related to antioxidant chemistry but more to the ability of these compounds to sequester iron in biological systems. Excessive availability of iron is known to be increased in certain disease states, and iron is a catalyst in the production of ROS via the Fenton reaction [35].

Besides potential effects on BP, ACE inhibitors interfere with the renin-angiotensin system and reduce the risk of CVD and stroke by multiple beneficial effects on vascular cells. Increased oxidative stress is associated with endothelial dysfunction, and endothelial dysfunction is related to hypertension. Activity of the ACE enzyme increases oxidative stress and reduces availability of nitric oxide. Therefore, ACE inhibitors are able to exert antioxidant effects by suppression of angiotensin II production [19,23, 36–38].

ACE activity and BP were not affected by the amounts of blueberries given in this study. This is in contrast to Aviram et al. [21] who found that 50 ml of 1.5 mmol total polyphenols per day for two-weeks resulted in a 36% reduction in ACE activity and a 5% reduction in systolic BP. Although the plasma effect of the polyphenol administration was not directly stated in this study, it was implied that the subjects had adhered to the protocol by analysis of serum total polyphenol analysis. We were unable to measure changes in plasma polyphenols due to having no suitable assay and may not have reached similar plasma polyphenol concentrations as Aviram et al. [21]. Therefore, it is possible that the concentration of polyphenols delivered by the blueberry ingestion was less than that administered by Aviram et al. [21]. It may simply require a nutraceutical concentration of polyphenols to be administered to see beneficial effects upon ACE activity and BP. In support of our findings, Hodgson et al. [31] found that eight-weeks of a 55 mg daily isoflavonoid supplement did not reduce BP in subjects with high-normal pressures. Additionally, changes in urinary F_2-isoprostanes were also not affected by the flavonoids.

LH are highly reactive compounds and interact with proteins, amino acids, amines and DNA [39]. Additionally, LH originate primarily from oxidation of omega-3 and omega-6 fatty acids found in lipoproteins [40]. In the present study, BB had significantly lower concentrations of LH after three-weeks compared ared to CON. Thus, the consumption of blueberries as administered in the present study effectively suppressed LH concentrations. This finding is supported by results from a previous study in which we found that 250 g of blueberries per day for one week significantly suppressed LH compared to 1250 mg vitamin C or placebo in athletes running to exhaustion in the heat [7]. If LH predominately originate from plasma lipoproteins, then our findings indicate that blueberry supplementation resulted in an increased lipid protection in the plasma compartment.

Another possibility is that there is some evidence to suggest that consumption of a meal containing oxidized or oxidizable lipids can increase plasma concentrations of LH and oxidatively modified LDL and that concurrent antioxidant consumption will inhibit this postprandial oxidative stress [41]. Therefore, the gut certainly represents a possible location for the polyphenol effect. Relevant to the anti-atherosclerotic effects of polyphenols by inhibition of LDL oxidation, this finding helps explain how regular fruit consumption can reduce the risk of CVD [10,25].

Since F_2-isoprostanes were not affected by blueberry supplementation, it is suggestive that primary generation of these compounds occurs in extravascular tissue and not from plasma lipoproteins. F_2-isoprostanes have been found to originate from arachidonic fatty acids esterified in phospholipid [24]. Parthasarthy et al. [42] suggested that oxidized phospholipids may be extravascular in origin. If F_2-isoprostanes are extravascular in origin, then the blueberry supplementation may not have been long enough or administered in large enough amounts to exert effects in extravascular compartments.

Generally, F_2 isoprostanes and LH increase concurrently in response to an increased oxidative stress [8,28]. Our outcomes in the current study are similar to a previous study in which we observed that daily blueberry ingestion (250 g) for one week prior to running in the heat to exhaustion did not affect post exercise F_2 isoprostanes but did reduce LH 128 and 53% compared to one week daily vitamin C ingestion (1250 mg) and placebo, respectively [7].

In summary, blueberries (250 g) ingested daily for three-weeks or acutely (250 g) did not affect ACE activity, BP, plasma antioxidant capacity, or F_2-isoprostanes. Interestingly, LH was significantly reduced by the three-week ingestion period. This suggests that regular ingestion of modest amounts of blueberries may reduce the risk of CVD.

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Acknowledgements

This work was funded by a grant from Appalachian State University and National Institutes of Health Grants (to J.D. Morrow) DK-48831, GM-15431, CA-77839, and RR00095. J.D. Morrow is the recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research. Fischer/Nycom Laboratory.

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