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Direct Vasoactive and Vasoprotective Properties of Anthocyanin Rich Extracts

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Running Head: polyphenolic vasoreactivity and vasoprotection

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Abstract

Reactive oxygen species (ROS) play a critical role in the impairment of nitric oxide mediated vascular functions and overall pathogenesis associated with cardiovascular disease. Plant pigment anthocyanins are exceptionally potent oxygen radical scavengers that produce beneficial effects in diseases outside the cardiovascular system. We examined for the first time the potential coronary vasoactive and vasoprotective properties of three anthocyanin enhanced extracts prepared from Chokeberry (Ck), Bilberry (B), or Elderberry (E). Coronary arterial rings were isolated from 64 pigs and incubated in sterile tissue culture media overnight for use in one of 4 separate *in vitro* isometric force recording studies. Ck and B but not E produced dose and endothelium-dependent vasorelaxation. (%maximal relaxation at 5mg total anthocyanins/L: Ck=68$\pm$11, B=59$\pm$10). Coronary vascular tone, endothelium dependent vasorelaxation to A23187 and vasorelaxation to DEA NONOate were not affected by exposure of rings to any extract at 0.05 mg total anthocyanins/L for 5 or 30 minutes. Chokeberry extract at 0.05 mg total anthocyanins/L showed the greatest protection against loss of A23187 relaxation following exposure to ROS from pyrogallol. (Ck, % Maximal relaxation and -logED_{50} to A23187 respectively, mean$\pm$sem: Ck alone, 93±5%, 7.91±0.1; Pyrogallol alone, 76±7%*, 7.46±0.06*; Pyrogallol+Ck, 98±1%, 7.82$\pm$0.06, Control: 99$\pm$1%, 7.86± 0.07. * = $p<0.05$ vs. Control). Neither the extracts nor pyrogallol affected responses to DEA NONOate. Thus anthocyanin enhanced extracts produce endothelium dependent relaxation in porcine coronary arteries. Extract concentrations too low to directly alter coronary vascular tone protect coronary

arteries from ROS without altering vasorelaxation to endogenous or exogenous NO. These results suggest that such extracts could have significant beneficial effects in vascular disease.

Keywords: Antioxidant, Endothelium, Nitric Oxide, Superoxide

Introduction

The endothelial nitric oxide (NO) system plays a pivotal role in vascular physiology and pathology. NO is a potent vasodilator agent with anti-hypertensive, anti-thrombotic, anti-atherogenic and anti-smooth muscle proliferative properties [44]. However, the endothelial NO system is impaired in conditions associated with atherosclerosis, hypertension, diabetes and ischemia-reperfusion injury [10]. These conditions are also associated with excess vascular and extravascular production of reactive oxygen species (ROS), such as superoxide [14,15, 21, 23]. ROS impair endothelial NO functions through direct damage to the endothelium as well as by chemical quenching of NO [22]. Clearly factors that can enhance or protect the endothelial NO system, or scavenge and inactivate ROS, have the potential for far reaching beneficial impacts on cardiovascular disease.

It has been suggested that such protective and anti-oxidant effects may be responsible for the decreased incidence and risk of cardiovascular disease associated with consumption of fruits and vegetables [34]. However, extensive studies attempting to link this observation to specific plant nutritional components, such as anti-oxidant vitamins, have failed to demonstrate significant

correlations [6,20]. This has led to the suggestion that some previously overlooked, non-nutritional, component of plants is the real source of the plant's cardioprotective effect [32]. In this regard recent evidence indicates that plant anthocyanins may have strong potential as cardioprotective agents. Anthocyanins are water soluble, glycosylated, non-acetylated polyphenolic compounds that make up the red, blue and purple pigments of fruits [7,13]. These pigments are absorbed in intact (glycosylated) form from the human GI tract after oral consumption [25, 27-29, 47] and have been shown to have tumor suppressive, anti-inflammatory, anti-viral and anti-diabetic properties [16, 19, 36, 37, 42, 43].

Of potential importance to cardiovascular disease, anthocyanins are potent anti-oxidant and ROS scavengers [11,18, 33, 35, 40, 46, 48, 52]. They have been shown to inhibit oxidation of lipids [35] and LDLs [45] as well as protect cultured endothelial cells from oxidant injury [51]. A recent study indicates that the single anthocyanin, cyanindin-3-*0* glucose chloride, has a greater efficacy in protecting against oxidant degradation of LDLs than vitamins C, E and beta-carotene combined [45]. The anthocyanin concentration of fruit is highly correlated with the ORAC (oxygen radical absorbance capacity) [5,11, 33, 40, 46, 48, 52] with chokeberry, bilberry and elderberry having the highest natural intrinsic total anthocyanin content and ORAC of dozens of fruits and vegetables tested [48, 52 and Artemis International, Inc. Fort Wayne, Indiana. internal data analysis].

We have obtained novel powdered pigment extracts from Chokeberry (*Aronia melanocarpa)*, Bilberry (*Vaccinium myrtillus*) and Elderberry (*Sambucus nigra)* in order to examine their potential vasoactive and vasoprotective properties in coronary arteries. These extracts have been processed to

enrich their total anthocyanin content while preserving the naturally different individual anthocyanin profiles within in each. We have designed three experiments to test the hypotheses that, 1) the extracts are directly vasoactive, 2) the extracts directly modify endothelium dependent and independent responses to NO, and 3) the extracts protect coronary arteries from oxidant injury. This report represents the first study of any kind on the vascular properties of these preparations.

Materials and Methods

In vitro analyses of the direct vasoactive or vasoprotective properties of three berry pigment extracts were performed using isolated coronary artery rings from mature female pigs. Extracts of Elderberry (*Sambucus nigra)*, Bilberry (*Vaccinium myrtillus*) and Chokeberry (*Aronia melanocarpa)* were obtained from Atermis International Inc., Fort Wayne, Indiana. The extracts we received were in the form of lyophilized powders that had their free sugars removed to enhance the total anthocyanin content of each extract on a percent dry weight basis. The total anthocyanin and total polyphenolic concentration of each extract is listed in Table 1. The extracts also exhibit different anthocyanin profiles. Chokeberry contains 4 different anthocyanins with approximately 90% of its total anthocyanin content due to cyanidin-3-galactoside and cyanidin-3-arabinoside. These cyanidin glycosides are not present in elderberry. In contrast, over 90% of the total anthocyanin content in elderberry is due to cyanidin-3-glucoside and cyanidin-3-sambubioside which are not present in chokeberry. Bilberry contains approximately 1-15% of 15 different anthocyanins including those in

the cyanidin, peonidin, delphinidin, petunidin and malvidin classes.

Hearts from 8 female pigs were obtained from local slaughterhouses immediately after death and placed in physiological salt solution (PSS) at 4C^{o}; (PSS, mmol/l: NaCl 130, KCl 4.7, $NaHCO_3$ 14.9, dextrose 5.5, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.17, $CaCl_2 \cdot 2H_2O$ 1.60, $CaNa_2$ EDTA 0.03). Hearts then were transferred to our laboratories where the left anterior descending and circumflex coronary arteries were carefully dissected free from the surrounding tissue and washed with sterile PSS. Using sterile procedures arteries were cleaned of excess fat and connective tissue and cut into 2-4mm wide rings. Rings were transferred under sterile conditions into separate petri dishes containing sterile, HEPES buffered, serum and phenol red free, Dulbecco's Modified Eagles Media with antibiotic and then placed in an O_2/CO_2 incubator overnight utilizing standard procedures for cell and tissue cultures as described previously [2]. As such, rings were kept in a humidified environment with temperature and CO_2 concentration precisely controlled at 37 C^{o} and 5.0%, respectively.

After the incubation period, rings were removed from the dishes and washed with PSS. The endothelium was removed from half of the rings by gentle rubbing of the intima with a forceps. Next, all rings were suspended between triangular stainless steel hooks and a stationary support rod, transferred in pairs to artery baths containing PSS at 37C^{o}, pH 7.4, bubbled with 95% O_2-5% CO_2 and connected to FT.03 force transducers (Grass Instruments, Quincy MA) for isometric force recording (Grass P7 and Gould 2400S recorders). Rings were suspended at a passive tension of 10g, previously determined to produce maximal force generation in all treatment groups, and allowed to equilibrate for not less than 90 minutes. Maximal contractile force was determined in all rings by

exposure to PSS containing equimolar replacement of NaCl with 130 mM KCl. Next, prostaglandin synthesis was blocked in the rings by exposure to 10^{-5} M meclofenamic acid for 30 minutes followed by repeated rinses with PSS. All rings were then contracted to 35-45% of their individual KCl maximum with U46619 (approximately 1-2 x10^{-8}M).

Stock solutions of Elderberry (*Sambucus nigra)*, Bilberry (*Vaccinium myrtillus)* or Chokeberry (*Aronia melanocarpa)* extracts were normalized for total anthocyanin (Ta) concentration in DD H_2O and used immediately for dose-response determinations in the ring preparations. Rings were exposed to cumulative full log M Ta additions of one of the extracts over a concentration range of 0.005 B 5 mg Ta/L and any final changes from initial tone recorded. After determination of these relationships the presence or absence of a functional endothelium in the rings was determined by the presence or absence, respectively, of relaxation of the rings to A23187. In rings from four pigs the involvement of NO in any observed relaxation instead was tested by exposing rings to 100 uM -NO_2- l-arginine at the end of the last dose of pigment extract.

In separate experiments using coronary rings from 10 additional female pigs, the effect of the acute presence of berry extracts upon endothelium-dependent and independent NO mediated vasorelaxation was examined. For these experiments eight artery baths containing two rings each were used with each pig serving as its own control. All rings were incubated in vehicle control media overnight, suspended in artery baths, exposed to KCl and meclofenamate and precontracted with U46619 as described in the previous section. Next, two baths each were exposed to Elderberry, Bilberry and Chokeberry extract respectively for 5 minutes at a concentration too low to alter

vascular tone (0.05 mg/L,). Rings in two additional artery baths were not exposed to any agents during this time and served as controls. Rings were then exposed to cumulative 2 log M additions of either A23187 or DEA NONOate. A23187 is a direct, receptor independent activator of ecNOS [31]. In porcine coronary artery rings pre-treated with meclofenamate, A23187 produces dose-dependent relaxation that is entirely mediated by endothelial NO [2, 3]. (Acetylcholine, the most common receptor mediated activator of ecNOS, is not an appropriate agonist of endothelium dependent relaxation of coronary arteries from pigs because it produces marked endothelium independent contraction, with little or no relaxation of any type in porcine coronary arteries [8]). DEA-NONOate spontaneously releases NO when exposed to pH< 8.0 and thus serves as a source of NO for the rings. This source has the advantages of avoiding required intracellular bioconversion to produce NO, as is the case with nitroglycerin and does not possess the secondary, non-cGMP mediated vasodilator properties of sodium nitroprusside. In a separate group of 13 female pigs, the procedures used for acute exposure of coronary arteries to berry extracts described above was repeated with the exception that the rings were exposed to the berry extracts for 30 minutes prior to the additions of A23187 or DEA NONOate.

In additional studies coronary arterial rings from 33 additional female pigs were used to examine whether impaired endothelium dependent or independent coronary vasorelaxation following exposure to O_2^- is altered by concurrent exposure to berry extracts. Rings were incubated in vehicle control media overnight, suspended in artery baths for isometric force recording and exposed to KCl and meclofenamate as described above. Rings were then divided into four treatment

groups. Group 1 consisted of rings exposed to superoxide through the auto-oxidation of 400:M pyrogallol [24] added to the artery bath for 15 minutes. This treatment impairs, but does not abolish, endothelium dependent, NO mediated vasorelaxation and thus allows one to test whether any agent exacerbates or attenuates superoxide mediated damage to the artery [2]. Group 2 consisted of rings exposed to pyrogallol, as in Group1, but with the simultaneous presence of one if the berry pigment extracts at a concentration of 0.05mg Ta/L. Group 3 consisted of rings exposed to the extract only for 15 minutes whereas the Group 4 received no additional treatments during the 15 minute exposures in groups 1-3 and thus served as controls. Following these exposures all rings were rinsed repetitively with PSS and precontracted with U46619. Dose-response relationships to A23187 or DEA NONOate were then determined as described above.

Data Analysis

Responses to all agents were recorded as the average response of two rings per treatment type per pig. Sensitivity of the coronary rings to an agent was expressed as the -log M ED_{50} value as determined by Probit transformation and linear regression of the dose-response relationship. Differences among the means of treatment groups were tested by a paired t-test with the Bonferroni correction for multiple comparisons. Any alterations in responses to A23187 were compared with responses to DEA-NONOate in coronary artery rings from the same pig. This comparison was made to determine if any alteration in endothelium dependent relaxation caused by a treatment was a reflection of altered endothelial function related to NO or, instead, was a reflection of altered arterial smooth muscle responsiveness to NO.

Reagents

DEA-NONOate used in this study was obtained from Cayman Chemicals, Inc., Ann Arbor, MI. All other agents used in these experiments were obtained from Sigma Chemical Co., St. Louis, MO.

Results

Responses of the rings to direct application of cumulative doses of each berry extract are shown in Figure 1. Chokeberry and bilberry, but not elderberry, extracts produced dose-dependent relaxation in isolated rings with endothelium. Responses to chokeberry extract produced the greatest degree of relaxation. Rings without endothelium did not relax to any dose of the berry extracts. In rings in which the contribution of NO to this relaxation was examined, the relaxations to either chokeberry or bilberry was abolished by 100 uM NO_2 - l- arginine An example of this effect is shown in Figure 2.

The presence of any berry extract at a concentration too low to cause direct changes in arterial tone (0.05mg Ta/L) did not alter sensitivity of rings to either A23187 or DEA NONOate (Table 2). This lack of effect was seen when the extracts were added either 5 or 30 minutes prior to the administration of either vasodilator. Precontraction of rings with U46199 did not differ among any groups. Maximum relaxation to each agent was similarly unaffected by acute exposure to any of the extracts.

The effect of berry pigment extracts on the impairment of endothelium dependent vasorelaxtion to A23187 following exposure to superoxide from pyrogallol is shown in Figure 3.

Exposure of the artery rings to 400 μM pyrogallol for 15 minutes resulted in a significant decrease in maximal relaxation and sensitivity to A23187 (Figure 3 and Table 3) without altering responses to DEA NONOate (Table 4). This impairment was abolished by concurrent exposure to 0.05 mg Ta/L chokeberry extract in arterial rings from all 12 pigs used in that determination. Similar impairment in sensitivity was abolished in rings from 9 of 12 pigs used in the bilberry determination and 8 of 12 pigs used in the elderberry determination. Sensitivity of rings exposed to bilberry and pyrogallol simultaneously was not significantly different from controls but also not significantly different from rings treated with pyrogallol alone. Sensitivity of rings exposed to elderberry and pyrogallol simultaneously was significantly different from controls and from rings treated with pyrogallol alone. Precontraction of rings with U46619 was not different among any groups nor did prior exposure to pyrogallol affect the concentration of U46619 needed to precontract arteries to 35-45% of their KCl maximal contraction.

Discussion

We observed that anthocyanin rich extracts from chokeberry and bilberry, but not elderberry, produced dose dependent relaxation of coronary arteries with the chokeberry extracts exhibiting the highest potency. These differences in vasorelaxation among the extracts can not be explained on the basis of a generic, non-selective, effect of their total anthocyanin content because each dose of an extract was adjusted to the same total anthocyanin concentration. Also, it is unlikely that these differences were due to different total phenolic content in the extracts; arteries exposed to elderberry

extract were exposed to total phenolic levels similar to those in the other extracts but did not relax. These results suggest that some component or combination of components of the chokeberry and bilberry extracts, but not found in elderberry, may possess vasodilatory properties. These differences could be related to different anthocyanin profiles within each extract. However, the experiments required for a definitive answer to this postulate is beyond the scope of our current study.

Relaxation of coronary arteries to chokeberry and bilberry preparations was totally endothelium dependent. It is unlikely this was mediated through endothelial prostaglandin synthesis because such synthesis was blocked in our arterial rings. In arteries from 4 of the pigs we tested for the role of NO in these relaxations and found that such relaxation could be abolished by the application of 100uM NO_2-l –arginine. These observations suggest that the endothelial NO system may be involved in the relaxation response of coronary arteries to chokeberry and bilberry extracts.

The literature on vasoactive properties of anthocyanins is limited. Fruit pigment preparations have been shown to produce endothelium dependent relaxation of arteries but these determinations have been largely confined to pigments contained in red wine or grapes [1, 9, 26, 30, 39]. Such preparations contain some anthocyanins but also contain significant amounts of other polyphenolics that are minor or non existent components of our berry pigment preparations. However, Xu et al [49] have shown that eNOS in BAEC is upregulated following a 6 hour exposure to 0.1 uM cyanidin-3-glucoside and that a 12 minute exposures of these cells to the same compound phosphorylates NOS and enhances NOS activity [50]. Such mechanisms may underlie the endothelial dependent vasodilatory properties of our extracts. However, is not certain whether this

specific cell culture effect can be extrapolated to NO mediated relaxation of our coronary arteries because cyanidin-3-glucoside is not present in chokeberry and is a minimal component of bilberry preparations.

It would be premature to try to extrapolate the endothelium dependent relaxation property of our berry pigment extracts to potential human health benefits. Although anthocyanins are absorbed intact across the human GI tract, little is known about the effective plasma concentrations of anthocyanins following oral consumption. [25, 27-29, 47]. Cao et al [4] have reported that plasma concentrations of total anthocyanins are approximately 100nM following oral consumption of elderberry extracts or blueberries and that this amount reflects primarily just two cyanidin glycosides. This concentration is similar to the concentration of cyanidin glycosides in the chokeberry and bilberry extracts that relaxed coronary artery rings. However, no studies have been performed yet to determine the human bioavailability of anthocyanins or other components from the specific berry preparations used in our study.

Results from several studies suggest that anthocyanins and other polyphenolics may have non-cardiovascular health benefits [16, 19, 32, 36, 37, 42, 46]. However, the health benefit of any compound would be seriously compromised if it interfered with coronary arterial function. Furthermore, agents can exist in plasma in concentrations too low to directly alter vascular tone, but nevertheless alter arterial reactivity because they modify the response of arteries to another agent [2,3]. Our experiments indicate that none of the berry preparations, at a total anthocyanin

concentration too low to directly modify arterial tone, altered endothelium dependent or independent NO mediated coronary vasorelaxation acutely.

NO mediated vasorelaxation can be enhanced by scavengers of superoxide [3, 17, 41]. Because anthocyanins and polyphenolics are potent ROS scavengers one might postulate that these compounds would affect coronary arterial relaxation mediated by NO. However, for such an effect to occur, basal arterial generation of superoxide would have to be significant enough to affect the bioavailability of NO and do so to the extent it impaired NO mediated relaxation. This is an unlikely scenario in our preparations. In previous studies we have shown that ascorbate, an O_2^- scavenger, does not alter relaxation to A23187 or DEA NONOate in isolated control coronary arteries even though it readily corrects impaired NO relaxations brought about by exposure to exogenously supplied superoxide [2]. Therefore, in our study, the failure of normal NO mediated coronary vasorelaxation to be altered in the acute presence of the berry extracts is not a false negative.

Pyrogallol is an auto-oxidizing source of O_2^- [24, 31] that has been used to induce damage from reactive oxygen species in *in vitro* artery bath and other preparations [2, 31]. In our study, acute exposure of isolated porcine coronary arteries to pyrogallol resulted in an impairment of subsequent endothelium dependent, NO mediated vasorelaxation by A23187 but not DEA NONOate. This impairment can not be due to quenching of NO by superoxide when the arteries are exposed to A23187 because pyrogallol was not present during A23187 exposure. These results are identical to those we have reported previously [2] and indicate that the loss of endothelium

dependent NO mediated relaxation to A23197 following exposure to pyrogallol results from superoxide mediated damage that is functionally confined to the coronary arterial endothelium.

It is clear that the berry extracts used in this study significantly attenuated the loss of A23187 mediated relaxation of coronary arteries exposed to pyrogallol. This effect was most prominent in arteries exposed to chokeberry extracts and least prominent in those exposed the elderberry extracts. It is most likely that the beneficial effects of these extracts are due to their oxygen radical absorbance capacity (ORAC) which would tend to reduce the effective concentration of superoxide or ROS from pyrogallol reaching the arterial endothelium. More importantly, this ability to prevent loss of endothelium dependent NO mediated relaxation was achieved at an extremely low total anthocyanin concentration that roughly reflects that seen in several studies to exist in human plasma after oral consumption of these compounds. However, because the total anthocyanin concentration to which all arteries were exposed was normalized for each of the extracts used in our experiment, the total polyphenolic concentration to which the vessels were exposed had to be slightly higher for the chokeberry and bilberry extracts compared to that obtained with the elderberry preparation. Because the ORAC is also correlated with phenolic concentration in a preparation as well as anthocyanin content the enhanced efficacy of the chokeberry extract compared to the other preparations tested may be related in part to its total phenolic content [33]. Nevertheless our results extend and support recent observations that suggest that red wine or anthocyanins protect vascular endothelium from oxidant injury [12, 38, 51].

It remains to be seen if our observations, which are limited to a specific type of radical damage and arterial function, are a reflection of a more general vascular protective effect of these berry derived compounds. Should our observations be representative of a broader ability of these berry preparations to prevent oxygen radical mediated damage to arteries the potential implications are significant. Excessive exposure of the vasculature to reactive oxygen species, radical mediated damage to arteries and impairment of the endothelial NO system are key components of the pathogenesis of major cardiovascular diseases. The ability of a common, safe, component of consumable foodstuffs to attenuate such pathological conditions could have far reaching implications for individuals suffering from cardiovascular disease. That these same compounds enhance the arterial NO system suggests they could also provide additional benefits with respect to several coronary arterial functions.

In summary we have undertaken the first studies to examine the potential vasoactive and vasoprotective properties of three anthocyanin rich preparations composed primarily of the pigments in chokeberry, bilberry and elderberry. These preparations possess endothelium dependent relaxation capacity in porcine coronary arteries. At a concentration too low to directly alter coronary vascular tone they do not alter coronary responses to endogenous or exogenous NO. However, this same low concentration has significant ability to prevent loss of endothelial dependent relaxation caused by exposure of arteries to exogenous reactive oxygen species.

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References

1. **Andriambeloson E, Magnier C, Hann- Archipoff G, Lobstein A, Anton R, Beretz A, Stoclet JC, Andriantsitohaina R**. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. J Nutr. 128: 2324-2333, 1998.

2. **Bell DR, Gochenaur KE, Hecht J**. O_2^- mediated impairment of coronary arterial relaxation is prevented by overnight treatment with 1nM β-estradiol. J Appl Physiol. 93: 1952-1958, 2002.

3. **Bell DR, Rensberger HJ, Koritnik DR, and Koshy A**. Estrogen pretreatment directly potentiates endothelium-dependent vasorelaxation of porcine coronary arteries. *Am J Physiol Heart Circ Physiol* 268: H377-H383, 1995.

4. **Cao G, Muccitelli HU, Sanchez-Moreno C, Prior RL**. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. Am J Clin Nutr 73: 920-926, 2000.

5. **Cao G, Verdon CP, Wu AHB, Wang H, Prior RL**. Automated oxygen radical absorbance capacity assay using COBAS FARA II. Clin Chem. 41: 1738-1744, 1995.

6. **Clarke R, Armitage J**. Antioxidant vitamins and risk of cardiovascular disease. Review of larger scale randomized trials. Cardiovasc Drugs Ther 16: 411-415, 2002.

7. **Clifford P**. Anthocyanins-nature occurrence and dietary burden. J Sci Food Agricul. 80: 1063-1072, 2000.

8. **Cristie MI, Griffith TM, and Lewis MJ**. A comparison of basal and agonist-stimulated release of endothelium-derived relaxing factor from different arteries. *Br J Pharmacol* 98: 397-406, 1989.

9. **Dell'Agli M, Busciala A, Bosisio E**. Vascular effects of wine polyphenols. Cardiovasc Res. 63: 593-602, 2004.

10. **Dillon GA, Vito JA**. Nitric oxide and endothelial dysfunction. In: *Nitric Oxide and the Cardiovascular system* edited by Vito JA.Totawa, NJ Humana, 2000

11. **Ehlenfeldt MK, Prior RL**. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. J Agric Food Chem. 49: 2222-2227, 2001.

12. **Fu W, Conklin BS, Lin PH, Lunsden AB, Yao Q, Chen C**. Red wine prevents homocysteine-induced endothelial dysfunction in porcine coronary arteries. J Surg Res 115: 82-91, 2003.

13. **Galvano F, La Fauci L, Lazzarino G, Fogliano V, Ritieni A, Caippellano S, Battistini NC, Tavazzi B, Galvano G**. Cyanindins: metabolism and biological properties. J Nutr Biochem 15: 2-11, 2004.

14. **Harrison DG and Ohara Y**. Physiologic consequences of increased vascular oxidant stresses in hypercholesterolemia and atherosclerosis: implications for impaired vasomotion. *Am J Cardio* 75: 75B-81B, 1995.

15. **Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, and Chayama K**. Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med* 346: 1954-1962, 2002.

16. **Hou D**. Potential mechanisms of cancer chemoprevention by anthocyanins. Curr Mol Med 3: 149-159, 2003.

17. **Jackson TS, Xu A, Vita JA, and Keaney JF**. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ Res 8*3: 916-922, 1998.

18. **Kahkonen MP, Heinonen M**. Antioxidant activity of anthocyanins and their aglycons. J Agric. Food Chem 51: 628-633, 2003.

19. **Knox YM, Hayashi K, Suzutani T, Ogasawara M, Yosida I, Shiina R, Tsukui A, Terahara N, Azuma M**. Activity of anthocyanins from fruit extract of *Ribes Nigrum L* against influenza A and B viruses. Acta Virologica 45: 209-215, 2001.

20. **Kromhout J**. Diet and the cardiovascular system. J Nutr Health Aging 5: 144-149, 2001.

21. **Langenstroer P and Pieper GM**. Regulation of spontaneous EDRF release in diabetic rat aorta by oxygen free radicals. *Am J Physiol Heart Circ Physiol* 32: H257-H265, 1992.

22. **Lynch SM, Frei B, Morrow JD, Roberts LJ, Xu A, Jackson T, Reyna R, Klevay LM, Vita JA, and Keaney JF**. Vascular superoxide dismutase deficiency impairs endothelial

vasodilator function through direct inactivation of nitric oxide and increased lipid peroxidation. *Arterioscler Thromb Biol* 17: 2975-2981, 1997.

23. **Ma X, Weyrich AS, Lefer D, and Lefer A**. Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ Res* 72: 403-412, 1993.

24. **Marklund S and Marklund G**. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 47: 469-474, 1974.

25. **McGhie TK, Ainge GD, Barnett LE, Cooney JM, Jensen DJ**. Anthocyanin glycosides fro berry fruit are absorbed and excreted unmetabolized by both humans and rats. J Agric Food Chem 51: 4539-4548, 2003.

26. **Mendes A, Desgranges C, Cheze C, Vercauteren J, Freslon JL**. Vasorelaxant effects of grape polyphenols in rat isolated aorta. Possible involvement of a purinergic pathway. Fund Clin Pharmacol 17: 673-681, 2003.

27. **Milbury PE, Cao G, Prior RL**, Blumberg J. Bioavailability of elderberry anthocyanins. Mech Aging Dev 123: 997-1006, 2002.

28. **Miyazawa T, Nakagawa K, Kudo M, Muraishi K, Someya K**. Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. J Agric Food Chem 47: 1083-1091, 1999.

29. **Murkovic M, Topiak H, Adam U, Pfannhauser W**. Analysis of anthocyanins in plasm for determination of their bioavailability. J Food Comp Anal 13: 291-296, 2000.

30. **Ndiaye M, Chataigneau T, Andriansitohaina R, Stocelt JC, Schini-Kerth VB**. Red wine polyphenols cause endothelium-dependent EDHF-mediated relaxations in porcine coronary arteries via a redox-sensitive mechanism. Biochem Biophys Res Comm 310: 371-377, 2003.

31. **Pearson PJ, Lin PJ, and Schaff HV**. Global myocardial ischemia and reperfusion impair endothelium-dependent relaxations to aggregating platelets in the canine coronary artery. A possible cause of vasospasm after cardiopulmonary bypass. *J Thor & Cardio Surg* 103: 1147-1154, 1992

32. **Prior R, Cao G**. Antioxidant phytochemicals in fruits and vegetables: Diet and health implications. Hort Sci 35: 588-591, 2000.

33. **Prior RL, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, M Lischner, Ehlenfeldt M, Kalt W, Krewer G, Mainland CM**. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity and variety of Vaccinium species. J Agric Food Chem 46: 2686-2693, 1998.

34. **Rajaram S, Sabate F**. Health benefits of a vegetarian diet. Nutrition 16: 531-533, 2000.

35. **Rameriz-Tortosa C, Andersen OM, Gardner PT, Morrice PC, Wood SG, Duthie SJ, Collins AR, Duthie GG**. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. Free Rad Biol Med. 31: 1033-1037, 2001.

36. **Rossi A, Serraino I, Dugo P, DiPaola R, Mondello L, Genovese T, Morabito D, Dugo G, Sautebin L, Caputi AP, Cuzzerea S**. Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation. Free Rad Res 37: 891-900, 2003.

37. **Seeram NP, Adams LS, Hardy ML, Heber D**. Total cranberry extract versus its phytochemical constituents: antiproliferative and synergistic effects against human tumor cell lines. J Agric Food Chem. 52: 2512-2517, 2004.

38. **Serraino I, Dugo L, Dugo P, Mondello L, Mazzon E, Dugo G, Caputi AP, Cuzzocrea S**. Protective effects of cyanidin-3-O-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure. Life Sci 73: 1097-1114, 2003.

39. **Stein JH, Keevil JG, Weibe DA, Aeschlimann S, Folts JD**. Purple grape juice improves endothelial function and reduces susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. Circulation 100: 1050-1055, 1999.

40. **Stintzing FC, AS Stintzing**, Carle R, Frei B, Wrolstad RE. Color and Antioxidant properties of cyanidin-based anthocyanin pigments. J Agric Food Chem. 50: 6172-6181, 2002.

41. **Taddei S, Virdis A, Ghiadoni L, Magagna A, and Salvetti A**. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* 97: 2222-2229, 1998.

42. **Tsuda T, Horio F, Uchida K, Aoki H, Osawa T**. Dietary cyanidin 3-O-β-D- glucoside- rich purple color prevents obesity and ameliorates hyperglycemia in mice. J Nutr 133: 2125-2130, 2003.

43. **Tsuda T, Ueno Y, Aoki H, Koda T, Horio F, Takahashi N, Kawada T, Osawa T**. Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes. Biochem Biophys Res Comm 316: 149-157, 2004.

44. **Vallence P**. Vascular nitric oxide in health and disease, In: *Nitric Oxide Biology and Pathobiology*. edited by Ignarro L. San Diego, CA, Academic Press 2000

45. **Vinson JA, Dabbagh YA, Serry MM, Jang J**. Plant flavanoids, especially tea flavanols, are powerful antioxidants using an *in vitro* oxidation model for heart disease. J Agric Food Chem. 43: 2800-2802, 1995.

46. **Wang H , Cao G, Prior RL**. Total antioxidant capacity of fruits. J Agric Food Chem 44: 701-705, 1996.

47. **Wu X, Cao G, Prior R**. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. J Nutr 132: 1865-1871, 2002.

48. **Wu X, Gu L, Prior RL, McKay S**. Characterization of anthocyanins and proanthocyanins in some cultivars of *Ribes, Aronia,* and *Sambucus* and their antioxidant capacity. J Agric Food Chem 52: 7846-7856, 2004.

49. **Xu J, Ikeda K, Yamori Y**. Upregulation of endothelial nitric oxide synthase by cyanidin-3-glucoside, a typical anthocyanin pigment. Hypertension 44: 217-222, 2004.

50. **Xu J, Ikeda K, Yamori Y**. Cyanidin-3-glucoside regulates phosphorylation of endothelial nitric oxide synthase. FEBS Letters 574:176-180, 2004.

51. **Youdim KA, Martin A, Joseph JA**. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. Free Rad Biol Med 29: 51-60, 2000.

52. **Zheng W, Wang SY**. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J Agric Food Chem 51: 502-509, 2003.

Figure 1 Relaxation responses of porcine coronary arterial rings with endothelium (A) and without endothelium (B) to cumulative log doses of Chokeberry , Bilberry or Elderberry extracts at equal total anthocyanin (Ta) concentrations. Data is expressed as the Mean ± SE of the mg force remaining following each dose as expressed per mg force of initial pre-contraction to U46619. Open squares = chokeberry; Open triangles = bilberry; + = elderberry. n = 8 in all groups.

Figure 2 Examples of the effect of inhibition of eNOS by 100uM ~~L~~ NO_2 –l- Arginine upon relaxation of coronary arterial rings with endothelium by Chokeberry (top), Bilberry (middle) and Elderberry (bottom).

Figure 3 Effect of Chokeberry (top, n = 9), Bilberry (middle, n = 12) and Elderberry (bottom, n = 12) extract on impaired coronary vasorelaxation from exposure to pyrogallol. Relaxation responses of porcine coronary arterial rings with endothelium were performed to cumulative 2 log doses of A23187. Rings were precontracted with U46619 to a similar percent of their individual KCl maximum prior to administration of A23187. Open circles = Control rings; Inverted solid triangles = rings exposed to 400 uM pyrogallol for 15 minutes prior to precontraction with U46619. Upright solid triangles = rings exposed to 400 uM pyrogallol plus 0.05 mg Ta/L of extract for 15 minutes prior to precontraction with U46619. Open squares = rings exposed to 0.05 mg Ta/L extract only for 15 minutes prior to precontraction with U46619. Data is expressed as the Mean + SE of the mg force remaining following each dose as expressed per mg force of initial pre-contraction to U46619. Maximum relaxation to A23187 was significantly impaired in rings treated with pyrogallol, $p<0.05$.

Table 1. *Total Phenolic and Anthocyanin Composition of Four Berry Extracts* [#]

	Total Anthocyanins (g/100g)	Total Phenolics (g /100g)
Chokeberry	11.6	39.9
Bilberry	12.1	35.7
Elderberry	17.7	24.8

Values provided by Artemis International, Inc., Fort Wayne, Indiana

Table 2. *Sensitivity and Maximal Relaxation of Porcine Coronary Arteries to A23187 & DEA NONOate after 5 minute and 30 minute Exposure to 0.05 mg total Anthocyanins/L from Berry Extracts. mean±sem.*

	A23187		**DEA NONOate**	
	Sensitivity (-logM ED_{50})	%Maximal Relaxation	Sensitivity (-logM ED_{50})	%Maximal Relaxation
5 minutes *(n=10)*				
Control	7.69±.09	96±2	8.79±.07	100±0
Chokeberry	7.74±.06	98±1	8.56±.08	100±0
Elderberry	7.67±.07	98±2	8.84±.07	100±0
Bilberry	7.55±.10	99±1	8.69±.07	100±0
30 minutes (n=13)				
Control	7.76±.06	100±0	8.78±.10	99±1
Chokeberry	7.72±.06	95±3	8.81±.07	99±1
Elderberry	7.80±.08	95±3	8.75±.09	100±0
Bilberry	7.92±.07	98±1	8.76±.08	99±1

Table 3. *Sensitivity & Maximal Relaxation of Porcine Coronary Arteries to A23187 mean±sem*

	Sensitivity(-logM ED_{50})	*Maximal Relaxation*
n=9		
Control	7.86±.07	99±1%
Chokeberry	7.91±.10	93±5%
Pyrogallol	7.46±.06*	76±7%*
Chokeberry&Pyrogallol	7.82±.06#	98±1%#
n=12		
Control	7.92±.06	100±0%
Bilberry	7.94±.07	98±1%
Pyrogallol	7.53±.02*	82±5%*
Bilberry&Pyrogallol	7.75±.08	95±3%
n=12		
Control	7.90±.06	99±1%
Elderberry	7.87±.07	97±1%
Pyrogallol	7.43±.09*	74±8%*
Elderberry&Pyrogallol	7.64±.07*#	86±6%

* = p<0.05 vs Control values. # = p<0.05 vs Pyrogallol

Table 4. *Sensitivity & Maximal Relaxation of Porcine Coronary Arteries to DEA NONOate mean±sem.*

	Sensitivity(-logM ED_{50})	*Maximum Relaxation*
n=9		
Control	8.53±.07	98±2%
Chokeberry	8.61±.07	99±2%
Pyrogallol	8.41±.06	96±2%
Chokeberry&Pyrogallol	8.42±.09	98±1%
n=12		
Control	8.55±.09	99±1%
Bilberry	8.64±.05	96±2%
Pyrogallol	8.41±.08	99±1%
Bilberry&Pyrogallol	8.46±.08	99±1%
n=12		
Control	8.50±.08	100±0%
Elderberry	8.56±.09	100±0%
Pyrogallol	8.40±.08	99±0%
Elderberry&Pyrogallol	8.44±.11	94±3%

Figure 1

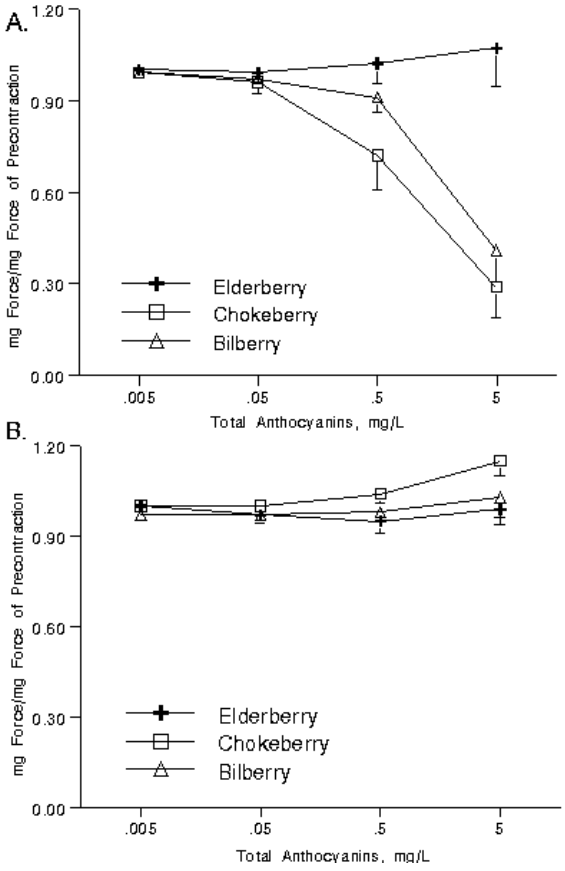

Figure 2

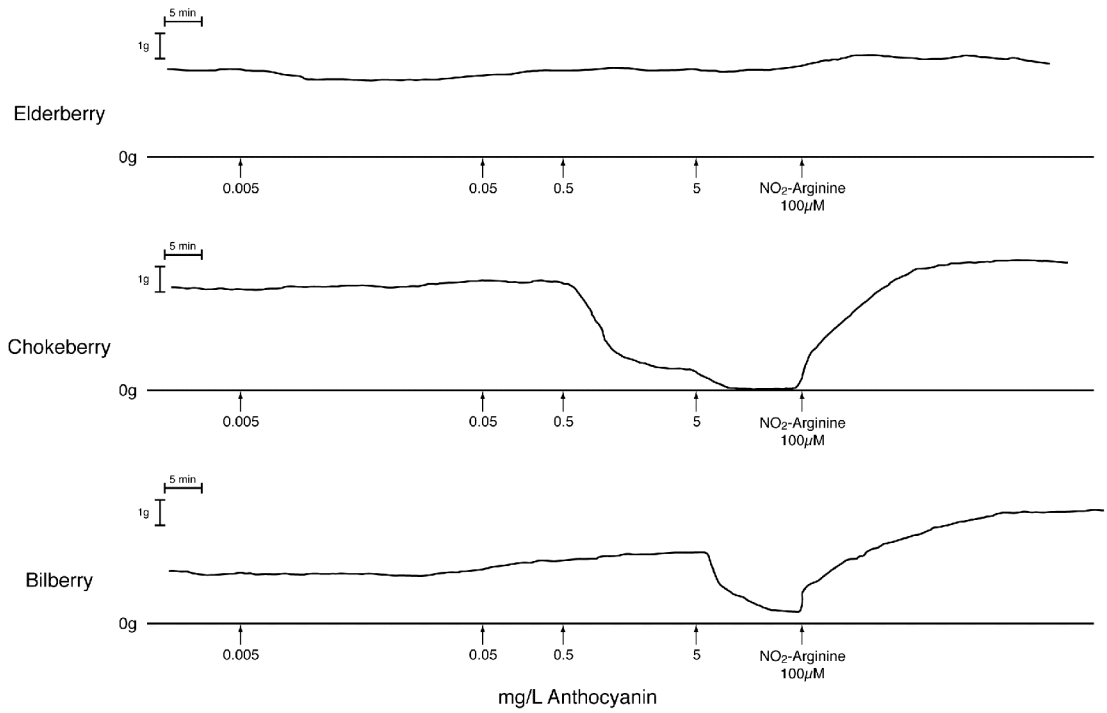

Figure 3