JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Effect of Black Currant Anthocyanins on the Activation of Endothelial Nitric Oxide Synthase (eNOS) in Vitro in Human Endothelial Cells

Indika Edirisinghe,[†] Katarzyna Banaszewski,[†] Jack Cappozzo,[†] Danielle McCarthy,[§] and Britt M. Burton-Freeman*[,†]

[†]Institute of Food Safety and Health, Illinois Institute of Technology, Moffet Campus, 6502 S. Archer Road, Bedford Park, Illinois 60501, United States

[§]GlaxoSmithKline, Nutritional Healthcare Future Group, 980 Great West Road, Brentford, Middlesex, TW8 9GS, United Kingdom

ABSTRACT: Polyphenols are known to induce vasodilatory function via activation of the redox-sensitive phosphatidylinositol-3 (PI3)/protein kinase B (Akt) pathway. Black currant fruits have appreciable amounts of polyphenolic compounds including cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside. It was hypothesized that black currant fruit extracts would cause activation of endothelial nitric oxide synthase (eNOS) through activation of redox-sensitive PI3 kinase/Akt signaling pathway. To test this hypothesis, human umbilical vein endothelial cells (HUVECs) were treated with different concentrations/times of black currant juice concentrates (Ben Gairn and Ben Hope) and the activation of Akt and eNOS was measured using immunoblotting. Vitamin C is also known to activate Akt and eNOS in in vitro models, and black currants are rich in vitamin C. Therefore, the effect of black currant extracts with and without coexisting vitamin C was investigated, using SPE columns to eliminate vitamin C content. The individual (and combined) effects of the major anthocyanins present in black currant juice samples with and without vitamin C were investigated and compared to the effects of the whole extract. Black currant juice samples (1 μL/mL) significantly increased the phosphorylation of Akt (p-Akt) and eNOS (p-eNOS) ($P < 0.05$). Activation of Akt and eNOS was abolished by incubation with wortmannin, a PI3K inhibitor, supporting the involvement of PI3K/Akt. Vitamin C alone significantly increased the p-Akt and p-eNOS ($P < 0.05$); however, removal of vitamin C from black currant did not significantly affect p-Akt and p-eNOS compared to black currant with vitamin C. Assessment of individual anthocyanins also showed significant effects on p-Akt and p-eNOS. In summary, in the present study data suggested that black currant concentrates, Ben Gairn and Ben Hope, activated eNOS via Akt/PI3 kinase pathway in vitro in HUVECs and that the effect was not dependent on vitamin C.

KEYWORDS: Akt, PI3 kinase, vitamin C, polyphenols, black currant

INTRODUCTION

Research supports the contribution of polyphenols derived from fruits and vegetables in lowering the risk for developing chronic diseases including cardiovascular diseases and cancers. These studies have indicated that, in addition to their antioxidant effects, polyphenols enhance the production of vasodilating factors such as nitric oxide.[1] Nitric oxide plays a key role in maintaining vascular integrity and endothelial function. Healthy endothelium maintains vascular tone and structure by regulating the balance between vasodilation and vasoconstriction.[2] On the contrary, in pathologic conditions, especially under oxidative stress and inflammation and particularly with the presence of cardiovascular risk factors, the endothelium undergoes structural and functional alterations, thus losing its protective role and becoming a proatherosclerotic structure.[2,3] In the earliest stages, changes in the endothelium are predominantly functional and termed accordingly "endothelial dysfunction".

Polyphenolic compounds are bioactive nonessential compounds found in fruits and vegetables and have been studied extensively in the past decade for their health-promoting properties when consumed by humans. Polyphenolic compounds vary widely in chemical structure, function, and composition in fruits and vegetables. They are grouped according to their chemical class, the largest subcategory being flavonoids, many of which have been studied for their antioxidant activity in vitro and in vivo.[4,5] However, the biological effects of polyphenols may extend well beyond the direct modulation of oxidative stress. Both antioxidant and prooxidant properties of polyphenols have been demonstrated with contrasting effects on cell physiological processes.[6] Due to their antioxidant/prooxidant activity, polyphenols may induce redox-sensitive kinases, resulting in downstream activation of several cell signaling molecules.[6−8] We and others have shown that extracts of strawberry, grape seed, tea, and cocoa that are rich in polyphenols induce endothelial dependent relaxation (EDR) mediated by the activation of endothelial nitric oxide synthase (eNOS).[7−10] Furthermore, we have demonstrated that EDR is mediated by activation of the redox-sensitive phosphatidylinositol-3 (PI3)/protein kinase B (Akt) pathway.[7,8]

Black currants are a rich source of antioxidants. Black currants contain appreciable amounts of the flavonoid subclass anthocyanins, including cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside.[11]

Received: March 18, 2011
Revised: July 11, 2011
Accepted: July 15, 2011
Published: July 15, 2011

Table 1. Vitamin C and Major Anthocyanins Present in the Black Currant Juice Concentrates before and after Extraction Using the SPE Column Cleanup Method

	cyanidin-3-*O*-glucoside (μg/mL)	cyanidin-3-*O*-rutinoside (μg/mL)	delphinidin-3-*O*-glucoside (μg/mL)	delphinidin-3-*O*-rutinoside (μg/mL)	vitamin C (mg/mL)
Ben Gairn	1795 ± 476	20880 ± 673	6756 ± 184	41533 ± 2912	7.51 ± 0.11
Ben Gairn (anthocyanin extracted)[a]	1398 ± 469	16873 ± 651	5538 ± 58	31148 ± 917	ND[b]
Ben Hope	2343 ± 1017	20045 ± 1458	5840 ± 87	29601 ± 2377	7.32 ± 0.02
Ben Hope (anthocyanin extracted)[a]	2237 ± 1085	18837 ± 969	5550 ± 95	25841 ± 716	ND

[a] Anthocyanin extraction: anthocyanins were extracted using the SPE column cleanup method as described under Materials and Methods. [b] ND, not detected, $n = 3$.

Apart from the anthocyanins present in black currant, vitamin C is also known to contribute to their antioxidant activity.[12] Because of the potential antioxidant action of the anthocyanins and vitamin C present in black currants and their juice, we hypothesized that black currant fruit extracts would cause an activation of eNOS through activation of the redox-sensitive PI3 kinase/Akt signaling pathway in vitro in human endothelial cells and therefore may have a role in maintaining endothelial function and reducing the risk for cardiovascular diseases. Studies were undertaken on human umbilical vein endothelial cells (HUVECs) to address three specific objectives: (1) to investigate the effect of black currant juice concentrates on the activation of eNOS and Akt in vitro in HUVECs; (2) to investigate the role of vitamin C on black currant-induced activation of Akt and eNOS in vitro in HUVECs; and (3) to investigate the individual effect of the major anthocyanins present in black currant samples on the activation of Akt and eNOS in vitro in HUVECs.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were of LC-MS grade and purchased from Sigma-Aldrich Co., St. Louis, MO. Cyanidin-3-*O*-glucoside and delphinidin-3-*O*-rutinoside were purchased from Chromadex (Irvine, CA). Cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-glucoside were purchased from Polyphenols (Sandnes, Norway). Black currant juice concentrates, of the Ben Gairn and Ben Hope varieties, were provided by GlaxoSmithKline, Brentford, U.K. The detailed anthocyanin profiles of the concentrates are given in Table 1. The dilutions of the juice concentrates were prepared in phosphate-buffered saline (PBS). HUVECs were purchased from Lonza, USA, and grown in EGM-2 (Lonza, DC) medium with 10% fetal bovine serum (FBS). Cells were grown to confluence (~90%) and starved for 6 h in serum-free medium before the cells were exposed to any treatments. Cell viability in response to different concentrations/times was investigated using the trypan blue exclusion test. Apoptosis in response to different concentrations/times was investigated using a Caspase 9 Colorimetric Activity Assay Kit (Millipore, Bedford, MA).

Preparation of Black Currant Concentrates. Freshly harvested black currants (*Ribes nigrum* 'Ben Hope' and 'Ben Gairn') were processed through roller mills and then fed directly into temperature-controlled mash heaters. The mash was heated to a controlled temperature of 50 °C for pectolytic enzyme hydrolysis. Sodium bisulfite was added as an aqueous solution to achieve a sulfur dioxide level of 150 mg/kg. Once the pectinase enzyme blend and sodium bisulfite had been added, the mash was maintained at 50 °C for 3 h. Juice was extracted from the enzyme-treated mash by pressing with a Bucher HPX5005i hydraulic press. On completion of the initial press, subsequent leaching was performed using evaporator condensate at 60 °C. Pressed juice was then heated through a tubular pasteurizer at a holding temperature of 103 °C for 30−45 s and then cooled to 35 °C. Pasteurized juice was then clarified by sedicantation and filtration (Flottweg sedicanter S4D-3/408 and Flottweg decanter centrifuge Z4D). It was then passed through a Velo evaporator (Velo TSE4000) and chilled to approximately 10 °C for storage.

Chemical Analysis of Black Currant Juice Concentrates. *LC-MS/MS Analysis of Black Currant Juice Concentrate Samples.* Black currant juice concentrate samples were extracted with acetone/water (70:30, v/v), evaporated to dryness under nitrogen, and dissolved in acetonitrile/water (5:95, v/v). Analysis of anthocyanins in black currant juice concentrate samples was performed using an Agilent Technologies 1200 series HPLC unit equipped with an Agilent Technologies 6460 triple Quadrupole LC-MS. The mobile phase was a gradient of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Chromatographic separation was achieved on an Agilent Poroshell 120 EC-C18 column. The four most abundant anthocyanins (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside) were detected in positive mode $(M + H)^+$, using an electrospray ionization (ESI) source. The analysis was carried out using a full MS^2 scan followed by MS^2 product ion scan with multiple reactions monitoring (MRM) fragmentation. Compounds in the black currant juice concentrates were quantified using commercially available standards (0.625−20 μg/mL) dissolved in mobile phase immediately prior to analysis. Stock solutions of anthocyanin standards were prepared in methanol and stored frozen at −80 °C in amber vials.

Ascorbic Acid Assay. Vitamin C (ascorbic acid) analysis was performed according to the method described by Sanchez Mata et al.[13] Briefly, 500 μL of each black currant juice sample was extracted with 10 mL of a buffer solution containing dithiothreitol (DDT), potassium phosphate (KH_2PO_4), and metaphosphoric acid (HPO_3). Samples were then diluted with 15 mL of reagent alcohol/water (50:50, v/v) and placed on a mechanical shaker for 18 min. A portion of the upper layer was filtered through a 0.2 μm PTFE syringe filter and analyzed by HPLC. The analysis was performed on Shimadzu Prominence HPLC with UV detection at 258 nm. The mobile phase consisted of 75% acetonitrile and 25% 0.15 mol/L acetate, pH 5.0. Chromatographic separation was obtained using a Luna 3u NH2 100A 150 × 2.0 mm column (Phenomenex, Torrance, CA). The concentration of vitamin C was determined using a standard curve. Ascorbic acid standards (0.625−20 μg/mL) were prepared fresh in a buffer solution (see above) and 50% alcohol solution in a 1:3 ratio.

Elimination of Vitamin C from Black Currant Juice Concentrates through an SPE Cleanup Method. Anthocyanins from black currant juice samples (Ben Hope and Ben Gairn) were extracted using acetone/water (70:30, v/v). Briefly, 200 μL of each sample was mixed with 10 mL of acetone/water (70:30, v/v) and vortexed for 1 min. The extracts were then filtered through 0.2 μm PTFE syringe filters into 15 mL glass tubes and evaporated to dryness under nitrogen. Samples were then dissolved

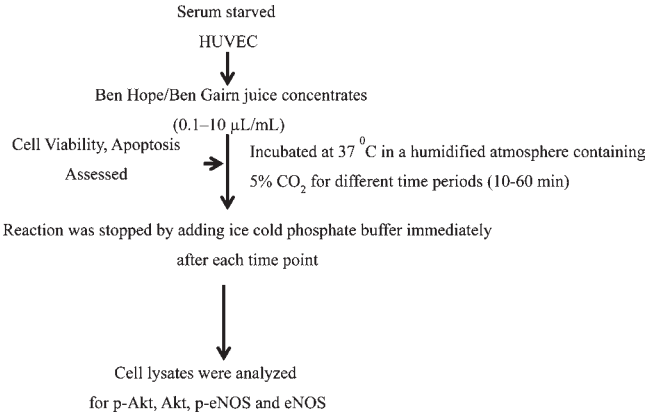

Figure 1. Schematic overview of steps involved in investigating the dose/time effect of black currant concentrates on the activation of eNOS and Akt in vitro in HUVECs.

in 10 mL of 3% formic acid in water and passed through preconditioned with water and methanol SPE cartridges (Bond Elut C18, Varian, Cary, NC). Samples were eluted with 3% formic acid in methanol, evaporated to dryness, and dissolved in 2 mL of ascorbic acid dilution buffer (mixture of 100 mL of ascorbic acid extraction buffer and 150 mL of alcohol solution). Eluted samples were filtered through 0.2 μm PTFE syringe filters. These samples were analyzed for vitamin C content using the above method. For anthocyanin analysis, sample extracts using the above method were dissolved in 1 mL of acetonitrile/water (5:95, v/v), filtered through 0.2 μm PTFE syringe filters/ and analyzed using LC-MS/MS.

Cell Culture Experiments. *Effect of Black Currant Juice Concentrates on the Activation of eNOS and Akt in Vitro in HUVECs.* The effect of Ben Gairn and Ben Hope black currant juice concentrate samples on the activation of eNOS and Akt was investigated using HUVECs. Confluent HUVECs were starved for 6 h in serum-free medium before the cells were treated with black currant juice concentrates. To identify the optimum time/concentration effects of Ben Gairn and Ben Hope black currant samples on the activation of Akt and eNOS, HUVECs were treated with different dilutions of Ben Gairn and Ben Hope black currant juice samples (0.1−10 μL/mL) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for different lengths of time (10−60 min). The incubation was stopped immediately after each time point (10, 15, 20, 30, 40, 50, and 60 min) by adding ice-cold PBS, the sample was washed twice with the same PBS, and cell lysates were prepared in cell lysis buffer (Cell Signaling Technology, Beverly, MA) (Figure 1).

It is known that anthocyanins activate Akt and eNOS via redox sensitive PI3 kinase pathway.[7] Therefore, wortmannin, a PI3 kinase inhibitor, was used to confirm the mechanism. Some HUVECs were treated with wortmannin (30 nmol/L) for 30 min before exposure to Ben Gairn or Ben Hope black currant juice samples. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 10 min after they had been treated with Ben Gairn or Ben Hope black currant juice samples (1 μL/mL). The reaction was stopped immediately by adding ice-cold PBS, the sample was washed twice with the same PBS, and cell lysates were prepared in cell lysis buffer (Cell Signaling Technology).

Cell lysates obtained from the above experiments were analyzed for total protein using bicinchoninic acid assay (BCA) (Pierce Biotechnology, Rockford, IL). Total proteins (30 μg) were separated on 7.5% SDS−polyacrylamide gels (Bio-Rad, Hercules, CA). Separated proteins were transferred electrophoretically onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with blocking buffer containing 5% blotto (Bio-Rad) in Tris-buffered saline solution and 0.1% Tween 20 (TBS-T) for 1 h. Phosphorylated Akt (Ser-473), phosphorylated eNOS (Ser 1177), Akt, and eNOS were detected after the membranes had been incubated with the respective primary antibodies (rabbit anti-p-eNOS-ser-1177, rabbit anti-eNOS, rabbit anti-p-Akt-ser-473, and rabbit anti-Akt, Cell Signaling Technology; dilution of 1:1000) overnight at 4 °C. Membranes were washed three times (10 min each) and incubated with the secondary antibody (peroxidase-labeled anti-rabbit IgG, 1:20000, Cell Signaling Technology) at room temperature for 60 min. Membranes were washed again three times (10 min each), and the specific protein bands were visualized using the enhanced chemiluminescence method (Amersham-Pharmacia Biotech, Beverly, MA). All four proteins were probed in the same blot, and the membranes were washed with stripping buffer (Pierce Biotechnology) for 30 min in 37 °C before they were incubated with the next primary antibody. β-Actin levels in the HUVECs cell lysates in response to various treatments were also measured, and the levels were not changed. Therefore, the phosphorylations of eNOS and Akt were expressed as p-Akt/total Akt and p-eNOS/total eNOS, respectively.

Effect of Vitamin C Present in the Black Currant Juice Concentrates on the Activation of Akt and eNOS in Vitro in HUVECs. Both Ben Gairn and Ben Hope concentrates contained significant quantities of vitamin C (Table 1). Vitamin C is also known to activate Akt and eNOS in in vitro models.[12] Therefore, this experiment was designed to identify the effect of anthocyanins, vitamin C, and the combination of both on the activation of Akt and eNOS in vitro in HUVECs. Vitamin C in the black currant samples was eliminated using the method described above. HUVECs were treated with black currant juice concentrate samples with and without vitamin C and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 10 min as described in Figure 1. The reaction was stopped immediately after 10 min of incubation by adding ice-cold PBS, the sample was washed twice with the same PBS, and cell lysates were prepared in cell lysis buffer (Cell Signaling Technology). Proteins were analyzed using a BCA assay, and p-Akt and eNOS were detected using the immunoblotting method described in the above section.

Individual Effect of Major Anthocyanin Present in Black Currant Juice Concentrates on the Activation of Akt and eNOS in Vitro in HUVECs. Cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside were identified as four major anthocyanins present in the black currant samples using LC-MS/MS technology (Table 1). Therefore, we used commercially available standard anthocyanin compounds present in the black currant (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside) to identify the individual effects of those "individual" compounds on the activation of Akt and eNOS and compared the activation responses with black currant juice samples (Ben Hope and Ben Gairn). Stock solutions of anthocyanin standards were prepared in methanol and stored frozen at −80 °C in amber vials. These stock samples were evaporated under a nitrogen stream and dissolved in PBS for cell culture experiments. The concentrations of the standards for cell culture work were calculated on the basis of the anthocyanin concentrations present in the black currant concentrates that produced maximum phosphorylation effects (p-Akt and p-eNOS). For Ben Hope, the concentrations of commercially available cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside used in the cell culture were 2.3, 20, 5.8, and 29 μg/mL, respectively. For Ben Gairn, the concentrations of the commercially available cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside used in the cell culture were 1.7, 20, 6.7, and 41 μg/mL, respectively. Serum-starved confluent HUVECs were treated with Ben Hope or Ben Grain black currant juice concentrates (1 μL/mL) with their major standard anthocyanins as calculated above for 10 min in a humidified atmosphere containing 5% CO_2 at 37 °C. p-Akt and p-eNOS were detected using the method described in the above section.

Anthocyanins detected in human blood after the consumption of foods containing appreciable amounts of anthocyanins are reported in the nanomoles per liter range.[14,15] Therefore, we investigated the effect of four major anthocyanins at the concentration of 100 nmol/L (cyanidin-3-*O*-glucoside, 45 ng/mL; cyanidin-3-*O*-rutinoside, 59.6 ng/mL; delphinidin-3-*O*-glucoside, 46.6 ng/mL; and delphinidin-3-*O*-rutinoside, 61.2 ng/mL) on the activation of Akt and e-NOS in vitro in HUVECs to mimic estimated circulating/physiological concentrations. Furthermore, we mixed vitamin C [7.5 μg/mL (~42 μmol/L)] with 100 nmol/L of each major anthocyanin to investigate the (potential) synergistic effect of each anthocyanin and vitamin C on the activation of Akt and e-NOS. The mixture of all four major anthocyanins with and without vitamin C was also tested for the activation of Akt and eNOS. p-Akt and e-NOS were measured using the immunoblotting method described above.

Image Processing and Statistical Analysis. The phosphorylation levels of Akt and eNOS with different treatments/times were compared with their own PBS-treated control experiments. The band density that represents the phosphorylation and expression of Akt and eNOS protein were determined by image J (image processing) software program (NIH, Bethesda, MD) by a single investigator. The identities of the bands were not known at the time of quantification. The Sigma plot 11 statistical program was used to analyze the data. Normality and equal variance tests were performed followed by ANOVA. $P < 0.05$ was considered to be significant. Results are given as the mean ± SD of at least three experiments.

RESULTS

Major Anthocyanins and Vitamin C in the Black Currant Samples. With LC-MS/MS profiling, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside were identified as the major anthocyanins present in both Ben Hope and Ben Gairn black currant juice concentrates (Figure 2). In MRM mode peak partial overlapping has no effect on quantitation accuracy due to its specificity, especially if standards are being used. These anthocyanins were quantified using respective standard curves and are reported in Table 1. Black currant samples also contained significant quantities of vitamin C and are reported in Table 1. We also carried out experiments to eliminate vitamin C in the black currant samples. After elimination of vitamin C from the black currant juice samples, they were quantified again for anthocyanins and vitamin C. Although we observed some reductions of anthocyanins after extraction procedure, vitamin C was not detected as analyzed using the HPLC method (Table 1).

Effect of Black Currant Juice Concentrates on PI3K/Akt and eNOS Activation in Vitro in HUVECs. HUVECs were treated with black currant juice concentrates (Ben Hope and Ben Gairn), and p-Akt and p-eNOS levels were measured as described under Materials and Methods. Both Ben Hope and Ben Gairn concentrate treatments resulted in significantly increased phosphorylation of Akt and eNOS in a dose-dependent manner ($P < 0.05$). The maximum p-Akt and p-eNOS were observed at 1 μL/mL in 10 min with both concentrates. HUVECs cell viability and apoptosis at the concentrations/times tested were not affected as measured by the trypan blue exclusion test and caspase 9 activity assay, respectively, compared to PBS-treated control experiments. An immunoblot representing the dose-dependent effect of Ben Hope black currant concentrate is given in Figure 3.

Polyphenolic compounds are known to activate eNOS via the PI3/Akt pathway.[7] Therefore, we investigated the effect of black currant concentrates on the activation of Akt and eNOS in the presence of a PI3 kinase inhibitor, wortmannin, in vitro in HUVECs. As expected, phosphorylation of Akt and eNOS was significantly attenuated in the presence of wortmannin compared to the cells treated without wortmannin in response to both black currant samples (Figure 4). These data support the involvement of PI3 kinase in the events of activating Akt and eNOS in response to black currant juice concentrates in vitro in HUVECs.

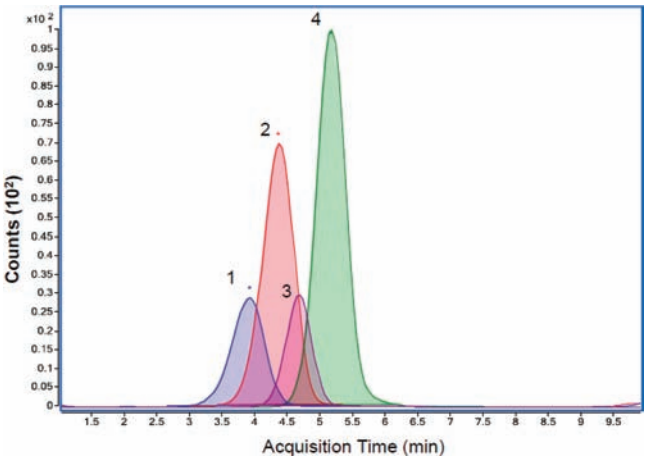

Figure 2. Analysis of anthocyanin compounds in black currant juice concentrates using LC-MS/MS. Identification of major anthocyanin compounds in black currant samples was made through multiple reaction monitoring (MRM) fragmentation analysis in both positive and negative ion mode. The chromatogram represents the Ben Hope sample. Four major peaks were identified as (1) delphinidin-3-*O*-glucoside, (2) delphinidin-3-*O*-rutinoside, (3) cyanidin-3-*O*-glucoside, and (4) cyanidin-3-*O*-rutinoside. Quantitative analyses are given in Table 1.

Effects of Black Currant Juice Concentrate with and without Vitamin C on the Activation of Akt and eNOS in Vitro in HUVECs. Black currant juice concentrates used in the present study contained significant quantities of vitamin C (Table 1). Therefore, experiments were carried out to investigate the importance of vitamin C on black currant-induced Akt and eNOS activation. Vitamin C was removed from the black currant samples as described under Materials and Methods. Subsequently, the vitamin C depleted samples were tested for vitamin C, and no trace amounts of vitamin C were identified (Table 1). HUVECs were treated with black currant juice samples with and without vitamin C as described under Materials and Methods (Table 2). Some cells were treated with vitamin C alone (7.5 μg/mL, corresponding concentration present in 1 μL/mL Ben Gairn sample). Significant differences were not observed in the levels of p-Akt and p-eNOS in response to black currant samples with and without vitamin C ($P > 0.05$). However, vitamin C alone significantly increased the levels of p-Akt and p-eNOS compared to PBS control ($P < 0.05$), but not to the same extent as black currant juice samples. The levels of p-Akt and p-eNOS in response to the black currant juice samples with and without vitamin C were significantly higher (~200 and 150%, respectively) compared to treatment with vitamin C alone ($P < 0.05$). An immunoblot representing the effects of Ben Hope concentrates with and without vitamin C is given in Figure 5.

Effects of Major Anthocyanins Present in the Black Currant Juice Concentrates on the Activation of Akt and eNOS in Vitro in HUVECs. The major anthocyanins present in the black currant juice concentrates were identified as cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and

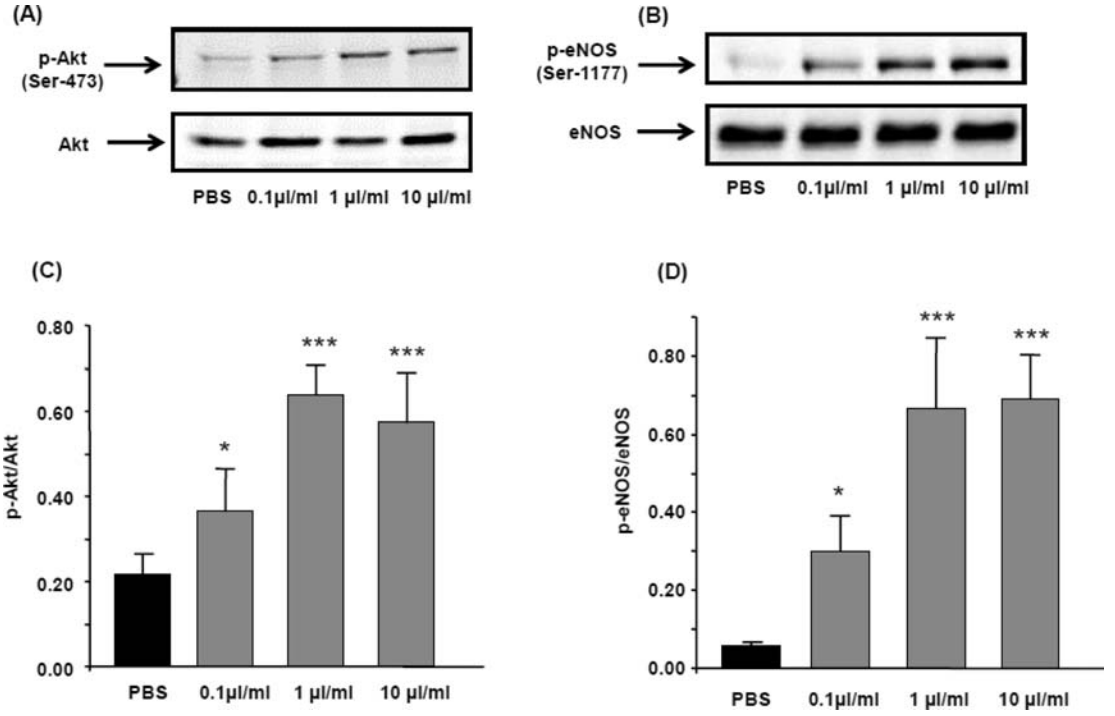

Figure 3. Dose-dependent effect of black currant juice concentrates on activation of Akt and eNOS in vitro in HUVECs. Black currant sample (Ben Hope)-induced Akt and eNOS phosphorylations were investigated using immunoblotting techniques as mentioned under Materials and Methods. Representative blots of the maximum Akt phosphorylation (p-Akt) and eNOS phosphorylation (p-eNOS) are given in panels A and B, respectively. The histograms shown in both panels C and D are those obtained after quantification of the blots using densitometry (n = 3) for p-Akt and p-eNOS, respectively. The ordinates are the relative ratios of the phosphorylated and nonphosphorylated form of each enzyme. (*)P < 0.05, (***) P < 0.001, significant compared to PBS control (n = 3).

delphinidin-3-*O*-rutinoside by LC-MS/MS (Table 1). The experiments were carried out to identify the effects of these major anthocyanins alone and in combination compared to the effects with Ben Hope and Ben Gairn black currant juice concentrate samples on the activation of Akt and eNOS in vitro in HUVECs. All four major anthocyanins (alone) showed significantly increased p-Akt and p-eNOS compared to PBS control at their corresponding concentrations found in Ben Gairn and Ben Hope black currant concentrates (1 μL/mL) that produced maximum p-Akt and p-eNOS (P < 0.05, Figure 6). The combination of all four major anthocyanins produced significantly increased p-Akt and p-eNOS compared to each individual anthocyanin alone (P < 0.01). Phosphorylated Akt and eNOS in response to the combination of all four major anthocyanins were similar to the responses produced by Ben Gairn and Ben Hope black currant concentrates (1 μL/mL).

Investigation of these same individual anthocyanin compounds at a dose estimated to mimic blood concentrations (~100 nmol/L) did not produce any significant effects on phosphorylation of Akt or eNOS compared to PBS control (P < 0.05, data not shown).

DISCUSSION

In the present investigation, we aimed to assess the potential role of black currants in maintaining endothelial function, a critical prerequisite for cardiovascular health. Studies were undertaken in vitro in HUVECs to address three specific objectives: (1) to investigate the effect of black currant juice concentrates on the activation of eNOS and Akt; (2) to investigate the role of vitamin C on black currant-induced activation of Akt and eNOS; and (3) to investigate the individual effect of the major anthocyanins present in black currant samples on the activation of Akt and eNOS.

Polyphenolic compounds cause an EDR of aortic rings in vitro[7,8] and have also been shown to activate eNOS[7,8] and up-regulate eNOS in cultured endothelial cells.[16] In humans, polyphenolic compound-rich extracts of fruits and vegetables have been shown to enhance flow-mediated vasodilation in the brachial artery, a functional measure of EDR in humans.[17] Therefore, we investigated whether black currant juice concentrates (Ben Gairn and Ben Hope) activate cell signaling pathways leading to nitric oxide (NO) release, including phosphorylation of Akt (on Ser473) and eNOS (on Ser1177) in vitro in HUVECs. Ben Gairn and Ben Hope black currant juice concentrates induced phosphorylation of Akt and eNOS as determined by immunoblotting. Prior exposure to a PI3K inhibitor, wortmannin, abolished the phosphorylation of Akt and eNOS in HUVECs, supporting the involvement of PI3 kinase/Akt in the activation of eNOS. Therefore, it can be suggested that incubation of endothelial cells with black currant juice concentrates phosphorylate eNOS through the PI3K/Akt pathway. A similar mechanism of eNOS activation has been observed in response to a grape seed extract,[8] grape skin extract,[18] purple grape juice,[19] strawberry powder,[7] and epigallocatechin-3-gallate,[9] a major flavonoid from the flavan-3-ol subclass found in green tea.

Previous studies have shown that vitamin C also causes an EDR when applied over concentrations ranging from 1 to 300 μmol/L.[12] In the present study, vitamin C levels in black currant juice concentrates were 42.6 and 41.4 μmol/L (7.5 and 7.3 mg/mL) for BH and BG, respectively, which provides

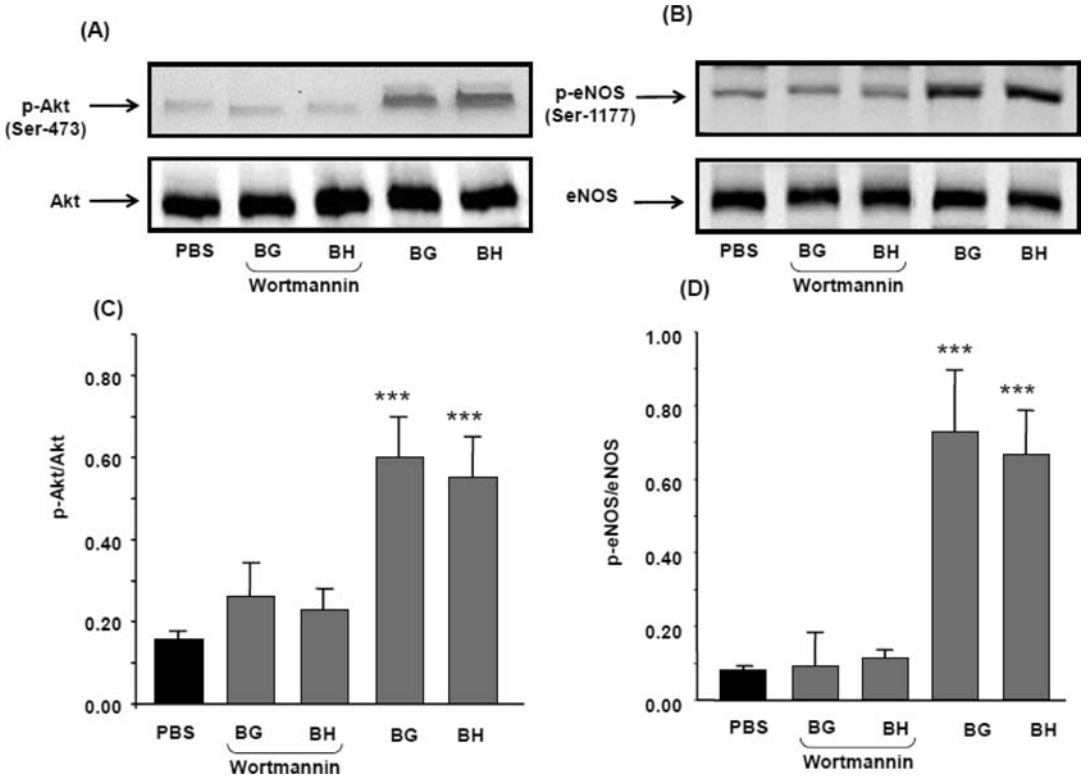

Figure 4. Black currant (Ben Gairn and Ben Hope) juice concentrates activated Akt and eNOS through PI3 kinase in vitro in HUVECs. Black currant sample (Ben Hope-BH and Ben Gairn-BG)-induced Akt and eNOS phosphorylations were investigated in HUVECs. The effects of a PI3 kinase inhibitor, wortmannin, on black currant-induced Akt and eNOS phosphorylation were also investigated. Representative immunoblots show the effect of BH and BG on phosphorylation of Akt (p-Akt) (A) and eNOS (p-eNOS) (B). The blot in lane 1 (control, PBS) is the control showing low concentration of phosphorylated Akt and eNOS. Blots in lanes 4 and 5 show evidence of increased levels of phosphorylated Akt and eNOS after incubation with BG and BH (1 μL/mL) for 10 min, respectively. The effects observed in lanes 4 and 5 are not evident in lanes 2 and 3 (pretreated with wortmannin). Wortmannin alone for 30 min did not show any increased levels of phosphorylated Akt and eNOS. The histograms in both panels C and D are those obtained after quantification of the blots using densitometry ($n = 3$) for p-Akt and p-eNOS, respectively. The ordinates are the relative ratios of the phosphorylated and nonphosphorylated forms of each enzyme. (***) $P < 0.001$, significant compared to control ($n = 3$).

Table 2. Experimental Approach Used To Investigate the Effect of Vitamin C Present in the Black Currant Juice Concentrates (Ben Hope, BH) on the Activation of Akt and eNOS in Vitro in HUVECs

	BH crude sample	anthocyanin extracts from BH	PBS control	PBS/use of the same extraction procedure[a]	vit C	vit C/use of the same extraction procedure[a]
stock solution concentration	100 μL/mL PBS	100 μL/mL PBS	1 mL PBS	1 mL PBS	0.75 mg/mL	0.75 mg/mL
final dilution in the cell culture (dilution factor)	×100	×100	×100	×100	×100	×100

[a] Extraction procedures: Anthocyanins were extracted using the SPE column cleanup method as described under Materials and Methods. PBS and vitamin C samples were extracted using the same procedures.

for ~42 μmol/L (~7.5 μg/mL) in cell culture. Therefore, we investigated the role of black currant juice concentrates on the activation of Akt and eNOS in vitro in HUVECs with and without vitamin C. Our approach included experiments to eliminate vitamin C from the samples followed by investigations to determine the effect on phosphorylation of Akt and eNOS in vitro in endothelial cells. Our methods indeed eliminated the vitamin C from the samples as confirmed by the HPLC method (Table 1). There were some anthocyanin losses in recoveries, especially in Ben Gairn, which could be attributed to the loss of stability due to ascorbic acid removal.[20] Although we observed that some compounds in the black currant concentrates were significantly reduced after SPE cleanup procedure, it did not affect the p-AKT and p-eNOS level compared to black currant juice concentrates without SPE cleanup. This may be because anthocyanin concentrations in SPE cleaned black currant juice concentrates are sufficient to produce the maximum level of phosphorylation (Akt and eNOS) in endothelial cells. Vitamin C at 7.5 μg/mL (42 μmol/L) increased the phosphorylation of Akt and eNOS compared to PBS-treated cells. Significant differences in p-Akt and eNOS were not observed with the black currant samples with and without vitamin C ($P < 0.05$); however, both produced greater phosphorylation of Akt and eNOS than vitamin C alone, suggesting that the effects of black currant juice concentrate are not dependent on vitamin C. Synergism between vitamin C and anthocyanins in the black currant samples was not observed,

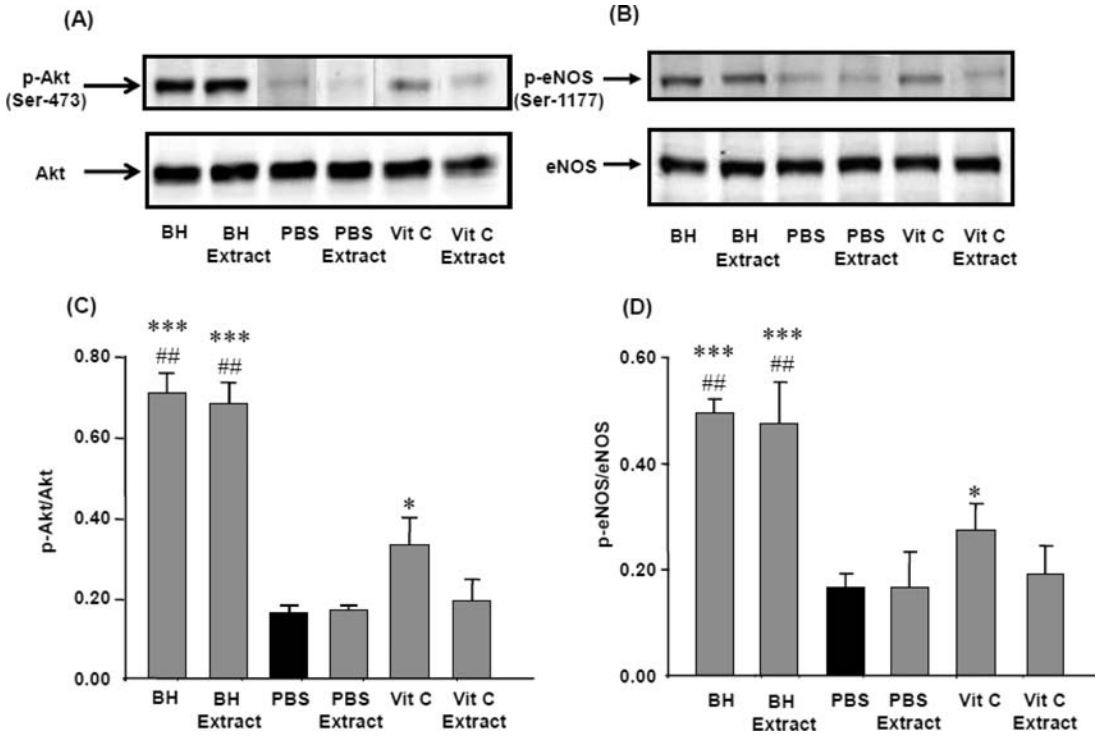

Figure 5. Effect of vitamin C depleted black currant juice concentrates on the activation of Akt and eNOS in vitro in HUVECs. Vitamin C in the black currant sample (Ben Hope, BH) was eliminated using the method described under Materials and Methods. RepresentativeiImmunoblots show the effect of BH and vitamin C depleted BH sample on phosphorylation of Akt (p-Akt) (A) and eNOS (p-eNOS) (B). Vitamin C (respective concentration found in BH sample) alone increased the level of p-Akt and p-eNOS significantly ($P < 0.05$). However, significant difference was not observed between BH samples and vitamin C depleted BH sample ($P > 0.05$). The histograms shown in both panels C and D are those obtained after quantification of the blots using densitometry ($n = 3$) for p-Akt and p-eNOS, respectively. The ordinates are the relative ratios of the phosphorylated and nonphosphorylated forms of each enzyme. (*) $P < 0.05$ and (***) $P < 0.001$, significant compared to control; (##) $P < 0.01$, significant compared to vitamin C ($n = 3$).

possibly due to the fact that anthocyanin concentrations in those samples produced the maximum level of phosphorylation (Akt and eNOS) in endothelial cells, and thus any additional effects due to additive or synergistic effects cannot be observed. Dose response experiments with various concentrations of black currant samples with and without vitamin C are required to identify and confirm synergies between anthocyanin and vitamin C in black currant.

We also carried out experiments to investigate the individual effects of major anthocyanins present in black currant juice samples on the activation of Akt and eNOS in vitro in endothelial cells. Doses for the individual anthocyanins were chosen on the basis of the maximum response given by black currant samples in vitro in endothelial cells. After a review of the literature, we also chose one dose (100 nmol/L) per individual anthocyanin to reflect plasma anthocyanin concentrations.[14] The effects of cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside did not significantly change ($P > 0.05$) phosphorylation of Akt or eNOS at the lowest/physiological dose (100 nmol/L) compared to PBS control; however, at higher doses reflecting fruit concentrations, Akt and eNOS were activated compared to PBS control. The purified anthocyanin compounds were prepared and stored in nitrogen-sealed amber-colored ampules, although they were exposed to air before they were added to the cell cultures. The stability of these compounds is not known, especially at lower doses, which may explain the limited (or lack of, 100 nmol/L) effect at the lower individual doses. The mixture of all four major anthocyanins present in black currant juice concentrates significantly increased the p-Akt and p-eNOS compared to the responses observed with each individual anthocyanin. Notably, the response of the mixture did not directly reflect the sum of all four anthocyanin responses. The mixture did, however, produce a response that was not different from the juice concentrates (at 1 μg/mL). This may not be surprising because the mixture of the anthocyanins represented the total concentration of major anthocyanins in the fruit juice concentrate at the dose (1 μg/mL) providing maximal phosphorylation of Akt and eNOS in endothelial cells. The data suggest that each anthocyanin alone can induce phosphorylation of Akt and eNOS, but there appears to be a phosphorylation threshold, because additivity was not observed. Furthermore, our dose response experiments with the juice concentrates indicate a threshold for phosphorylation of Akt and eNOS. In the present study, we have not investigated the dose response curves for different mixtures of anthocyanins. To determine if there are additive or synergistic relationships between the individual anthocyanins, additional experiments with much lower (including ineffective) doses of individual anthocyanins with different combinations would be required.

In the present study, we suggested redox changes in endothelial cells in response to the black currant anthocyanins and vitamin C as the potential mechanism of the action responsible for the activation of Akt and eNOS. However, we have not investigated the redox changes specifically in the endothelial cells upon exposure to various anthocyanins and vitamin C in the present study. It is possible that the other factors such as the

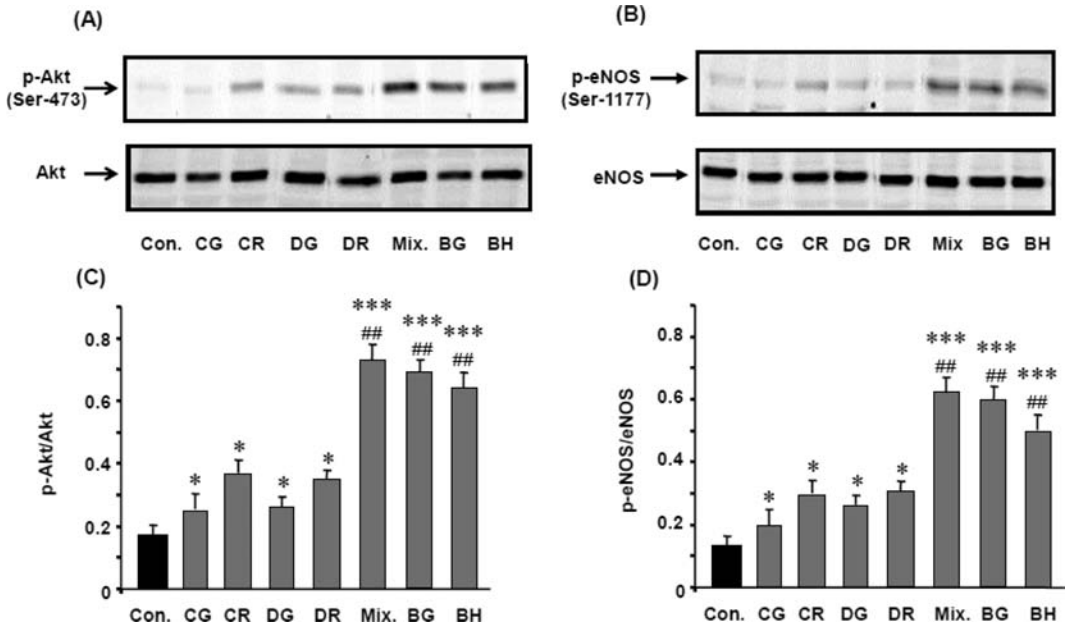

Figure 6. Effect of major anthocyanins present in black currant juice concentrates on the activation of Akt and eNOS in vitro in HUVECs. HUVEC cells were treated with commercially available cyanidin-3-*O*-glucoside (CG; 2.3 μg/mL), cyanidin-3-*O*-rutinoside (CR; 20 μg/mL), delphinidin-3-*O*-glucoside (DG; 5.8 μg/mL), and delphinidin-3-*O*-rutinoside (DR; 29.6 μg/mL) and a mixture of all four (Mix), and the levels of p-Akt and p-eNOS were compared with PBS control and Ben Gairn and Ben Hope concentrates as described under Materials and Methods. Representative blot shows that all four major anthocyanins significantly increased (A) p-Akt and (B) p-eNOS compared to PBS control at the corresponding concentration found in Ben Gairn and Ben Hope (1 μL/mL) that produced maximum p-Akt and p-eNOS ($P < 0.05$). The mixture of all four major anthocyanins produced significantly increased p-Akt and p-eNOS compared to each individual anthocyanin alone ($P < 0.01$). Phosphorylated Akt and eNOS in response to the mixture of all four major anthocyanins were similar to the ones produced by Ben Gairn and Ben Hope (1 μL/mL). The histograms shown in both panels C and D are those obtained after quantification of the blots using densitometry ($n = 3$) for p-Akt and p-eNOS, respectively. The ordinates are the relative ratios of the phosphorylated and nonphosphorylated form of each enzyme. (*) $P < 0.05$ and (***) $P < 0.001$, significant compared to control ($n = 3$); (##) $P < 0.01$, significant compared to CG, CR, DG, and DR ($n = 3$).

redox status of the endothelial media and endothelial cells vary from experiment to experiment, although we control the cell passages (from passage 3−5), the composition of the endothelial media, and the incubator conditions in all experiments.

The active compounds in vivo may not be the native anthocyanins found in black currants, which are tested in vitro in the present study; they are more likely to be metabolites.[15] Anthocyanins are extensively conjugated in the body, and nonconjugated metabolites most often account for a minor fraction of the circulating metabolites.[14,15] Very little is currently known regarding the biological activities of metabolites.[14,15] The available literature suggests that in vitro studies of anthocyanins and their metabolites must be considered carefully in light of their bioavailability in in vivo studies. A significant body of literature supports a role for oxidative stress in the pathogenesis of cardiometabolic diseases and a contribution of dietary anthocyanins to disease prevention and progression.[21] The complex relationships between antioxidant status and disease are still poorly understood, despite being extensively studied. For many years, plant anthocyanins and other antioxidants were thought to protect cell constituents against oxidative damage through scavenging of free radicals. However, this concept now appears to be an oversimplified view of their mode of action.[6] More likely, cells respond to anthocyanins mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions[6] through their antioxidant effects. Therefore, the biological effects of anthocyanins (polyphenols) may extend well beyond the modulation of oxidative stress. Detailed understandings of the molecular events underlying these various biological effects in in vivo systems are essential for evaluation of the overall impact on disease risk and progression.

In summary, the present study data showed that black currant concentrates, Ben Gairn and Ben Hope, phosphorylated eNOS via activation of the PI3 kinase/Akt pathway. Vitamin C alone significantly increased the phosphorylation of Akt and eNOS, although the magnitude of increase was significantly lower compared to those of black currant juice concentrates. The presence of vitamin C in the black currant concentrates did not have significant effects on phosphorylation of Akt and eNOS compared to black currant without vitamin C. Finally, the major individual anthocyanins of black currant fruit juice induce phosphorylation of Akt and eNOS, however, only partially when compared to the mixture of anthocyanins or the juice concentrates.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Institute of Food Safety and Health, Illinois Institute of Technology, Moffet Campus, 6502 S. Archer Road, Bedford Park, IL 60501. Phone: (708) 341-7078. Fax: (708) 563-1873. E-mail: bburton@iit.edu.

Author Contributions

B.B.-F., I.E., J.C., and D.M. designed the research. I.E. and K.B. conducted the research. I.E., B.B.-F., J.C., and K.B. analyzed the data and compiled the manuscript. All authors were involved

with review and the final submitted manuscript. All authors read and approved the final manuscript.

Funding Sources

Funding for this project was provided by GlaxoSmithKline, Nutritional Healthcare Future Group, GlaxoSmithKline, U.K.

Notes

D.M. is an employee of GlaxoSmithKline, Nutritional Healthcare Future Group, GlaxoSmithKline, U.K.

■ ACKNOWLEDGMENT

We thank Claire Chang, Sandhya Krishnankutty, and Archana Kangath, of the Institute of Food Safety and Health, for help in growing endothelial cells.

■ REFERENCES

(1) Stoclet, J. C.; Chataigneau, T.; Ndiaye, M.; Oak, M. H.; El Bedoui, J.; Chataigneau, M.; Schini-Kerth, V. B. Vascular protection by dietary polyphenols. *Eur. J. Pharmacol.* **2004**, *500*, 299–313.

(2) Hirata, Y.; Nagata, D.; Suzuki, E.; Nishimatsu, H.; Suzuki, J.; Nagai, R. Diagnosis and treatment of endothelial dysfunction in cardiovascular disease. *Int. Heart J.* **2010**, *51* (1), 1–6.

(3) Vanhoutte, P. M.; Shimokawa, H.; Tang, E. H.; Feletou, M. Endothelial dysfunction and vascular disease. *Acta Physiol. (Oxford)* **2009**, *196* (2), 193–222.

(4) Pandey, K. B.; Rizvi, S. I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell Longev.* **2009**, *2* (5), 270–278.

(5) Manach, C.; Mazur, A.; Scalbert, A. Polyphenols and prevention of cardiovascular diseases. *Curr. Opin. Lipidol.* **2005**, *16* (1), 77–84.

(6) Scalbert, A.; Johnson, I. T.; Saltmarsh, M. Polyphenols: antioxidants and beyond. *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl.), 215S–217S.

(7) Edirisinghe, I.; Burton-Freeman, B.; Varelis, P.; Kappagoda, C. T. Strawberry extract caused endothelium-dependent relaxation through the activation of PI3 kinase/Akt. *J. Agric. Food Chem.* **2008**, *56* (20), 9383–9390.

(8) Edirisinghe, I.; Burton-Freeman, B.; Kappagoda, C. T. Mechanism of the endothelium-dependent relaxation evoked by a grape seed extract. *ClinSci (London)* **2008**, *114*, 331–337.

(9) Lorenz, M.; Wessler, S.; Follmann, E.; Michaelis, W.; Düsterhöft, T.; Baumann, G.; Stangl, K.; Stangl, V. A constituent of green tea, epigallocatechin-3-gallate, activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMPdependent protein kinase-, and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. *J. Biol. Chem.* **2004**, *279*, 6190–6195.

(10) Karim, M.; McCormick, K.; Kappagoda, C. T. Effects of cocoa extracts on endothelium-dependent relaxation. *J. Nutr.* **2000**, *130*, 2105S–2108S.

(11) Nakamura, Y.; Matsumoto, H.; Morifuji, M.; Iida, H.; Takeuchi, Y. Development and validation of a liquid chromatography tandem mass spectrometry method for simultaneous determination of four anthocyanins in human plasma after black currant anthocyanins ingestion. *J. Agric. Food Chem.* **2010**, *58* (2), 1174–1179.

(12) Dudgeon, S.; Benson, D. P.; MacKenzie, A.; Paisley-Zyszkiewicz, K.; Martin, W. Recovery by ascorbate of impaired nitric oxidedependent relaxation resulting from oxidant stress in rat aorta. *Br. J. Pharmacol.* **1998**, *125*, 782–726.

(13) Sanchez Mata, M. C.; Camara Hurtado, M.; Diez Marques, C.; Torija Isasa, M. E. Comparison of high-performance liquid chromatography and spectrofluorimetry for vitamin C analysis of green beans (*Phaseolus vulgaris* L.). *Eur. Food Res. Technol.* **2000**, *210*, 220–225.

(14) Galvano, F.; Fauci, L.; Vitaglione, P.; Fogliano, V.; Vanella, L.; Felgines, C. Bioavailability, antioxidant and biological properties of the natural free-radical scavengers cyanidin and related glycosides. *Ann. Ist. Super. Sanita* **2007**, *43*, 382–393.

(15) Del Rio, D.; Costa, L. G.; Lean, M. E.; Crozier, A. Polyphenols and health: what compounds are involved? *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20* (1), 1–6.

(16) Wallerath, T.; Deckert, G.; Ternes, T.; Anderson, H.; Li, H.; Witte, K.; Förstermann, U. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* **2002**, *106*, 1652–1658.

(17) Plotnick, G. D.; Corretti, M. C.; Vogel, R. A.; Hesslink, R., Jr.; Wise, J. A. Effect of supplemental phytonutrients on impairment of the flow-mediated brachial artery vasoactivity after a single high-fat meal. *J. Am. Coll. Cardiol.* **2003**, *41*, 1744–1749.

(18) Madeira, S. V.; Auger, C.; Anselm, E.; Chataigneau, M.; Chataigneau, T.; Soares de Moura, R.; Schini-Kerth, V. B. eNOS activation induced by a polyphenol-rich grape skin extract in porcine coronary arteries. *J. Vasc. Res.* **2009**, *46*, 406–416.

(19) Anselm, E.; Chataigneau, M.; Ndiaye, M.; Chataigneau, T.; Schini-Kerth, V. B. Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS. *Cardiovasc. Res.* **2007**, *73*, 404–413.

(20) Özkan, M. Degradation of anthocyanins in sour cherry and pomegranate juices by hydrogen peroxide in the presence of added ascorbic acid. *Food Chem.* **2002**, *78*, 499–504.

(21) Zafra-Stone, S.; Bagchi, M.; Bagchi, D. Health benefits of edible berry anthocyanins: novel antioxidant and anti-angiogenic properties. *Antioxidant Measurement and Applications*; ACS Symposium Series 956; American Chemical Society: Washington, DC, 2007; 22, pp 337−351