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Aronia melanocarpa juice, a rich source of polyphenols, induces endothelium-dependent relaxations in porcine coronary arteries via the redox-sensitive activation of endothelial nitric oxide synthase

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ABSTRACT

This study examined the ability of *Aronia melanocarpa* (chokeberry) juice, a rich source of polyphenols, to cause NO-mediated endothelium-dependent relaxations of isolated coronary arteries and, if so, to determine the underlying mechanism and the active polyphenols. *A. melanocarpa* juice caused potent endothelium-dependent relaxations in porcine coronary artery rings. Relaxations to *A. melanocarpa* juice were minimally affected by inhibition of the formation of vasoactive prostanoids and endothelium-derived hyperpolarizing factor-mediated responses, and markedly reduced by N^{ω}-nitro-L-arginine (endothelial NO synthase (eNOS) inhibitor), membrane permeant analogs of superoxide dismutase and catalase, PP2 (Src kinase inhibitor), and wortmannin (PI3-kinase inhibitor). In cultured endothelial cells, *A. melanocarpa* juice increased the formation of NO as assessed by electron paramagnetic resonance spectroscopy using the spin trap iron(II)diethyldithiocarbamate, and reactive oxygen species using dihydroethidium. These responses were associated with the redox-sensitive phosphorylation of Src, Akt and eNOS. *A. melanocarpa* juice-derived fractions containing conjugated cyanidins and chlorogenic acids induced the phosphorylation of Akt and eNOS. The present findings indicate that *A. melanocarpa* juice is a potent stimulator of the endothelial formation of NO in coronary arteries; this effect involves the phosphorylation of eNOS via the redox-sensitive activation of the Src/PI3-kinase/Akt pathway mostly by conjugated cyanidins and chlorogenic acids.

Introduction

Several epidemiological studies have suggested an association between polyphenol-rich diets and a lower risk of cardiovascular diseases [1–4]. In particular, a reduced risk of cardiovascular diseases has been observed with the consumption of fruits and vegetables [5,6], red wine [4,7], green tea [8,9], cocoa and chocolate [10,11]. Several studies have indicated that red wine polyphenols and green tea catechins are able to induce potent endothelium-dependent relaxations by increasing the nitric oxide (NO) component and also, in some blood vessels, the endothelium-dependent hyperpolarization (EDH) component of the relaxation [12,13]. One of the underlying mechanism involved is the PI3-kinase/Akt-dependent activation of endothelial NO synthase by stimulating the phosphorylation of Ser1177 (an activator site) and the dephosphorylation of Thr495 (an inhibitor site) [14–16]. Besides red wine and tea, fruits and in particular berries are important sources of polyphenols. However, the literature on vasoactive properties of berries is relatively limited. Among various kinds of berries, *Aronia melanocarpa* is known to have one of the highest content of phenolic constituents including procyanidins, anthocyanins and phenolic acids [17–20].

A. melanocarpa (Michx.) Elliott (Rosaceae) also known as black chokeberry is a shrub native from North America, which is now also cultivated extensively in Europe [20,21]. The juice of

Abbreviations: AKT, protein kinase B; AUC, area under the curve; DHE, dihydroethidium; EDH, endothelium-dependent hyperpolarization; eNOS, endothelial nitric oxide synthase; EPR, electron paramagnetic resonance spectroscopy; GAE, gallic acid equivalents; HBSS, Hanks balanced salt solution; L-NA, N^{ω}-nitro-L-arginine; NO, nitric oxide; PEG-catalase, polyethyleneglycol-catalase; ROS, reactive oxygen species; SOD, superoxide dismutase; TIC, total ion current.

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A. melanocarpa is known to contain high concentrations of polyphenols up to 7 g/l [22]. The high content and the composition of the phenolic constituents of *A. melanocarpa* seem to be responsible for the wide range of its potential biological effects. It has been shown that the extract or the juice of *A. melanocarpa* has cardioprotective, gastroprotective, hepatoprotective, anti-diabetic, anti-inflammatory, anti-oxidative, anti-viral, anti-mutagenic, and anti-cancer activities [19–21,23]. *A. melanocarpa* fruit derivatives have also been shown to have a beneficial effect on several risk factors for cardiovascular disease. *A. melanocarpa* extract reduced blood pressure in patients with metabolic syndrome [23] or after myocardial infarction [24]. Moreover, a lipid-lowering effect of *A. melanocarpa* fruit juice has been observed in animals and humans [25,26]. Anthocyanins of *A. melanocarpa* decreased lipid peroxidation in sulfide-2-chloroethyl-3-chloropropyl-intoxicated mice [27]. In addition, an anti-platelet effect [28] as well as endothelium-dependent vasorelaxation has been observed in porcine coronary arteries [29–31]. In vitro studies have shown that *A. melanocarpa* fruit extract inhibits 7β-hydroxycholesterol-induced apoptosis of endothelial cells [32]. The aim of the present study was to determine whether *A. melanocarpa* is able to induce endothelium-dependent relaxations in porcine coronary artery rings and, if so, to characterize the endothelial factors and the underlying mechanism involved, and also to identify active polyphenolic compounds.

Methods

Preparation of A. melanocarpa juice

A. melanocarpa juice concentrate (66° Bx) containing cyanidin-3-galactoside (1149.7 mg/l), cyanidin-3-glucoside (53.8 mg/l), cyanidin-3-arabinoside (447.7 mg/l), and cyanidin-3-xyloside (55.9 mg/l) was kindly provided by Eckes-Granini (Nieder-Olm, Germany). Final *A. melanocarpa* juice (AMJ) was reconstituted by dilution to 15° Bx in distilled water. The final AMJ contained 7.15 g/l polyphenols expressed as gallic acid equivalents (GAE) measured by the Folin–Ciocalteu method [33]. Red wine phenolic extract dry powder (RWPs) was obtained from French red wine (Corbières A.O.C., vintage 2001, composed of a blend of Carignan, Grenache Noir, and Syrah). The extract was provided by Dr. M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France).

Chemicals

Unless indicated, all products were from Sigma–Aldrich (Saint-Quentin Fallavier, France), except wortmannin, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and the SOD mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) from Enzo Life Sciences (Lausen, Switzerland).

Vascular reactivity studies

Vascular reactivity studies were performed using isolated porcine coronary arteries as described previously [15]. Briefly, left circumflex coronary arteries were dissected from porcine hearts (obtained from the local slaughterhouse), carefully cleaned of connective tissue, and cut into rings (3–5 mm in length). Rings were suspended in organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs bicarbonate solution (mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25, and D-glucose 11, pH 7.4, 37 °C) under a resting tension of 5 g for the determination of changes in isometric tension. Rings were pre-contracted to similar levels (about 70% of the maximal contraction to KCl) by addition of an appropriateconcentration of the thromboxane mimetic U46619 (10–60 nM) before construction of a concentration-relaxation curve to *A. melanocarpa* juice. When it was needed, rings were incubated with an inhibitor for 30 min before the addition of U46619. The relaxations are expressed as relative responses, where 0% is the precontraction level to U46619 and 100% is the basal tension observed before addition of U46619.

Cell culture

Porcine coronary artery endothelial cells were isolated and cultured using the method previously described [15]. Briefly, endothelial cells were isolated from freshly dissected porcine coronary arteries by collagenase treatment (type I, Worthington, 1 mg/ml for 12 min at 37 °C), and cultured in flasks containing MCDB 131 medium (Invitrogen, Saint Aubin, France) supplemented with 15% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 μg/ml), and L-glutamine (2 mM) (all from Cambrex, Saint-Beauzire, France). All experiments were performed with confluent cultures of cells used at first passage. Cells were incubated with serum-free culture medium (with 0.1% fetal bovine serum) for 5 h prior to treatment.

Determination of NO formation

Determination of NO formation was assessed by electron paramagnetic resonance spectroscopy (EPR) after formation of $[Fe(II)NO(DETC)_2]$, a paramagnetic DETC iron complex with NO, in cultured endothelial cells. The EPR methodology was used as reported previously with minor modifications [34]. Confluent cultures of endothelial cells (first passage, ~1 million cells per well) were washed twice with Hanks balanced salt solution (HBSS; Lonza, Verviers, Belgium) buffered with 10 mM HEPES, and then they were incubated in a HBSS–HEPES solution in the presence of bovine serum albumin (20.5 mg/ml), 1.5 mM $CaCl_2$, 0.3 mM L-arginine and with or without L-NA for 30 min at 37 °C. Spin trap chemicals $FeSO_4$ (0.8 mM) and DETC (1.6 mM) were rapidly mixed to obtain a colloid form $[Fe(II)(DETC)_2]$, which was added to endothelial cells at a final concentration of 0.2 mM. After 5 min, the endothelial formation of NO was induced by the addition of either bradykinin (300 nM) or *A. melanocarpa* juice (16.8 μg GAE/ml) for 20 min. Thereafter, dishes were placed on ice, and the incubation medium was removed before addition of 0.2 ml of the HBSS–HEPES buffer. Cells were then collected and loaded into a calibrated tube. Tubes were rapidly frozen at 77 K for EPR measurements. EPR measurements were performed on an MS100 spectrometer (Magnettech Ltd., Berlin, Germany) under the following conditions: temperature 77 K, microwave frequency 9.34 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 1 mT. NO is not directly measured by EPR due to its short half-life. Therefore, NO is stabilized by its liaison with the DETC iron spin trap $[Fe(II)\ (DETC)_2]$, forming a paramagnetic complex $[Fe(II)NO(DETC)_2]$, which gives a characteristic triplet signal EPR spectra [35], with an amplitude directly proportional to the quantity of $[Fe(II)NO(DETC)_2]$ present in the sample. The quantification of the signal is based on the high of the third companion signal, which appears around 331 mT in our experimental settings, and is not modified by the present other products such as Cu^{2+}-DETC [35].

In situ detection of reactive oxygen species

The oxidative fluorescent dye DHE was used to evaluate the *in situ* formation of reactive oxygen species (ROS) using a method previously described [15,36]. Cultured coronary endothelial cells in Hanks' balanced salt solution were loaded with DHE (2.5 μM)

for 20 min before treatment either with solvent or *A. melanocarpa* juice (16.8 μg GAE/ml) for 10 min at 37 °C. As indicated, cells were incubated with either catalase (500 U/ml), SOD (500 U/ml), PEG-catalase (500 U/ml), or MnTMPyP (100 μM) for 30 min before addition of *A. melanocarpa* juice. Images were obtained with a Leica DM 4000 fluorescence microscope equipped with CY3 filter (Leica microsystem SAS, Nanterre, France) and analyzed using ImageJ software (National Health Institute, USA).

Western blot analysis

Western blot analyses were performed as previously published [15,37]. Briefly, *A. melanocarpa* juice-treated cells were washed twice with PBS and then lysed in extraction buffer. When it was necessary, cells were incubated with catalase (500 U/ml), SOD (500 U/ml), PEG-catalase (500 U/ml), MnTMPyP (100 μM), PP2 (10 μM) or wortmannin (30 nM) for 30 min before addition of *A. melanocarpa* juice. Total proteins (20 μg) were separated on 8% SDS–polyacrylamide gels at 80 V for 2.5 h. Separated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Amersham, Les Ulis, France) at 100 V for 120 min. Membranes were blocked with blocking buffer containing 3% bovine serum albumin, Tris-buffered saline solution (Euromedex, Souffelweyersheim, France) and 0.1% Tween 20 (TBS-T) for 1 h. To detect the phosphorylated proteins, membranes were incubated with the respective primary antibody (p-Src Tyr418, Biosource; p-Akt Ser473 and p-eNOS Ser1177, Cell Signaling Technology; and total eNOS, BD Biosciences, dilution of 1:1000) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit or anti-mouse IgG; Cell Signaling Technology, dilution of 1:5000 or 1:20,000, respectively) at room temperature for 60 min. Prestained markers (Euromedex) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham, Les Ulis, France).

Preparative HPLC

A. melanocarpa juice was fractionated using preparative RP-HPLC. Separations were carried out using a semipreparative RP-HPLC system comprising a Gilson 305 pump and a Gilson 115 UV detector (Gilson International-France, Roissy, France) fitted with a column C18-Nucleodur®, 250 × 21 mm; 10 μm, (Macherey–Nagel, Hoerdt, France), and eluted with 0.01% aqueous formic acid (A) and methanol (B) at a flow rate of 14 ml/min following the conditions: from 90% to 50% A for 80 min, and 100% of B for 5 min, followed by washing and reconditioning of the column. Fractions were monitored at 370 nm and each individual large peak (Fractions 1–10) was collected separately. All fractions were reduced to dryness using a rotary evaporator set at 40 °C.

Analytical RP-HPLC

All fractions were analyzed using an analytical RP-HPLC system comprising a Varian 9010 pump and a Varian ProStar® 330 diode array detector (Varian, Courtaboeuf, France) fitted with a column C18-Nucleodur®, 250 × 4.6 mm; 5 μm (Macherey–Nagel, Hoerdt, France) and eluted with water containing 0.1% TFA (A) and methanol (B) using the following gradient: from 90% to 50% (A) for 50 min, 50% (A) for 5 min and 100% (B) for 10 min and back to the initial conditions. The flow rate was 1 ml/min at 25 °C.

LC–MS analysis

Identification of major phenolic compounds in fractions was carried out by LC–MS technique on an Agilent 1200 SL HPLC (Agilent, Massy, France) chain fitted with a 30 × 1 mm; 1.9 μm i.d. Hypergold column (ThermoScientific, Courtaboeuf, France), coupled to a HCT ultra mass spectrometer (Bruker Daltonics, Wissembourg, France) and eluted with acetonitrile (A) and water (B) using the following gradient: from 98% to 2% A in 6 min, followed by washing and reconditioning of the column. All analyses were done in positive ionization using data dependent MS^2. A combination of co-chromatography with authentic standards, when available, absorbance spectra and mass spectra, using MS^2, were used to confirm the identity of compounds previously reported in the literature [20]. Quantitative estimations of proportions are based on the area under the curve for total ion signal on MS analysis.

Statistical analysis

Values are expressed as means ± SEM. Statistical evaluation was performed using ANOVA followed by Fischer's protected least significant difference test for vascular reactivity studies, and using Student's *t*-test for paired data for Western blot, DHE and EPR experiments. Values of $P < 0.05$ were considered statistically significant.

Results

A. melanocarpa juice induces endothelium-dependent NO-mediated relaxations in coronary artery rings

As shown in Fig. 1, *A. melanocarpa* juice induced concentration-dependent relaxations in coronary artery rings with endothelium but only minor ones in those without endothelium (Fig. 1). When compared based on their total phenolic content (expressed as μg of GAE, Fig. 1), *A. melanocarpa* juice exhibited a significantly greater vasorelaxation than red wine polyphenols with EC50 of 1.29 ± 0.1 and 3.35 ± 0.38 μg GAE/ml, respectively. Relaxations to *A. melanocarpa* juice were significantly reduced by about 62% by L-NA (300 μM, a competitive inhibitor of NO synthase), not affected by inhibitors of endothelium-dependent hyperpolarization-mediated responses, charybdotoxin plus apamin (100 nM each), and markedly reduced by the combination of L-NA and charybdotoxin plus apamin (Fig. 2). These findings indicate that *A. melanocarpa* juice

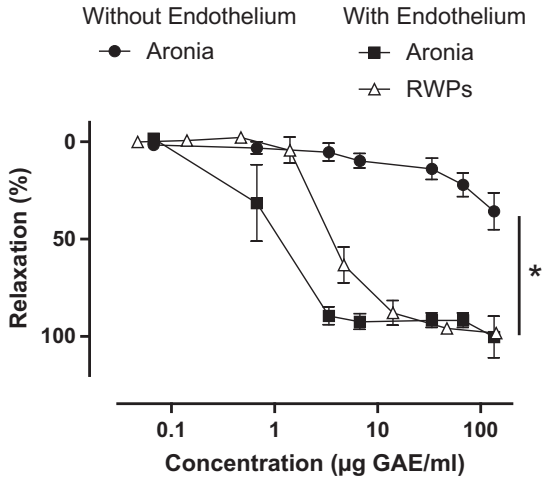

Fig. 1. Characterization of endothelium-dependent relaxations to *Aronia melanocarpa* juice in porcine coronary artery rings. Endothelium-denuded and intact rings were contracted with U46619 before the addition of increasing concentrations of *Aronia melanocarpa* juice (Aronia) or red wine polyphenols (RWPs). Results are shown as mean ± SEM of 4 independent experiments. *$P < 0.05$ versus control.

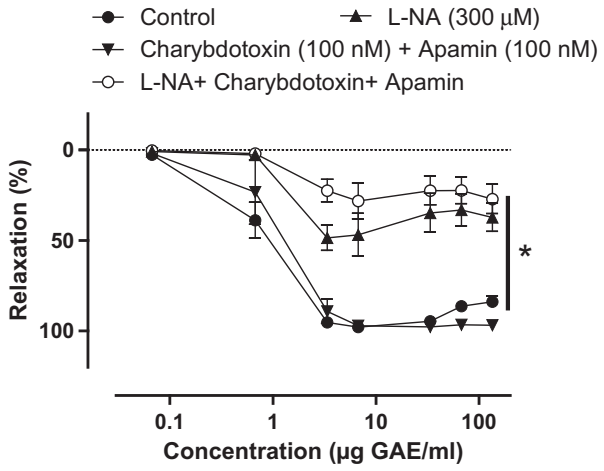

Fig. 2. Characterization of the endothelium-dependent relaxations to *Aronia melanocarpa* juice in coronary artery rings. Intact rings were incubated with either the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NA, 300 μM), inhibitors of EDH using the combination of charybdotoxin plus apamin (100 nM each), or both for 30 min before the contraction was induced with U46619 and the subsequent relaxation to *Aronia melanocarpa* juice. Experiments were performed in the presence of indomethacin (10 μM) to prevent the formation of vasoactive prostanoids. Results are shown as mean ± SEM of 5 independent experiments. $^{*}P < 0.05$ versus control.

induces endothelium-dependent relaxations of coronary arteries, which are mainly mediated by NO.

A. melanocarpa juice-induced relaxations are redox-sensitive and involve Src kinase and the PI3-kinase/Akt pathway

Previous studies have shown that the grape-derived products-induced NO-mediated relaxations are critically dependent on a redox-sensitive event and involve the Src/PI3-kinase/Akt pathway leading to the phosphorylation of eNOS [14,15]. Therefore, experiments were performed to determine whether this signaling pathway is also involved in *A. melanocarpa* juice-induced relaxations. The Src kinase inhibitor, PP2, and the PI3-kinase inhibitor, wortmannin, significantly reduced the endothelium-dependent relaxations to *A. melanocarpa* juice (Fig. 3; relaxations amounted to 97.7 ± 1.6, 62.7 ± 9.1 and 47.8 ± 10.5% at 6.73 μg GAE/ml of AMJ for control, PP2 and wortmannin, respectively). Endothelium-dependent relaxations to *A. melanocarpa* juice were also markedly reduced by the membrane permeant SOD mimetic, MnTMPyP, and the cell permeable PEG-catalase whereas native SOD and native catalase did not have such effects (Fig. 4; relaxations amounted to 92.3 ± 4.1, 87.0 ± 7.3, 88.8 ± 5.4, 6.8 ± 5.3 and 22.8 ± 14.9% at 6.73 μg GAE/ml of AMJ for control, SOD, catalase, MnTMPyP and PEG-catalase, respectively). Previous studies have shown that MnTMPyP affected neither the NO-mediated endothelium-depen-

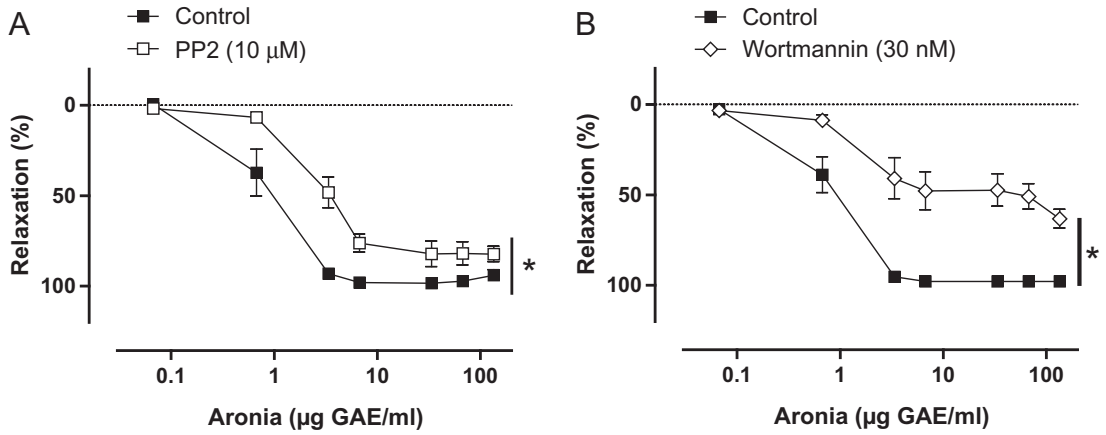

Fig. 3. The role of Src kinase and the PI3-kinase/Akt pathway in the *Aronia melanocarpa* juice-induced endothelium-dependent relaxations. Coronary artery rings with endothelium were incubated with (A) PP2 (10 μM), a Src kinase inhibitor or (B) wortmannin (30 nM), a PI3-kinase inhibitor for 30 min before the contraction was induced with U46619 and the subsequent relaxation to *Aronia melanocarpa* juice. Results are shown as mean ± SEM of 5 independent experiments. $^{*}P < 0.05$ versus control.

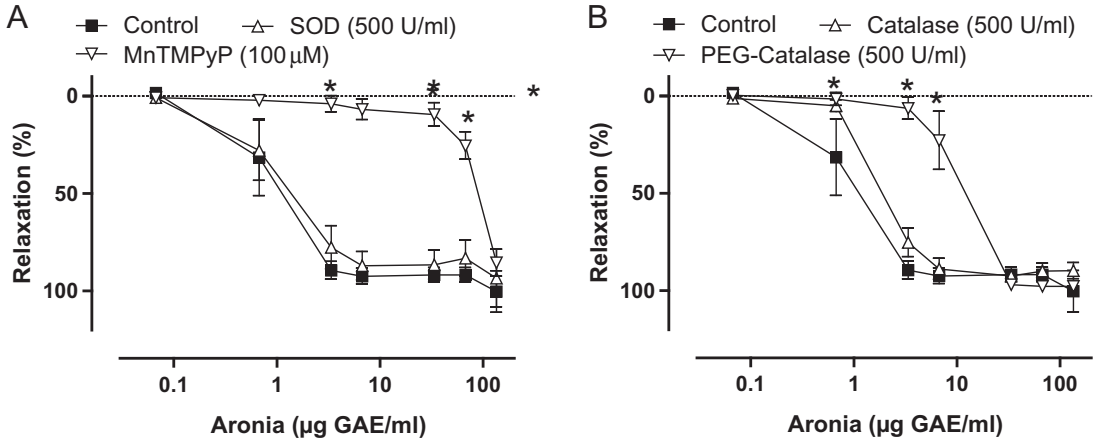

Fig. 4. The endothelium-dependent relaxation to *Aronia melanocarpa* juice is a redox-sensitive event in coronary artery rings. Coronary artery rings with endothelium were incubated with either (A) MnTMPyP (100 μM), a cell permeable superoxide dismutase (SOD) mimetic, or native SOD (500 U/ml), (B) the membrane permeant analog of catalase polyethyleneglycol-catalase (PEG-catalase) (500 U/ml) or native catalase (500 U/ml) for 30 min before the contraction was induced with U46619 and the subsequent relaxation to *Aronia melanocarpa* juice. Results are shown as mean ± SEM of 4 independent experiments. $^{*}P < 0.05$ versus control.

dent relaxation of coronary artery rings nor the formation of NO in cultured coronary artery endothelial cells in response to bradykinin [14]. Altogether, these findings suggest that the *A. melanocarpa* juice-induced NO-mediated relaxation is a redox-sensitive event involving superoxide anions and hydrogen peroxide, and the Src/PI3-kinase/Akt pathway.

A. melanocarpa juice stimulates the formation of NO in endothelial cells

Next, experiments were performed to provide direct evidence that *A. melanocarpa* juice induces NO formation in endothelial cells using electron paramagnetic resonance spectroscopy after formation of [Fe(II)NO(DETC)$_2$], a paramagnetic DETC iron complex with NO. Exposure of cells to *A. melanocarpa* juice induced about a 2.5-fold increase in the formation of NO, which was similar to that induced by a physiological activator bradykinin (Fig. 5). Both stimulatory effects were abolished by L-NA indicating the involvement of NO (Fig. 5).

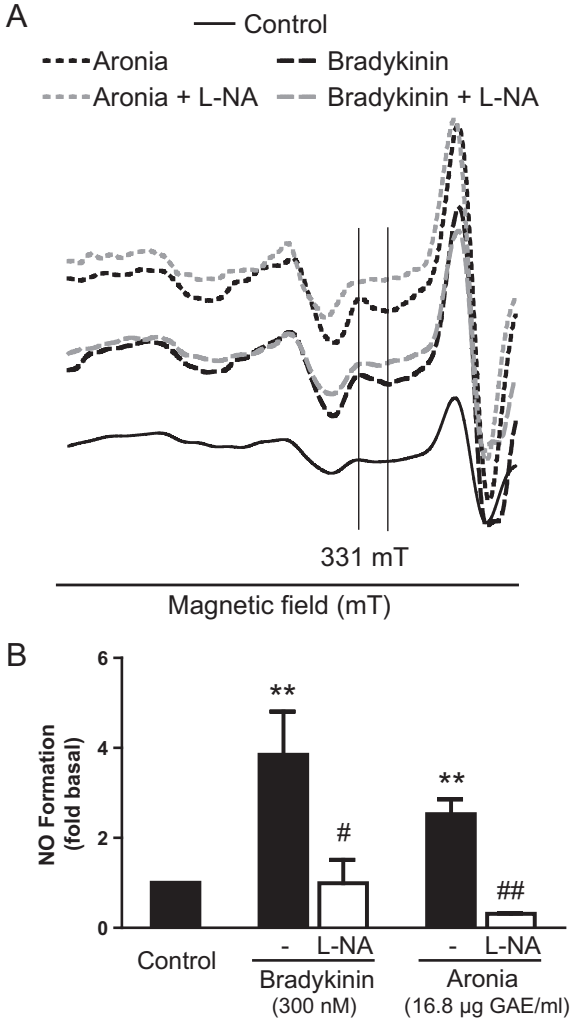

Fig. 5. *Aronia melanocarpa* juice stimulates the formation of NO in cultured endothelial cells. Cells were exposed to either solvent or L-NA (300 µM) for 30 min before the addition of *Aronia melanocarpa* juice (16.8 µg GAE/ml) or bradykinin (300 nM), a potent endothelium-dependent vasodilator for 20 min. The formation of NO was assessed by electron paramagnetic resonance spectroscopy after formation of [Fe(II)NO(DETC)$_2$], a paramagnetic DETC iron complex with NO. (A) Representative original data showing the third companion used for quantification (vertical bars). (B) Corresponding cumulative data. Results are shown as mean ± SEM of 3 independent experiments. $^{**}P < 0.01$ versus control. $^{\#\#}P < 0.01$ versus *Aronia melanocarpa* juice. $^{\#}P < 0.05$ versus bradykinin.

A. melanocarpa juice induces the redox-sensitive Src/PI3-kinase/Akt-dependent phosphorylation of eNOS

To better characterize the signaling pathway involved in eNOS activation in response to *A. melanocarpa* juice, levels of p-Src, p-Akt and p-eNOS were assessed in endothelial cells by immunoblotting. *A. melanocarpa* juice increased within 3 min signals of p-Akt and p-eNOS, and these effects increased gradually at least up to 30 min (Fig. 6A). *A. melanocarpa* juice also caused the phosphorylation of Src (Fig. 8). The stimulatory effect of *A. melanocarpa* juice was concentration-dependent (Fig. 6B). *A. melanocarpa* juice-induced phosphorylation of Akt and eNOS was inhibited by the Src kinase inhibitor, PP2, and the PI3-kinase inhibitor, wortmannin (Fig. 7), and also by both MnTMPyP and PEG-catalase, but not by native SOD and catalase (Fig. 8). In addition, the stimulatory effect on Src was also abolished by MnTMPyP and not significantly affected by PEG-catalase, native SOD and catalase (Fig. 8). These data indicate that ROS, especially superoxide anions, act as upstream mediators of Src kinase whereas both superoxide anions and hydrogen peroxide are involved in the activation of the PI3-kinase/Akt pathway leading to eNOS phosphorylation in response to *A. melanocarpa* juice.

A. melanocarpa juice stimulates the formation of ROS in endothelial cells

To provide further evidence that *A. melanocarpa* juice is able to cause an intracellular pro-oxidant response, the *in situ* formation of ROS was assessed in coronary artery endothelial cells using the redox-sensitive fluorescent probe DHE. Exposure of cells to *A. melanocarpa* juice markedly increased the fluorescent signal, and this effect was abolished by MnTMPyP and PEG-catalase, but not by native SOD and catalase (Fig. 9).

The A. melanocarpa juice-induced phosphorylation of Akt and eNOS in endothelial cells involves conjugated cyanidins and chlorogenic acids

A. melanocarpa juice is known to contain a mixture of polyphenolic compounds and, in particular, procyanidins, anthocyanins and flavonols [20,38]. In order to identify active polyphenolic compounds, *A. melanocarpa* juice wassubjected to fractionation using a semi-preparative HPLC [39]. Ten different fractions of *A. melanocarpa* juice were prepared and their ability to induce the phosphorylation of Akt and eNOS in endothelial cells was evaluated. Fractions 1–6 induced the phosphorylation of both Akt and eNOS whereas fractions 7–10 were inactive (Fig. 10). The analysis of the different fractions by analytical HPLC and LC–MS techniques has lead to the tentative identification of several chlorogenic acids, cyanidin glycosides and derivatives of quercetin (Table 1). The data indicate that the active fractions contained predominantly conjugated cyanidins and chlorogenic acids.

Discussion

Endothelial cells lining the luminal surface of all blood vessels have a key role in the control of vascular tone in part via potent vasodilator mechanisms involving NO and EDH. Numerous studies have reported the ability of several sources of polyphenols such as red wine and green tea to stimulate the endothelial formation of NO [12–16]. Such an effect is likely to contribute to their beneficial effect on the cardiovascular system. In our previous study [31], comparing 13 different fruit juices or purees, *A. melanocarpa* and

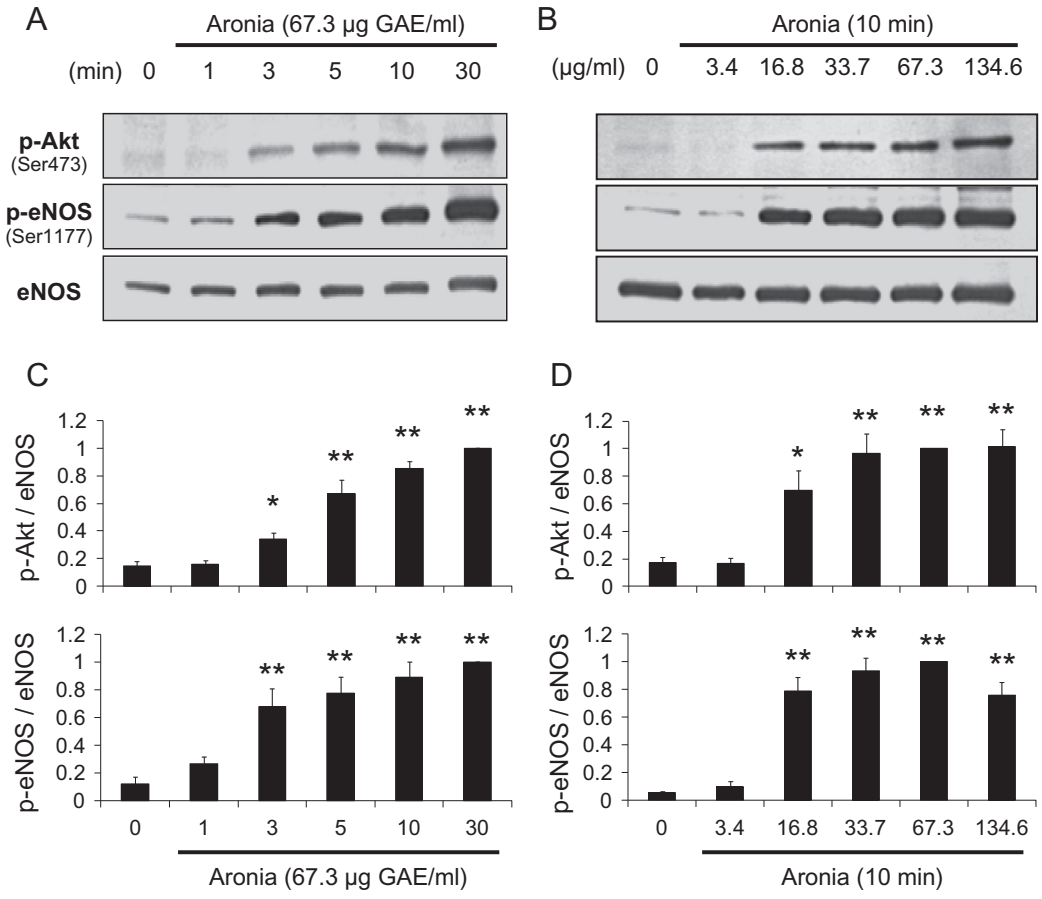

Fig. 6. *Aronia melanocarpa* juice induces a time- and concentration-dependent phosphorylation of Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Cells were exposed to *Aronia melanocarpa* juice for the indicated times and concentrations at 37 °C. Thereafter, the level of p-Akt and p-eNOS was determined by Western blot analysis. (A and B) Representative immunoblots, and (C, D) corresponding cumulative data. Results are shown as mean ± SEM of 4 independent experiments. $^{*}P < 0.05$ versus control. $^{**}P < 0.01$ versus control.

blackcurrant were identified as the most potent inducers of endothelium-dependent relaxations in isolated coronary arteries. The present study indicates further that the *A. melanocarpa* juice-induced relaxations were markedly reduced by L-NA indicating a major role for NO, as shown previously by Bell et Gochenaur [29]. Moreover, the present findings provide also direct evidence that *A. melanocarpa* juice activates eNOS as indicated by the L-NA-sensitive formation of NO and the enhanced phosphorylation of eNOS at Ser1177 in endothelial cells. Interestingly, *A. melanocarpa* juice induced the endothelial formation of NO to a level similar as that induced by the physiological agonist bradykinin.

NO-mediated relaxations induced by red wine polyphenols, grape juice, and tea catechins in coronary arteries have been shown to be redox-sensitive events involving the intracellular formation of ROS [14–16,40,41]. Therefore, the role of ROS in *A. melanocarpa* juice-induced relaxation was assessed. Both membrane permeant analogs of either SOD or catalase strongly reduced relaxations to *A. melanocarpa* juice whereas native SOD and native catalase were inactive. These findings in addition to previous ones indicating that MnTMPyP affected neither NO-mediated relaxations in coronary artery rings nor the formation of NO in cultured coronary artery endothelial cells in response to bradykinin [14,16,41], suggest a key role of both intracellular superoxide anions and hydrogen peroxide in the *A. melanocarpa*-induced NO-mediated relaxation. In addition, MnTMPyP and PEG-catalase also prevented the *A. melanocarpa* juice-induced phosphorylation of eNOS at Ser1177. Moreover, direct evidence that *A. melanocarpa* juice is able to cause a modest MnTMPyP and PEG-catalase-sensitive pro-oxidant response in endothelial cells was obtained using the redox-sensitive fluorescent probe dihydroethidium.

In contrast, polyphenols and polyphenol-rich sources have been shown to have antioxidant effects *in vivo*. Indeed, polyphenols reduced the increased oxidative stress in the vascular wall associated with endothelial dysfunction in many cardiovascular diseases, such as hypertension, and aging [42–46]. Moreover, the beneficial antioxidant effect of polyphenols on the pathological increased oxidative stress seems to be due to an indirect antioxidant effect such as the induction of antioxidant enzymes and inhibition of NADPH oxidase, rather than to their direct antioxidant properties (scavenging of ROS). Indeed, we, as well as others, have previously reported in several models of cardiovascular diseases that endothelial dysfunction is associated with an increased vascular expression of NADPH and increased oxidative stress throughout the arterial wall, and that treatment with polyphenol-rich sources prevented both responses [44–47]. However, in healthy arteries, polyphenols and polyphenol-rich sources induce a formation of ROS specifically in the endothelial layer, but not in the underlying smooth muscle cells [37,48]. Similarly, polyphenols and polyphenol-rich sources induced ROS formation in cultured endothelial cells, leading to the activation of the Src/PI3-kinase/Akt pathway and eNOS with the subsequent formation of NO and vasorelaxation for review, see [12].

The endothelial specific mechanism underlying the pro-oxidant effect of polyphenols is unkown. In our previous studies, the redox-sensitive NO-mediated relaxations to red wine polyphenols and tea catechins were not affected by pharmacological inhibitors of the

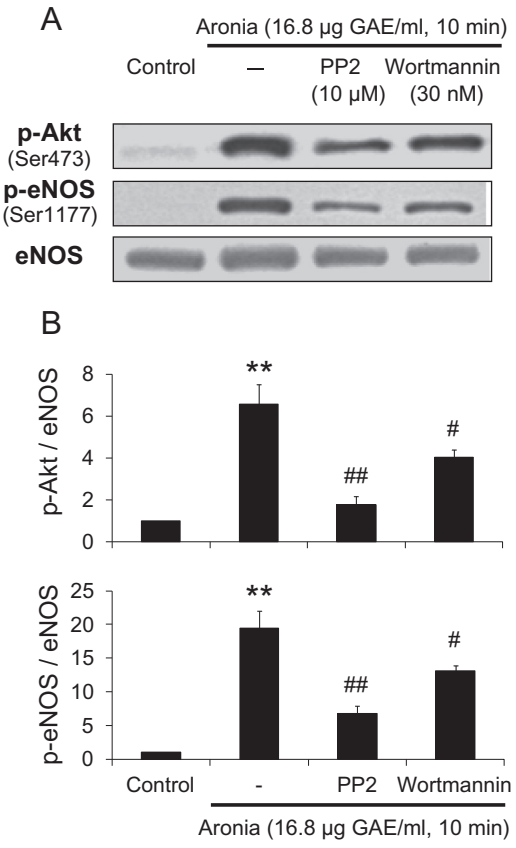

Fig. 7. Role of the Src kinase and the PI3-kinase/Akt pathway in *Aronia melanocarpa* juice-induced phosphorylation of Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Cells were incubated either with solvent, PP2 (10 μM) or wortmannin (30 nM) for 30 min before the addition of *Aronia melanocarpa* juice (16.8 μg GAE/ml). The level of p-Akt and p-eNOS was determined by Western blot analysis. (A) Representative immunoblots, and (B) corresponding cumulative data. Results are shown as mean ± SEM of 4 independent experiments. $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ versus *Aronia melanocarpa* juice. $^{\#\#}P < 0.01$ versus *Aronia melanocarpa* juice.

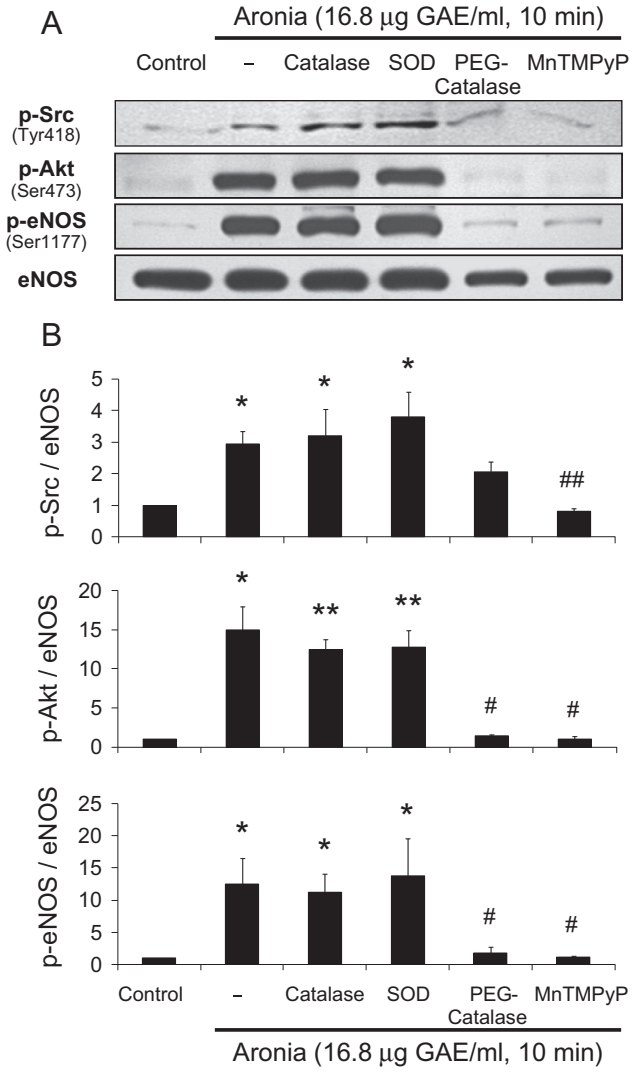

Fig. 8. Role of a redox-sensitive event in the *Aronia melanocarpa* juice-induced phosphorylation of Src at Tyr418, Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Cells were incubated with either solvent, catalase (500 U/ml), SOD (500 U/ml), PEG-catalase (500 U/ml) or MnTMPyP (100 μM) for 30 min before the addition of *Aronia melanocarpa* juice (16.8 μg GAE/ml). The level of p-Src, p-Akt and p-eNOS was determined by Western blot analysis. (A) Photos show representative immunoblots, and (B) corresponding cumulative data. Results are shown as mean ± SEM of 4 independent experiments. $^{*}P < 0.05$ versus control. $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ versus *Aronia melanocarpa* juice. $^{\#\#}P < 0.01$ versus *Aronia melanocarpa* juice.

mitochondrial function, xanthine oxidase, and cytochrome P450, indicating that they are most likely not involved [14,16,41]. Moreover, the observation that NO-mediated relaxations to red wine polyphenols were similar in aortas from gp91phox knockout and wild-type mice also does not support a role for NADPH oxidase [14]. Alternatively, other types of ROS generating enzymes may be involved and possibly also the auto-oxidation of the phenolic structure. Indeed, it has been shown that some polyphenols produce superoxide anions and hydrogen peroxide following auto-oxidation leading to the formation of semi-quinones and quinones catalyzed by metal ions such as Cu^{2+} and Fe^{2+} [16,49,50]. Moreover, the hydroxyl functions, and especially the conjugated hydroxyls groups on B and D rings, have been shown to be involved in both the antioxidant and pro-oxidant properties of flavonoids [16,51,52].

ROS have been identified as important intracellular mediators activating various redox-sensitive protein kinases to induce biological responses. Indeed, hydrogen peroxide is a potent activator of the PI3-kinase/Akt [53], and the Src family of kinases are redox-sensitive kinases [54] which have been shown to act as up-stream activators of the PI3-kinase/Akt pathway [55]. In addition, the redox-sensitive Src/PI3-kinase/Akt pathway has been shown to mediate the stimulatory effect of grape-derived polyphenols on the endothelial formation of NO [14,15]. Therefore, the role of the redox-sensitive Src/PI3-kinase/Akt pathway on the endothelial formation of NO to *A. melanocarpa* juice has been studied. The present findings indicate that *A. melanocarpa* juice induces activation of the PI3-kinase/Akt pathway in a Src-dependent and redox-sensitive manner in endothelial cells. Moreover, the Src/PI3-kinase/Akt pathway mediates activation of eNOS at Ser1177 and the subsequent relaxation of coronary arteries since these responses are inhibited by PP2 and wortmannin. These observations are consistent with previous ones with grape juice [15]. Altogether, these findings suggest that the intracellular formation of superoxide anions and hydrogen peroxide is an early event, which triggers the cascade of events leading to an enhanced endothelial formation of NO by increasing the phosphorylation of eNOS via the Src/PI3-kinase/Akt pathway.

According to previous studies, both procyanidins and anthocyanins are strong activators of eNOS and this effect is critically dependent on the polyphenol structure. Indeed, the red wine

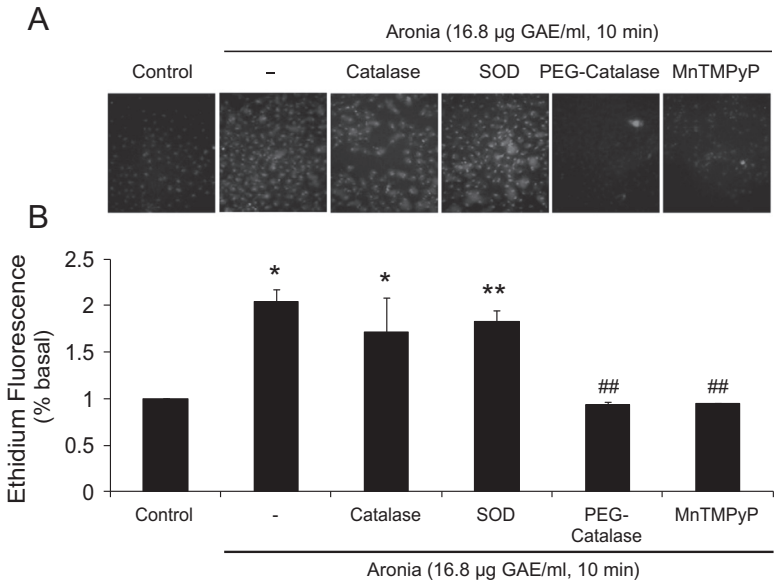

Fig. 9. *Aronia melanocarpa* juice induces the formation of ROS in cultured endothelial cells. Dihydroethidium (DHE, 2.5 μM)-loaded cells were exposed to either solvent, catalase (500 U/ml), SOD (500 U/ml), PEG-catalase (500 U/ml) or MnTMPyP (100 μM) for 30 min before the addition of *Aronia melanocarpa* juice (16.8 μg GAE/ml). Ethidium fluorescence was detected using an epifluorescent microscope. (A) Representative original photos showing ethidium fluorescence in cells. (B) Corresponding cumulative data. Results are shown as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ versus control. $^{**}P < 0.01$ versus control. $^{\#\#}P < 0.01$ versus *Aronia melanocarpa* juice.

polyphenol petunidin-O-coumaroyl-glucoside is able to activate eNOS whereas the closely related structure malvidin-O-coumaroyl-glucoside is inactive [16]. Similarly, green tea polyphenols (−)-epicatechin-3-*O*-gallate and (−)-epigallocatechin-3-*O*-gallate activate eNOS while (−)-epicatechin and (−)-epigallocatechin are inactive [16]. It has been shown that almost 25% of the total

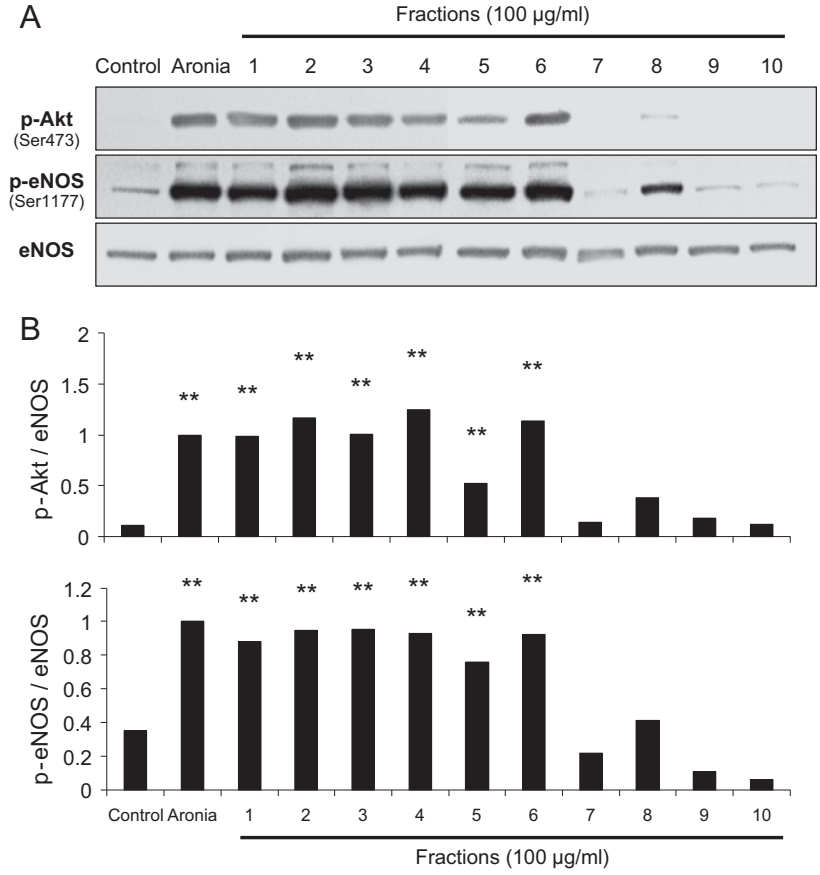

Fig. 10. Effect of 10 different *Aronia melanocarpa* juice fractions on the phosphorylation of Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Cells were exposed to each fraction (100 μg/ml) for 10 min at 37 °C. Thereafter, the level of p-Akt and p-eNOS was determined by Western blot analysis. (A) Photos show representative immunoblots, and (B) corresponding cumulative data. Results are shown as mean ± SEM of 6 independent experiments. $^{**}P < 0.01$ versus control.

Table 1
Tentative identification of phenolic compounds present in *Aronia melanocarpa* juice fractions by HPLC and LC–MS analysis.

Fraction	Tentative identification	t_r (min)	λmax (nm)	$[M]^+$ (*m/z*)	MS^2 ions (*m/z*)	Percentage area (TIC)
1	Neochlorogenic acid	27	325	355	163	69%
	Cyanidin-3-arabinoside	45	525	419	391–279	9%
	Cyanidin-3-xyloside	47	525	419	391, 307, 289	22%
2	Neochlorogenic acid	27	325	355	163	100%
3	Cyanidin-3-galactoside	43	525	449	391, 368	72%
	Cyanidin-3-arabinoside	45	525	419	391, 338	20%
4	Chlorogenic acid	38	325	355	163	4%
	Cyanidin-3-arabinoside	45	525	419	305, 391	92%
	Cyanidin-3-xyloside	47	525	419	391	4%
5	Chlorogenic acid	38	325	355	163	68%
	Cyanidin-3-glucoside	45	525	449	355, 391	12%
	Cyanidin-3-xyloside	47	525	419	391	20%
6	Cyanidin-3-arabinoside	45	525	419	387, 287, 207	84%
	Cyanidin-3-xyloside	47	525	419	391, 279, 201	16%
7	Cyanidin rutinoside	45	525	595	517, 391	n.p.
	Cyanidin-3-arabinoside	45	525	419	391, 309	n.p.
8	Quercetin-hexoside	43	355	517	487, 303	46%
	Quercetin-diglycoside	48	355	627	487, 303	38%
9	Quercetin-diglycoside	48	355	627	465, 303	75%
10	Quercetin-rutinoside	55	355	611	597, 465, 303	90%

All retention times (t_r) and λmax were obtained with analytical HPLC. Mass spectra were obtained with LC–MS and the percentage of compounds in each fraction is given as percentage of the area under the curve (AUC) for Total Ion Current (TIC) trace. n.p., not performed due to co-elution of compounds in LC–MS analysis.

polyphenols in *A. melanocarpa* are anthocyanins, which makes it one of the richest plant sources of anthocyanins [38]. Especially in *A.melanocarpa*, most of the anthocyanins are cyanidin based glycosides including cyanidin 3-galactoside, cyanidin 3-arabinoside, cyanidin 3-xyloside, and cyanidin 3-glucoside [20]. The fractionation of *A. melanocarpa* juice and the evaluation of the effect of the fractions on eNOS activation indicate that cyanidin-3-galactoside, cyanidin-3-arabinoside, cyanidin-3-xyloside, cyanidin-3-glucoside, neochlorogenic acid, and chlorogenic acid are present in the active fractions whereas cyanidin-rutinoside, quercetin-hexoside, quercetin-diglycoside, and quercetin-rutinoside are in the inactive ones. A previous study has indicated that cyanidin-3-glucoside induces phosphorylation of eNOS [56]. The possibility that other conjugated cyanidins like cyanidin-3-galactoside and cyanidin-3-xyloside activate eNOS is also suggested by the present findings. In addition, the derivatives of chlorogenic acids may also be able to activate eNOS since fraction 2 contained mostly neochlorogenic acid and this fraction induced the phosphorylation of eNOS.

Although it is important to study the biological functions of natural polyphenols and understand the underlying mechanisms, the bioavailability of these compounds is also a key point. Despite the fact that polyphenols can undergo hydrolysis by intestinal enzymes and/or the colonic microflora before absorption [57,58], intact glycosides are major circulating forms of anthocyanins [59] possibly due to the instability of the aglycone form, anthocyanidins, and to the existence of a specific mechanism of absorption or metabolism. Several studies have shown that, in rats and mice, anthocyanins can be absorbed from the stomach [60–62]. Passamonti et al. have proposed that glycosides of anthocyanins are transported by bilitranslocase at the gastric level, because they are good ligands for this carrier [63]. Moreover, published studies indicate that the bilitranslocase transporter is involved in the cellular uptake of anthocyanins in endothelial cells [64,65], and that blockade of the bilitranslocase transporter by specific antibodies significantly inhibited the anthocyanin-induced relaxation in rat aortic rings [66]. Such a hypothesis is also supported by the fact that the *A. melanocarpa* juice-induced phosphorylation of Akt and eNOS in endothelial cells is significantly inhibited by an antibody directed against bilitranslocase (Ziberna, Kim, Schini-Kerth & Passamonti, unpublished data).

In addition, anthocyanins can undergo metabolization by intestinal and hepatic cells to generate glucuronides, methyl and sulfo-conjugates, which may contribute to the biological effect [67]. Cyanidin-based anthocyanins can also undergo cleavage of the sugar moiety followed by ring fission of the released cyanidin, which produces phenolic acids such as 3,4-dihydroxybenzoic acid (protocatechuic acid) by the microflora in the colon [68,69]. The resulting smaller molecules can be absorbed and reach blood flow where they can be, in turn, metabolized by the liver and organs before urinary excretion [70]. Recent studies indicate that the colic catabolites and their metabolites are found in amounts largely superior to the flavonoid metabolites in urine after oral intake of flavonoids for review, see [71,72]. However, the possibility that these phenolic acids contribute to the biological action remains to be determined.

In conclusion, the present findings indicate that *A. melanocarpa* juice is a powerful endothelium-dependent vasodilator of coronary arteries by stimulating the endothelial formation of NO. They further indicate that the intracellular formation of ROS in particular superoxide anions and hydrogen peroxide, is a crucial and early event leading to activation of eNOS via the Src/PI3-kinase/Akt pathway. Thus, the beneficial effect of *A. melanocarpa* juice consumption on the endothelial and platelet function might be, in part, due to an enhanced formation of NO. Among a great variety of polyphenolic compounds which induce these activities, both conjugated cyanidins and chlorogenic acids appear to be active polyphenols present in *A. melanocarpa* juice.

Acknowledgments

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