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ARTICLE

Effect of *Vaccinium myrtillus* and Its Polyphenols on Angiotensin-Converting Enzyme Activity in Human Endothelial Cells

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This study investigates if the connection between *Vaccinium myrtillus* and angiotensin-converting enzyme (ACE) might be an explanation of the pharmacological effects on circulation. Cultured endothelial cells from human umbilical veins were incubated with bilberry 25E extract. The main anthocyanidins combined in myrtillin chloride and separately in cyanidin, delphinidin, and malvidin, respectively, were examined concerning their effects on ACE. After 10 min of incubation with bilberry 25E, a significant, dose-dependent inhibition of ACE activity was seen, and after incubation with myrtillin chloride a significant inhibition was seen. No effect was seen with the anthocyanidins. The effect seems to be dependent on this specific mixture of anthocyanins in the bilberry. *V. myrtillus* may thus have the potential to prevent and protect against cardiovascular diseases.

KEYWORDS: Angiotensin-converting enzyme; *Vaccinium myrtillus*; anthocyanidins

INTRODUCTION

Vaccinium myrtillus L. (Ericaceae), commonly known as bilberry, grows on moors and peaty soil in hilly and mountainous regions in Europe, Asia, and North America. *V. myrtillus* has been traditionally used since the Middle Ages as an antidiabetic, adstringent, and antiseptic for diarrhea. Today, *V. myrtillus* is more important for the beneficial effect on blood circulation, especially in vision disorders such as retinopathy caused by diabetes or hypertension and venous insufficiency (*1*−*3*).

The dark blue, sometimes velvet-black, berries of *V. myrtillus* contain anthocyanidins (i.e., aglycons), and different anthocyanins have been isolated from *V. myrtillus*, mainly galactosides and glycosides of delphinidin, cyanidin, and malvidin (*4*). The anthocyanidins are considered to be responsible for the main pharmacological effects, due to their antioxidative (*5*, *6*) and free radical scavenging properties (*7*).

Significant interaction between biological systems and anthocyanins have been reported: enzyme inhibition (*2*, *6*), antibacterial properties, protein stabilization (*6*), antioxidative properties (*7*), anticancer activity (*8*, *9*), free radical scavenging (*10*), improvement of vision (*11*), antiplatelet effects (*12*), increased nitric oxide production (*13*, *14*), and regulation of Ca^{2+} homeostasis (*15*). Inhibition of proteolytic enzymes, anti-inflammatory, antiulcer, and antiatheroscleotic activities, and reduced fluid retention have also been reported (*16*).

The renin−angiotensin system (RAS) is one of the most important mechanisms in the body concerning the regulation of blood pressure, fluid, and electrolyte balance by maintaining extracellular fluid volume and arterial pressure despite wide variations in electrolyte intake.

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The angiotensin-converting enzyme (ACE; also known as ACE I), a carboxypeptidase, is involved in the RAS, where it rapidly converts the physiologically inactive angiotensin I to the physiologically active octapeptide angiotensin II. Angiotensin II increases systemic blood pressure and renal perfusion pressure.

ACE is mainly present in endothelial cells. A very small amount is present in vascular smooth muscle cells or other cells. The endothelial ACE converts angiotensin I to angiotensin II. Angiotensin II then diffuses to the vascular smooth muscle cells and stimulates its receptors on the vascular smooth muscle cells, thereby affecting smooth muscle contraction and proliferation. Inhibiting endothelial ACE thus leads to attenuated smooth muscle proliferation.

The two active sites of ACE contain a Zn^{2+} atom, and ACE inhibitors are designed on the basis of the ability to bind to the Zn^{2+} (*17*). ACE inhibitors are first-line treatment of patients with hypertension and heart failure.

We have previously shown that proanthocyanidins, for example, condensed tannins, inhibit ACE activity (*18*), and because there is a connection between the biosynthesis of condensed tannins and anthocyanidins, we were interested to see if the anthocyanidins also exhibit similar pharmacological effects.

The aim of this study was to examine whether *V. myrtillus* has any effect on ACE activity and if this could explain the beneficial properties of *V. myrtillus* anthocyanidins on the circulation. The three main anthocyanidins, cyanidin, delphinidin, and malvidin, were also investigated, together in myrtillin chloride and separately.

MATERIALS AND METHODS

The study on cultured endothelial cells from human umbilical veins (HUVEC) was approved by the regional ethics committee at the Faculty of Health Sciences, Linkoping, Sweden (Dnr 03-602).

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***V. myrtillus* Extraction.** *V. myrtillus*, bilberry extract 25E containing anthocyanosides standardized for 25% anthocyanidin, was used for preparation of solution. Solution was made of 1 g *V. myrtillus* extract in 20 mL of sterile phosphate-buffered saline (PBS) for 15 min in a boiling water bath. The solution was filtered twice, first through a standard filter and then through a sterile filter 0.2 μm (Millipore). The obtained filtrate was considered to be 50 mg/mL and was frozen in −20 °C in aliquots. This method was chosen as being close to household use of bilberry.

The bilberry extract is soluble in water. The anthocyanidins, cyanidin chloride, delphinidin chloride, malvidin chloride, and these compounds combined in myrtillin chloride dry power are insoluble in water and were therefore dissolved in dimethyl sulfoxide (DMSO).

Cultured Endothelial Cells from HUVEC. Human umbilical cords were obtained after normal vaginal delivery (after informed consent from the mothers) and kept in sterile bottles containing PBS, penicillin, streptomycin, and gentamicin. Endothelial cells were then isolated by treatment with 0.5 mg/mL collagenase for 20 min at 37 °C as described previously (*19*). In short, the collagenase + cell perfusate was washed twice and then resuspended in culture medium [Dulbecco's modified Eagle's medium (DMEM) with normal glucose] supplemented with nonessential amino acids (NEAA 1:100), oxalacetic acid (1.2 mM; to stabilize insulin), insulin (0.24 IE/mL), penicillin (5 U/mL), streptomycin (0.5 μg/mL), Hepes (10 mM), endothelial cell growth factor (ECGF, 30 μg/mL), and 17% inactivated fetal calf serum (FCS). Resuspended HUVEC were seeded in 25 cm^2 tissue culture flasks coated with 0.2% gelatin and kept in an incubation chamber. Medium was replaced every 48−72 h. At confluence, cells were harvested with trypsin-EDTA for 5−10 min and then reseeded 1:2. Second passage was seeded in a 96-well microtiter plate, and allowed to reach confluence. Then, medium was removed and replaced with medium without FCS to avoid discrepancies in results due to ACE present in the serum. Cells were treated with *V. myrtillus*, myrtillin chloride, delphinidin, cyanidin, or malvidin. *V. myrtillus* extract was dissolved in PBS; all other drugs were dissolved in DMSO. The effects of a traditional ACE inhibitor were also tested by adding different concentrations of enalaprilat to the cells.

Corresponding volumes of PBS or DMSO were used as controls. After 10 min of incubation, with drugs, ACE activity was analyzed as described below.

ACE Activity. After stimulation of the cells with drugs, ACE activity was analyzed with a commercial radioenzymatic assay (ACE-direct REA, Buhlmann Laboratories, Allschwil, Switzerland) with the following modifications. Blank and standard sera were added to wells with corresponding volumes of medium without FCS. The synthetic substrate ^{3}H-hippuryl-glycyl-glycine was then added directly to all of the wells of the microtiter plate. Cells were incubated with substrate for 2 h in 37 °C to allow for the ACE present to cleave the substrate to ^{3}H-hippuric acid. Then, 150 μL of medium + substrate was transferred from each well into scintillation vials containing 50 μL of 1 M HCl (to stop the enzymatic reaction). After acidification, the ^{3}H-hippuric acid is separated from unreacted substrate by extraction with the scintillation liquid included in the kit, and the samples were counted in a scintillation counter.

The possible quenching effects of the extract on the results were tested by measuring tubes with radioactive labeled substrate, bilberry extract, and scintillation liquid. Results showed a reduction in cpm of 3.3% by the extract compared to PBS control.

Chemicals. All chemicals for culturing cells were obtained from Life Technologies, Carlsbad, CA, USA, except endothelial cell growth factor, which was bought at Boehringer-Mannheim, Mannheim, Germany, and heparin (Heparin LEO), from LEO Pharma AB, Malmo, Sweden. Myrtillin chloride and the anthocyanidins were purchased from Extrasynthese, Genay, France. *V. myrtillus* bilberry extract 25E was a kind gift from Ferrosan, Copenhagen, Denmark. Enalaprilat (Renitec) was obtained from Merck Sharp & Dohme, Haarlem, The Netherlands.

Viability. A viability test was performed by incubating HUVEC with *V. myrtillus* extract 0.1 mg/mL, myrtillin chloride, cyanidin, delphinidin, and malvidin 0.05 mg/mL, respectively, for 24 h. Then, Trypan Blue 0.05% was added for 5 min at room temperature before counting the cells. The CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) and the colorimetric assay MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium] were also used to investigate viability/quality of the cells after incubation with bilberry extract 25E.

Calculations. Results are presented as mean ± standard error of the mean (SEM). One unit (U) of ACE activity is defined as the amount of enzyme required to release 1 μmol of hippuric acid per minute and liter. Statistical calculations were done with Graph Pad Prism 3.0. One-way analysis of variance (ANOVA) for repeated measures was performed followed by Dunnett's post test. Statistical significance is denoted as follows: ∗, $p < 0.05$; ∗∗, $p < 0.01$; and ∗∗∗, $p < 0.001$.

IC_{50} values were calculated with nonlinear regression (Graph Pad Prism 5.0) and are given in log mg/mL with 95% confidence intervals in parentheses.

RESULTS

The results of this study showed significant and dose-dependent inhibition (∗∗∗, $p < 0.001$) of the ACE activity with *V. myrtillus* extract in the dosages 0.00625, 0.0125, 0.025, 0.05, and 0.1 mg/mL (**Figure 1**).

Myrtillin chloride showed (∗, $p < 0.05$) significant inhibition on the ACE activity in the dosages 0.025 and 0.05 mg/mL (**Figure 2**).

There was no significanct effect on the ACE activity when cells were incubated with the anthocyanidins, cyanidin, delphinidin, and malvidin (**Figures 3** and **4**).

Log IC_{50} values for bilberry, myrtillin, and enalaprilat are shown in **Table 1**. Log IC_{50} values for other substances were not tested because these substances did not significantly affect ACE activity.

Viability. Cell viability after exposure to the *V. myrtillus* extract 0.1 mg/mL was 99.9 ± 0%, $n = 3$. PBS control was 99.7 ± 0.3%, $n = 3$. Myrtillin chloride, cyanidin, delphinidin, and malvidin, 0.05 mg/mL, were 99.9 ± 0%, respectively, $n = 2$. DMSO control was 99.9 ± 0%, $n = 2$. The cell viability/quality performed with

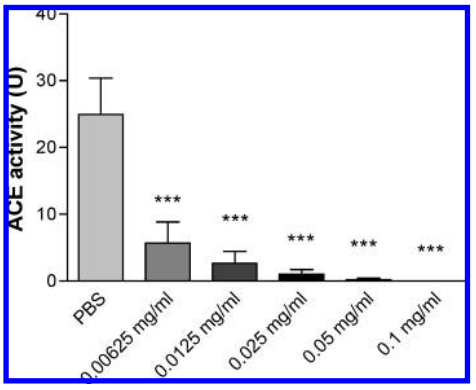

Figure 1. ACE activity in HUVEC after 10 min ($n = 6$) of incubation with *Vaccinium myrtillus* extract. Statistical significance is denoted as ∗∗∗, $p < 0.001$, compared to PBS.

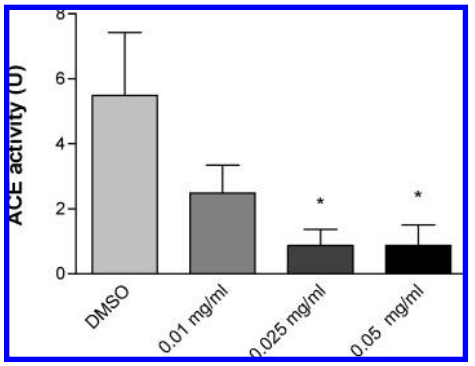

Figure 2. ACE activity in HUVEC after 10 min ($n = 6$) of incubation with myrtillin chloride. Statistical significance is denoted as ∗, $p < 0.05$, compared to DMSO.

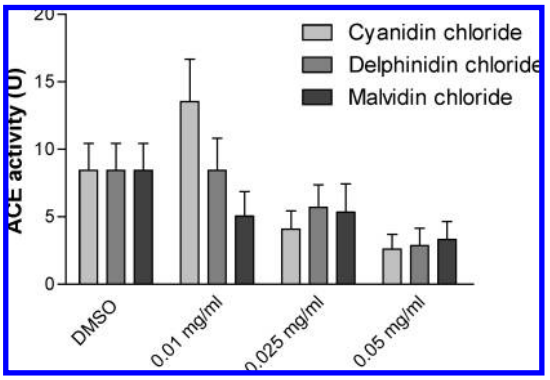

Figure 3. ACE activity in HUVEC after 10 min (n = 6) of incubation with cyanidin, delphinidin, and malvidin, respectively, compared to DMSO.

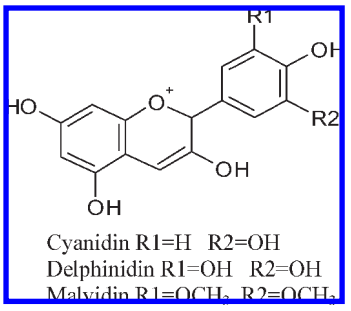

Figure 4. Molecular structures of the anthocyanidins (flavylium caution derivatives) used: cyanidin, delphinidin, and malvidin.

Table 1. Log IC_{50} Values for Bilberry, Myrtillin, and Enalaprilat[a]

	log IC_{50} (mg/mL)
bilberry extract	−2.6 (−3.83 to −1.38)
myrtillin	≈ −2.02
enalaprilat	−8.54 (−9.41 to −7.67)

[a] Due to lack of fitness and great variance, the regression analysis for myrtillin resulted in an approximated value and no confidence interval.

MTS after exposure to *V. myrtillus* extract 0.1 mg/mL was A_{490} = 1.548 compared to PBS A_{490} = 1.558.

DISCUSSION

The aim of this study was to examine whether *V. myrtillus* affects ACE activity and if this could be part of the explanation of the beneficial properties of *V. myrtillus* and its anthocyanins on circulation.

As shown in **Figure 1**, the *V. myrtillus* extract exhibited a significant inhibition of ACE activity. The results of this study show a more pronounced effect of the anthocyanins combined in the bilberry plant compared to the anthocyanidins combined in myrtillin chloride as shown in **Figure 2** and to the anthocyanidins separately (**Figure 3**). The reason that *V. myrtillus* extract is a stronger inhibitor of ACE activity than myrtillin chloride and the anthocyanidins might be because compounds other than the anthocyanidins cause the inhibition. According to the manufacturer (Ferrosan AB) bilberry extract 25E contains 100% dried bilberry *V. myrtillus*, wild bilberry picked "by hand in the arctic northern nature". Bilberry contains anthocyanins (glycosides of anthocyanidins) and anthocyanidins (aglycones) of delphinidin, cyanidin, petunidin, peonidin, and malvidin (presented in order of concentration) (*20*). Bilberry extract also contains the flavonol quercetin, the stilbene resveratrol, ferulic acid, and coumaric acid (*21*). A previous study from our laboratory showed a significant inhibition of ACE activity by quercetin (*22*), and a concentration of 30 mg/kg quercetin in bilberry has been reported (*23*).

Significant amounts of research have been performed on red wine polyphenols, especially delphinidin (*12*, *14*, *15*), and several studies refer to delphinidin and its beneficial effect on the cardiovascular system (*13*, *14*, *24*−*26*). These studies refer to the antioxidative activities, nitric oxide production, and vasorelaxation, not to the ACE activity, as in our study. In our study we used the chloride salt of the anthocyanidins and myrtillin chloride. Studies have shown that ACE might be dependent on chloride and that high doses of chloride might interfere with ACE (*27*).

Information about the pharmacokinetics of anthocyanins in human is sparse.

Data available on bilberry flavonoids and their pharmacokinetics consider unmetabolized flavonoids. During metabolism of the anthocyanins and the anthocyanidins various metabolites are formed; glucuronides and methylated and sulfated derivatives of anthocyanins have been identified (*28*, *29*). Therefore, no reliable/complete data on plasma concentrations in humans exist. However, preliminary data from our laboratory show that the ACE inhibitory effect of a flavonoid-related compound is actually attenuated when the glycated form is used (data not shown).

Anthocyanins are shown to be absorbed in their glycosylated forms in the human gastrointestinal tract (*30*). The anthocyanins are distinguished from other flavonoids as a separate class by virtue of their ability to form flavylium cations. This ability makes it possible for the anthocyanins to exist in various forms.

In our study the *V. myrtillus* extract was dissolved in PBS, whereas myrtillin chloride and the anthocyanidins were dissolved in DMSO, which probably is of importance. Myrtillin chloride and the anthocyanidins are insoluble in water. Anthocyanins exist in protonated, deprotonated, hydrated, and isomeric forms, and the relative proportion of these molecules is dependent on pH. The stability of anthocyanins is affected by factors such as pH, temperature, oxygen, ascorbic acid, light, and metals (*31*). The ability to bind metal ions may play an important role in the pharmacological mechanism as an ACE inhibitor. The pharmacological mechanism of the allopatic ACE inhibitors is their ability to bind to the Zn^{2+} at the active site of ACE. Flavonoids are known to have chelate-binding capacity to metal ions such as Zn^{2+} and Fe^{2+} (*16*).

The anthocyanins are soluble in water, and the aglycone anthocyanidins are lipophilic (*16*). In vitro and in situ studies show that anthocyanidins most likely can enter the brain and that the flavonoids even exhibit high permeability (*32*).

In conclusion, the inhibitory effect seen in this study by *V. myrtillus* on ACE activity may be one explanation of the beneficial effects seen of this plant on the cardiovascular system. As vascular factors contribute to the development of various ocular diseases such as diabetic retinopathy, glaucoma, and macular degeneration, the results of this study might be an explanation of the effects of *V. myrtillus* on vision. At least, this study might be an inspiration for further investigations of *V. myrtillus* and the cardiovascular system. This in vitro study needs to be repeated in vivo to confirm the results on ACE.

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