DOI 10.1002/mnfr.200500134 Mol. Nutr. Food Res. 2006, *50*, 44 – 51

Anthocyanidins decrease endothelin-1 production and increase endothelial nitric oxide synthase in human endothelial cells

Maria Claudia Lazzè[1], Roberto Pizzala[1], Paola Perucca[1], Ornella Cazzalini[1], Monica Savio[1], Luca Forti[2], Vanio Vannini[1] and Livia Bianchi[1]

[1]Dipartimento di Medicina Sperimentale, Sez. Patologia Generale, Università di Pavia, Pavia, Italy
[2]Dipartimento di Chimica, Università di Modena e Reggio Emilia, Modena, Italy

Epidemiological and intervention studies correlate anthocyanin-rich beverages and a low incidence of coronary heart diseases. Since endothelin-1 (ET-1) and nitric oxide (NO) produced by endothelial NO synthase (eNOS) are vascular tension regulators secreted by endothelial cells, we studied the influence of two anthocyanidins, namely cyanidin (CY) and delphinidin (DP), on the regulation of ET-1 and eNOS in cultured human umbilical vein endothelial cells (HUVECs). Aglycon anthocyanidin forms, such as CY and DP, may be present *in vivo* after the first deglycosylation step occurring in the jejunum and in the liver. DP showed a major action compared to CY inducing a significant dose-dependent inhibitory effect on both protein and mRNA levels of ET-1. CY and DP both increased the protein level of eNOS, but DP showed the major effect raising eNOS protein in a dose-dependent manner. To correlate the vasoprotective effect of CY and DP with their antioxidant activity, we analysed also the antioxidant effect of anthocyanidins both *in vitro* and in HUVECs. In particular, we examined the effect of anthocyanidins on endothelial heme oxygenase-1 (HO-1), an inducible stress protein. In all tests, DP showed a higher antioxidant activity than CY. Finally, the antiproliferative effect induced by DP was detected in HUVECs. DP and CY differ in the number and position of hydroxyl groups in their structure; therefore, the greater biological activity by DP, compared with CY, seems to be due to the presence of the three hydroxyl groups on the B ring in the molecular structure of DP.

Keywords: Antioxidant / Anthocyanidins / Atherosclerosis / Endothelial cells / Vascular regulators

Received: August 4, 2005; revised: October 6, 2005; accepted: October 12, 2005

1 Introduction

Epidemiological evidence suggests that cardiovascular diseases such as atherosclerosis and hypertension can be decreased by moderate consumption of red wine due to its high content of phenolic compounds [1, 2]. Anthocyanins are polyphenols widely distributed in vegetables, fruits, beverages such as wine, tea or bilberry extract, and there are several reports supporting their beneficial effects [3, 4]. The common aglycon forms, anthocyanidins, are cyanidin, delphinidin, peonidin, petunidin, malvidin and pelargonidin. They differ from each other by having different substituents in the B ring. In plants, anthocyanidins occur as the glycosylated forms, anthocyanins and the prevalent sugar moieties are glucose, rhamnose, galactose and fructose [5]. Some studies regarding metabolism of flavonoids show that also the aglycon forms may be present *in vivo*, because they could be produced from the glycosylated forms by β-glucosidase reactions in the intestine [6, 7].

It has been suggested that anthocyanins play an important role in the prevention of human diseases associated with oxidative stress, *e.g.*, coronary heart disease and cancer [8]. The antioxidant properties of anthocyanins have been demonstrated by both *in vitro* and *in vivo* experiments [5, 9 – 14].

Correspondence: Prof. Roberto Pizzala, Dipartimento di Medicina Sperimentale, Sez. Patologia Generale, Piazza Botta 10, 27100 Pavia, Italy
E-mail: roberto.pizzala@unipv.it
Fax: +39-0382-303-673

Abbreviations: BrdU, bromodeoxyuridine; **CY**, cyanidin; **DP**, delphinidin; **DPPH**, 2,2-diphenyl-1-picrylhydrazyl radical; **EC_{50}**, antioxidant concentration necessary to decrease the initial amount of DPPH by 50%; **eNOS**, endothelial nitric oxide synthase; **EQ**, efficient quantity; **ET-1**, endothelin-1; **HO-1**, heme oxygenase-1; **HUVECs**, human umbelical vein endothelial cells; **PBT**, PBS containing 1% BSA and 0.2% Tween-20

It is well known that the vasomotor functions of endothelium are impaired during the course of vascular disorders including atherosclerosis and hypertension [15]. Key regulators of vasomotor function are the vasocostrictor endothelin (ET) and the vasodilator nitric oxide (NO). Endothelins are 21-amino acid isopeptides (ET-1, ET-2 and ET-3), which has been identified in a variety of tissues, including lung, kidney, brain, pituitary gland and peripheral endocrine tissues as well placenta [16]. ET-1, in contrast to ET-2 and ET-3, is also produced by endothelial cells and is probably the major isoform in the cardiovascular system [17, 18]. NO is a diatomic free radical produced from L-arginine by constitutive and inducible NO synthases in numerous mammalian cells and tissues. The constitutive endothelial NO synthase (eNOS) is present in endothelium and it is known to play an important role in the dynamic control of the vascular tone [19].

Numerous studies have shown that oxidative stress interferes with vascular function, so that it appears to be causally linked to the pathogenesis of atherosclerosis [20, 21]. The depletion of bioavailable NO [22] and regulation of ET-1 [23] are one of the mechanism by which oxidative stress seems to influence vascular function.

We have previously demonstrated that the two aglycon anthocyanins, delphinidin (DP) and cyanidin (CY) exert a higher protective activity against oxidative DNA damage than glycosylated anthocyanins [10]. This is in accord with data reported by Youdim *et al.* [24], who demonstrated that sugar moiety impairs cellular bioavailability. An antiproliferative effect induced by DP and CY in normal human fibroblasts and a pro-apoptotic effect in human tumour cell lines was also detected [25].

In the present work, we studied the effect of CY and DP on ET-1 and eNOS expression in human umbilical vein endothelial cells (HUVECs). We analysed also the antioxidant effect of anthocyanidins in order to correlate the vasoprotective effect of CY and DP with their antioxidant activity. In particular, we examined the antioxidant activity of anthocyanidins *in vitro* by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method [26] and the citronellal thermo-oxidation inhibition test [27]. In addition, antioxidant activity was assessed in HUVECs by measuring lipid peroxidation inhibition after Fe^{2+}/ascorbate treatment and by evaluation of endothelial heme oxygenase-1 (HO-1), an inducible stress protein that degrades heme to the vasoactive molecule carbon monoxide and the antioxidant biliverdin [28].

Finally, the antiproliferative effect of these two compounds was evaluated.

2 Materials and methods

2.1 Chemicals

DP and CY were obtained from Extrasynthese (Genay, France). All chemicals used, were purchased from Sigma (St. Louis, MO), unless otherwise specified.

Anthocyanidins were dissolved in DMSO for the cell treatments, in methanol for the DPPH reduction assay, or in DMF for the citronellal thermo-oxidation method.

2.2 Cell culture and treatment

HUVECs, kindly provided by Dr. Jeanette A. M. Maier [29], were cultured in M199 containing 5 U/mL heparin (Roche), 5 μg/mL endothelial cell growth factor (Roche), 10% foetal bovine serum, 1 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin (all from Gibco-Invitrogen).

Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The treatments were performed for 24 h with different concentrations of DP or CY with the concentration of DMSO never exceeding 0.4%. Negative controls consisted of untreated cells and cells that received the solvent alone.

2.3 Cell viability

Cells were plated in 24-well tissue culture plates at the concentration of 2×10^5 cells/well, treated for 24 h with DP or CY at the concentrations of 50, 100, 200 μM and cell viability was determined by the 3-[4,5-dimethylthiazolil-2yl]-2,5-diphenyl-tetrazoliumbromide colorimetric assay [30], as reported previously.

2.4 Measurement of ET-1 concentration

Cells were plated in 6-well tissue culture plates at a concentration of 5×10^5/well. Confluent cultures were treated for 24 h with CY and DP (50 and 100 μM, respectively) in serum-free medium. At the end of the incubation period, cell supernates were collected and subjected to the ET-1 ELISA quantitative assay (Biomedica) following the manufacturer's instructions and using a microtiter plate reader (Bio-Rad-Mod.550) for the absorption measurements at 450 nm.

2.5 RT-PCR expression of ET-1 and β-actin

Total RNA was extracted using Qiagen RNeasy kit (Qiagen) as instructed by the manufacturer. The first strand cDNA

was synthesized using AMV-RT (Eurobio). For the semi-quantitative PCR the Multiplex PCR (Qiagen) was used according to the manufacturer's instructions. The sequence for the β-actin primers is as follows: 5′-TTGCCGATCC-GCCGCCCGTCCACA-3′ and 5′TAAGGTGTGCACTTT-TATTCAACT-3′; for the ET-1: 5′TTGCCGGATATCATG-GATTATTTGCTCATGATTTTCTCT-3′ and 5′GAATT-CCCTAGGTCACCAATGTGCTCGGTTGTGGGTCACA-TA-3′.

The PCR products were electrophoresed on a 2% agarose gel, and bands corresponding to RT-PCR products were visualized by UV light. Gene Ruler 1-kb DNA Ladder (MBI Fermentas) was used as size marker. Digitalized images of the bands were acquired by UMAX power Look II^{TM} scanner (UMAX Data System) and the densitometric analysis was performed on a Macintosh computer using the public domain NIH-Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image) with the aid of the bundled gel plotting macros.

2.6 Western blot analysis of eNOS protein

For the determination of eNOS protein expression, total protein was isolated from confluent HUVECs treated with anthocyanidins (50 and 100 μM) for 24 h. Briefly, cell lysate was prepared in cell lysis buffer containing 10 mM Tris-HCl, pH 7.5, $MgCl_2$ 2.5 mM, 0.5% NP-40, 1 mM PMSF, protease inhibitor cocktail (Complete, EDTA-free; Roche) and by sonication on ice twice for 20 s (60 W). The protein content of the lysate was determined using the Bradford protein assay. After heating the samples for 3 min at 90°C, 80 μg of protein was loaded and run in 7.5% SDS-PAGE and transferred to PVDF membranes in a semidry apparatus. The membranes were blocked in 5% milk in PBS containing 0.2% Tween-20 for 1 h at room temperature and then incubated overnight with anti-eNOS rabbit polyclonal antibody (NOS3-C-20; Santa Cruz Biotechnology, CA) diluted 1 : 1000 in PBS containing 0.2% Tween-20. After several washes in PBS containing 0.2% Tween-20, the membrane was incubated for 30 min in the presence of donkey anti-rabbit biotinylated IgG (Amersham) diluted 1:2000 in PBS containing 0.2% Tween-20. The membrane was then washed in PBS containing 0.2% Tween-20 and finally incubated for 30 min with streptavidine conjugated to horseradish peroxidase (Amersham) diluted 1 : 2000 in PBS containing 0.2% Tween-20. Visualization of immunoreactive bands was performed by an ECL system (Amersham). The eNOS protein bands were normalized using the respective actin protein bands. Actin was detected by an mAb (1 : 500) and an anti-mouse IgG antibody conjugated to horseradish peroxidase (1 : 2000, Amersham).

2.7 Antioxidant activity

2.7.1 General remarks

The antioxidant activity of anthocyanidins was evaluated *in vitro* by the DPPH method [26] and the citronellal thermo-oxidation inhibition test [27]. In addition, antioxidant activity was assessed in HUVECs by measuring lipid peroxidation inhibition after Fe^{2+}/ascorbate treatment and by endothelial HO-1 evaluation.

2.7.2 DPPH reduction method

Antioxidant solution in methanol (50 μL) was added to 2950 μL of a 0.1 mM DPPH solution in methanol [26]. The exact initial DPPH concentration in the reaction medium was calculated from a calibration curve. The decrease in absorbance was determined at 515 nm at 0 min, at 30 s, every 1 min for 15 min, and every 2 min until the reaction reached a plateau (about 30 min). Antiradical activity was expressed as the antioxidant concentration necessary to decrease the initial amount of DPPH by 50% (EC_{50}).

2.7.3 Citronellal thermo-oxidation method

In this test, the aldehyde (−)-citronellal is used as the oxidation substrate. It is subjected to heating and intensive oxygenation in chlorobenzene, and its disappearance with the consequent formation of its degradation products are monitored by GC [27]. Fifteen mililiters of a chlorobenzene solution, containing 150 μL of dodecane and 150 μL of tridecane as internal standard, was poured into a two-necked flask equipped with a condenser to prevent evaporation. Anthocyanidins dissolved in DMF, were added to the chlorobenzene solution to reach final concentrations ranging from 30 to 120 μM. The mixture was then heated at 80°C and intensively oxygenated by bubbling in O_2 at a flow rate of 10 mL/min. At time 0, 300 μL of (−)-citronellal (Fluka) were added to the reaction medium. Immediately and at periodic intervals, 1-μL samples were withdrawn and analysed by GC. The antioxidant power of anthocyanidins was measured by determining the efficient quantity (EQ), *i.e.*, the concentration required each compound to double the half-life with respect to control reaction (citronellal without antioxidant).

2.7.4 Lipid peroxidation assay

Five million cells were preincubated for 2 h with anthocyanidins at the concentration of 100 μM and then treated for 2 h with 200 μM/1 mM Fe^{2+}/ascorbate in order to induce lipid peroxidation. After treatment, lipid peroxidation was measured by a fluorometric method for the determination of hexanal in supernates using 1,3-cyclohexanedione reagent and HPLC according to Yoshino *et al.* [31] and Cabré *et al.* [32].

2.7.5 Assay for endothelial heme oxygenase quantification

Heme oxygenase was determined with the StressXpress Human HO-1 ELISA kit (Stressgen). At the end of 24-h treatment with DP or CY (50 and 100 μM), confluent cells were harvested and cell lysate was prepared and subjected to the ELISA kit according to the manufacturer' instructions. For the final absorption measurements at 450 nm, a microtiter plate reader (Bio-Rad-Mod.550) was used.

2.8 Cell-cycle analysis

Cell-cycle distribution was assessed by determining bromodeoxyuridine (BrdU) incorporation versus DNA content. HUVECs were incubated with 30 μM BrdU during the last hour of 24-h treatment with 100 or 200 μM anthocyanidins, harvested and fixed in cold 70% ethanol. Fixed cells were washed in PBS, resuspended in 2 N HCl for 30 min at room temperature, pelletted, and then resuspended in 0.1 N sodium tetraborate for 15 min. The samples were washed in PBS, incubated for 15 min in PBS containing 1% BSA and 0.2% Tween-20 (PBT), and then for 60 min in 100 μL of anti-BrdU mAb (Becton Dickinson) diluted 1:20 in PBT. After two washes with PBT, cells were incubated for 30 min with 100 μL of FITC-conjugated anti-mouse antibody (Amersham) diluted 1 : 100 in PBT, then washed twice and resuspended in PBS containing 5 μg/mL propidium iodide and 1 mg/mL of RNase A. Cells were analysed with a Coulter Epics XL (Coulter Corp.) flow cytometer. Ten thousand cells were measured for each sample. Computer statistical analysis of mean fluorescence intensity and graphic representation were performed with the XL2 software (Coulter).

To verify the reversibility of the effect on cell cycle, cells were treated with anthocyanidins for 24 h, washed in PBS and fresh medium was added for 24 or 48 h.

2.9 Statistical analysis

Results are expressed as mean ± SD. Statistical significance was calculated in all experimental sets using the Student's *t*-test.

3 Results

3.1 General observations

Preliminary experiments were carried out to determine the cytotoxicity of CY and DP on HUVECs by the 3-[4,5-dimethylthiazolil-2yl]-2,5-diphenyl-tetrazoliumbromide test. Up to the concentration of 200 μM anthocyanidins did not affect cell viability (data not shown).

3.2 Effect of anthocyanidins on ET-1 secretion and expression

The effect of CY and DP on the vasoconstrictor ET-1 was studied analysing both protein secretion and gene expression.

On the protein level, ET-1 expression was monitored by ELISA. Figure 1 shows that DP at both 50 and 100 μM concentrations significantly decreased ET-1 secretion by about 46 and 70%, respectively. In contrast, CY exerted a lesser effect reducing the ET-1 secretion by about 38% only at 100 μM concentration.

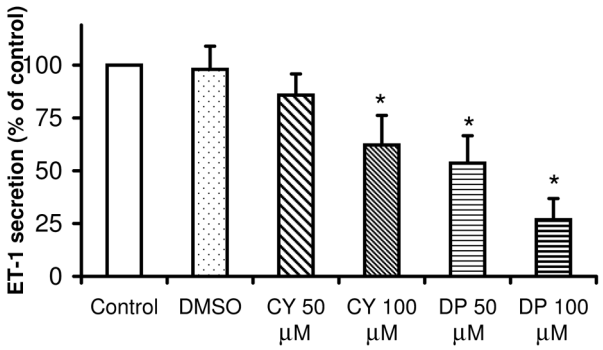

Figure 1. Effect of anthocyanidins on ET-1 release from HUVECs. Results (means ± SD) are expressed as a percentage of basal release from three independent experiments. * Significantly different compared to control ($p < 0.05$ by Student's *t*-test).

Anthocyanidins exerted the same effect also on the m-RNA level: DP induced a significant reduction of prepro-ET-1 m-RNA expression by about 45 and 52% after the treatment with 50 and 100 μM, respectively; CY reduced prepro-ET mRNA expression only by 17% at 100 μM concentration (Fig. 2).

3.3 Effect of anthocyanidins on eNOS protein

To examine whether CY and DP activate eNOS in HUVECs, we analysed the expression of this protein by Western blot. DP showed a higher effect significantly increasing eNOS protein by 58 and 78% at concentrations of 50 and 100 μM, respectively. In contrast, in cells treated with CY, eNOS protein increased by 46% only at 100 μM concentration (Fig. 3).

3.4 Antioxidant activity of anthocyanidins

The antioxidant activity of anthocyanidins was evaluated *in vitro* by the DPPH method and the citronellal thermo-oxida-

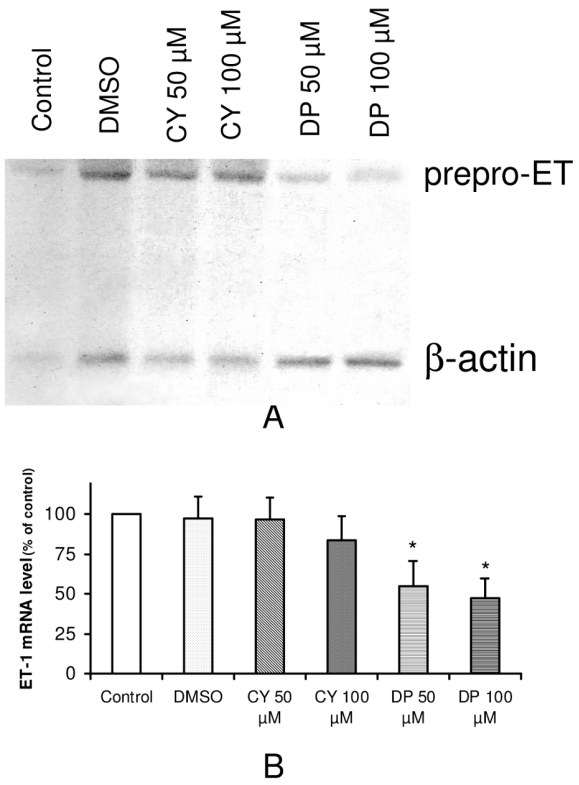

Figure 2. Effect of anthocyanidins on prepro-ET mRNA expression. (A) The blot shown is representative of three independent experiments with similar results. β-Actin RNA was used to normalize the RNA applied in each lane. (B) The panel shows densitometric analysis of three different blots. Results (means ± SD) are expressed as a percentage of basal expression from three independent experiments. *Significantly different compared to control ($p < 0.05$ by Student's *t*-test).

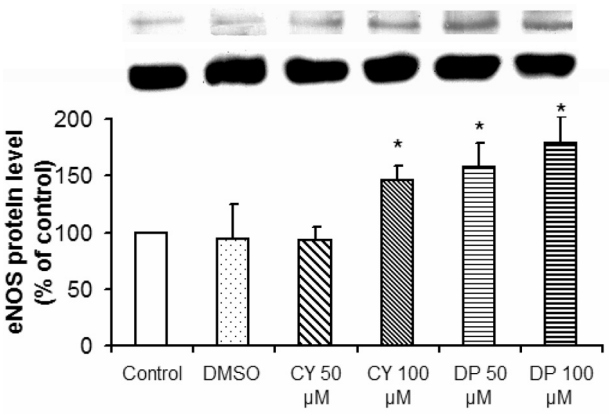

Figure 3. Effect of anthocyanidins on eNOS protein in HUVECs. Western blot analysis was performed using a polyclonal anti-eNOS antibody and an anti-actin mAb, for normalization. The blot shown in the upper panel is representative of three independent experiments with similar results. The lower panel shows densitometric analysis of three different blots. Bars represent mean ± SD. *Significantly different compared to control ($p < 0.05$ by Student's *t*-test).

tion inhibition test and in HUVECs by measuring lipid peroxidation inhibition and endothelial heme oxygenase induction.

Table 1 reports the results expressed as the EC_{50} (DPPH assay) or EQ (citronellal test) of each compound used: the stronger the antioxidant, the smaller the EC_{50} or EQ value. In both tests, DP showed a higher antioxidant activity than CY.

Figure 4 shows the effects of DP and CY on hexanal production induced by Fe^{2+}/ascorbate in HUVECs. Incubation of the cells for 2 h in the presence of 200 µM/1 mM Fe^{2+}/ascorbate significantly increased membrane lipid peroxidation, raising the hexanal production to 145.4 nmol/5×10^6 cells, from the level of 15 nmol/5×10^6 cells measured in untreated control samples. DP and CY at 100 µM concentration inhibited the hexanal production by about 43.4 ($p < 0.05$) and 14%, respectively.

Induction of HO-1 by anthocyanidins in HUVECs was analysed at protein level by ELISA. Figure 5 shows that DP at both concentrations significantly induced HO-1 (1.5-fold increase). In contrast, induction of HO-1 protein by CY was significant only at 100 µM concentration (1.2-fold increase).

Table 1. Antioxidant activities of CY and DP. The antioxidant power is expressed by EC_{50} and EQ as described in Section 2. Each value is the mean of three independent experiments ± SD

Compound	DPPH EC_{50} (µM)	Citronellal EQ (µM)
CY	17.8 ± 1.1	77 ± 4.3
DP	12.7 ± 1.2	50 ± 3.2

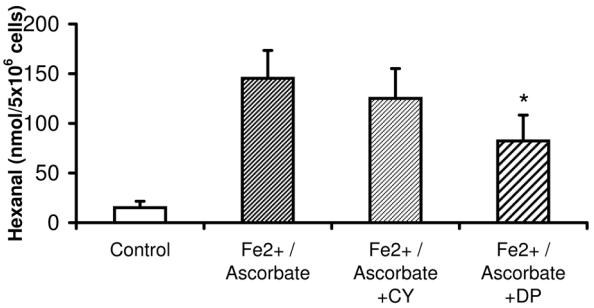

Figure 4. Effect of preincubation with 100 µM anthocyanidins on Fe^{2+}/ascorbate-induced lipid peroxidation, evaluated as the production of hexanal in HUVECs. Results are mean values of independently reproduced experiments; $p < 0.05$ with respect to cells treated with Fe^{2+}/ascorbate alone.

3.5 Effect of anthocyanidins on cell cycle

To investigate the effect of anthocyanidins on cell proliferation, the distribution in each phase of the cell cycle was analysed by determining the DNA content with flow cytometry.

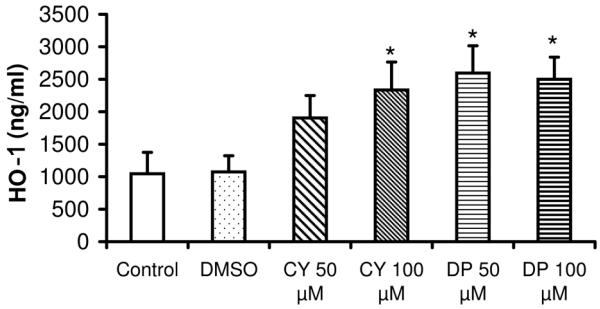

Figure 5. Effect of anthocyanidins on heme oxygenase protein expression in HUVECs. Results are mean values of independently reproduced experiments; *Significantly different compared to control ($p < 0.05$ by Student's *t*-test).

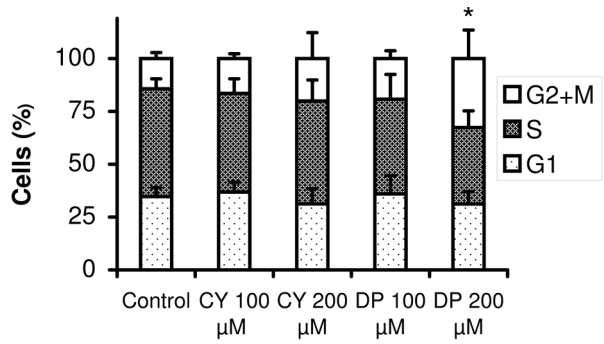

Figure 6. Cell-cycle analysis of HUVECs treated with anthocyanidins for 24 h. Cell-cycle distribution was determined in samples stained with propidium iodide and measured by flow cytometry, as described in Section 2. Mean values of the percentage of cells in each phase of cell cycle were obtained from three independent experiments. * Significantly different compared to control ($p < 0.05$ by Student's *t*-test).

As reported in Fig. 6, a significant reduction ($p < 0.05$) of cells in S phase and an accumulation in the G2/M phase were observed in HUVECs treated with 200 μM DP. In contrast, no significant effect was detected in HUVECs treated with 100 μM DP and with CY at both concentrations.

The reduction by about 30% of the number of cells in S phase induced by DP at the highest concentration was confirmed by cytometric evaluation of BrdU incorporation. However, the results showed that DNA synthesis was not affected (Fig. 7). The inhibitory effect on cell growth induced by DP was reversible, as 48 h after removal of the compound, the percentage of cells in S phase returned to the control value (data not shown).

4 Discussion

A high intake of polyphenolic compounds present in fruits, vegetables or red wine is reported to have beneficial effects on the cardiovascular system [33, 34]. As ET-1 and NO produced by eNOS are vascular tension regulators secreted by endothelial cells, we focused on whether two polyphenolic micronutrients, the anthocyanidins DP and CY, could influence the regulation of ET-1 and eNOS in cultured HUVECs.

Recent studies demonstrated that ethanol-free red wine extracts, and in particular *trans*-resveratrol, a polyphenolic phytoalexin present in this beverage, strongly decrease ET-1 synthesis in cultured aortic endothelial cells by suppressing transcription of the ET-1 gene [35, 36]. In the present study, we provide the experimental evidence that also anthocyanidins, *i.e.*, the aglycon forms of anthocyanins contained in red wine, are able to reduce ET-1 expression. In particular, DP showed a major action compared to CY inducing a significant dose-dependent inhibitory effect on ET-1 both on protein and on mRNA level.

Many studies suggest that resveratrol can increase eNOS protein expression and the subsequent NO production [37,

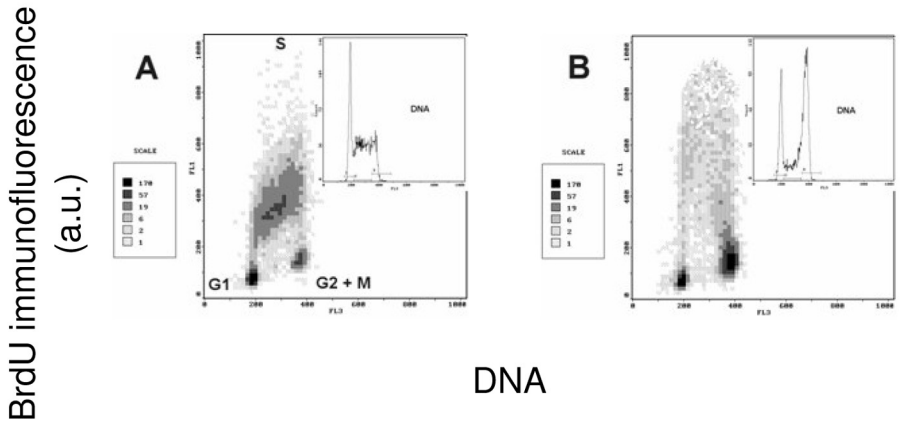

Figure 7. Effect of anthocyanidins on DNA synthesis in HUVECs. Two-parameter dot blots of BrdU incorporation versus DNA content in control cells and in samples treated with 200 μM DP for 24 h. Results shown are from one out of three independent experiments. Control cells (A) and HUVECs treated with 200 μM DP for 24 h (B).

38]. In contrast, there are still conflicting opinions about the effect of anthocyanidins. Leikart *et al.* [39] demonstrated that, with the exception of resveratrol, none of the compounds of a red wine polyphenol extract (including anthocyanidins) showed any influence on eNOS. In contrast, recent studies showed that anthocyanidins such as CY and its glycoside derivative cyanidin-3-glucoside are able to enhance eNOS expression in endothelial cell lines [38, 40]. Data presented here indicate that both CY and DP increased the protein level of eNOS: particularly DP showed the major effect raising eNOS protein in a dose-dependent manner.

Previously, we reported that anthocyanidins protect against DNA damage induced by oxidative agents in rat smooth muscle and hepatoma cells [10]. In the present study we confirmed the antioxidant action of CY and DP both *in vitro* (by DPPH and citronellal assays) and in HUVECs. In all these experiments DP showed a major antioxidant action compared to CY. To study the antioxidant activity of *trans*-resveratrol as well, Stivala *et al.* [41] used the DPPH and citronellal assays. Comparison of the EC_{50} and EQ values indicates clearly that anthocyanidins show a higher antioxidant activity than *trans*-resveratrol. As the mechanism by which oxidative stress influences vascular function seems to be the depletion of bioavailable NO [22] and regulation of ET-1 [23], we can suppose that the mechanism by which polyphenols, such as anthocyanidins, exert their protective action against cardiovascular diseases is related to their significant antioxidant properties. In particular, the inhibition of hexanal production by anthocyanidins showed here could be protective against the atherosclerotic lesion, as Cabré *et al.* [32] demonstrated that the induction of tissue factor by aldehydes produced during lipid peroxidation might contribute to the severity of atherogenesis.

Furthermore, we demonstrated that anthocyanidins (in particular DP) could significantly induce HO-1 protein. Recently, numerous studies have shown that the induction of HO-1 is an important cellular protective mechanism against oxidative injury [42]. Physiological concentrations of bilirubin, the production of which follows HO-1 stimulation, have been shown to protect endothelial cells against hydrogen peroxide-mediated injury and prevent protein oxidation in human blood plasma [43]. Therefore, we can hypothesize that the cardiovascular protection mechanism of anthocyanidins can also be associated with HO-1 induction.

Finally, we showed that DP, at the highest concentration used, namely 200 μM, has a reversible antiproliferative effect in HUVECs. We have previously demonstrated an inhibitory effect of anthocyanidins on cell cycle also in different normal and tumour human cell lines [25] and in rat smooth muscle cells (unpublished observations). Since both endothelial and smooth muscle cells are involved in the formation of the atherosclerotic plaques [44], also these effects may contribute to a cardiovascular protective action of anthocyanidins.

In agreement with our recent studies [10, 25], in all experiments DP showed a stronger effect compared to CY, confirming that the presence of three hydroxyl groups on the B ring in its structure may be important for its greater biological activity.

Anthocyanidins are present in nature as glycosylated forms and have been found to be absorbed unmodified from the diet [45, 46]. The biological relevance of the aglycon forms comes from experimental findings supporting the hypothesis that deglycosylation is a rate-limiting step for absorption of dietary flavonoid glycosides in the small intestine [7]. These authors demonstrate that human β-glucosidases present in the epithelial cells of the small intestine are able to deglycosylate flavonoid glycosides during passage across the gut wall. Moreover, a study in rats fed with cyanidin-3-glucoside (C3G) showed that in the intestines C3G is probably hydrolysed by β-glucosidase to CY which is in fact detected in jejunum [6].

Depending on the nutrition habits, the daily intake of anthocyanins in humans has been estimated to be 180–215 mg/day [47]. According to the fact that the absorption rate of anthocyanins was about 1% after oral administration [48], the concentrations of DP and CY adopted in our study, corresponding to 17–34 μg/mL, should be reached *in vivo.* More recently, Kroon *et al.* [49] stated that the polyphenol concentrations tested *in vitro* should be of the same order as the maximum plasma concentrations attained after a polyphenol-rich meal, which are in the range of 0.1–10 μM. Altogether, this suggests that the concentration of anthocyanidins used in our study might be reached in plasma after dietary ingestion of an anthocyanin-enriched meal or with anthocyanin dietary supplementation.

In conclusion, the results of this study demonstrate that (i) DP and, at a lesser extent, CY decrease ET-1 production and induce eNOS protein in HUVECs; (ii) DP and, at a lesser extent, CY have an important antioxidant activity as demonstrated by DPPH and citronellal tests, inhibition of lipid peroxidation, and HO-1 induction; (iii) all these findings may explain the mechanism by which anthocyanidins could protect against cardiovascular disease.

Altogether, our data suggest that anthocyanidins might play a role in the protection from cardiovascular diseases attributed to red wine components and other anthocyanin rich vegetables and fruits and may prove helpful in the development of new natural drugs to improve endothelial function and thereby prevent hypertension and coronary atherosclerosis.

The authors want to thank Dr. L. A. Stivala for critical review of the manuscript and Dr. M. R. Cramarossa for technical assistance in antioxidant tests in vitro. This work was supported partially by the EU research project "Anthocyanin Bioactivities" (QLK1-1999-00124) and by Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR – Italy) "Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale (PRIN)" Bando 2003 Prot. 2003051182_006.

5 References

[1] Gronbaek, M., Sorensen, T. I., *Br. Med. J.* 1996, *313*, 365.

[2] German, J. B., Walzem, R. L., *Annu. Rev. Nutr.* 2000, *20*, 561 – 593.

[3] Harborne, J. B., Grayer, R. J., The Anthocyanins, in: Harborne, J. B. (Ed.), *The Flavonoids,* Chapman and Hall, London 1988, pp. 1 – 20.

[4] Mazza, G., *Crit. Rev. Food Sci. Nutr*. 1995, *35*, 341 – 371.

[5] Kahkonen, M. P., Heinonen, M., *J. Agric. Food Chem*. 2003, *51*, 628 – 633.

[6] Tsuda, T., Horio, F., Osawa, T., *FEBS Lett*. 1999, *449*, 179 – 182.

[7] Nemeth, K., Plumb, G. W., Berrin, J. G., Juge, N. *et al.*, *Eur. J. Nutr*. 2003, *42*, 29 – 42.

[8] Duthie, G. G., Duthie, S. J., Kyle, J. A. M., *Nutr. Res. Rev.* 2000, *13*, 79 – 106.

[9] Kong, J M., Chia, L. S., Goh, N. K., Chia, T. F., Brouillard, R., *Phytochemistry* 2003, *64*, 923 – 933.

[10] Lazzè, M. C., Pizzala, R., Savio, M., Stivala, L. A. *et al.*, *Mutat. Res*. 2003, *535*, 103 – 115.

[11] Tsuda, T., Shiga, K., Ohshima, K., Kawakishi, S., Osawa, T., *Biochem. Pharm.* 1996, *52*, 1033 – 1039.

[12] Tsuda, T., Horio, F., Kitoh, J., Osawa, T., *Arch. Biochem. Biophys*. 1999, *368*, 361 – 366.

[13] Tsuda, T., Kato, Y., Osawa, T., *FEBS Lett.* 2000, *484*, 207 – 210.

[14] Ramirez-Tortosa, C., Andersen, Ø. M., Gardner, P. T., Morrice, P. C. *et al.*, *Free Radic. Biol. Med*. 2001, *31*, 1033 – 1037.

[15] Luscher, T. F., *Eur. Heart J.* 1992, *13*, Suppl. D, 50 – 55.

[16] Stojilkovic, S., Catt, K. J., *Trends Pharmacol. Sci.* 1992, *13*, 385 – 391.

[17] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y. *et al.*, *Nature* 1988, *332*, 411 – 415.

[18] Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y. *et al.*, *Proc. Natl. Acad. Sci. USA* 1989, *86*, 2863 – 2867.

[19] Li, H., Wallerath, T., Förstermann, U., *Nitric Oxide* 2002, *7*, 132 – 147.

[20] Griendling, K. K., Alexander, R. W., *Circulation* 1997, *96*, 3264 – 3265.

[21] Harrison, D. G., *Clin. Cardiol.* 1997, *20*, Suppl. 2, 11 – 17.

[22] Hink, U., Li, H., Mollnau, H., Oelze, M. *et al.*, *Circ. Res.* 2001, *88*, E14 – 22.

[23] Ruef, J., Moser, M., Kübler, W., Bode, C., *Cardiovasc. Pathol*. 2001, *10*, 311 – 3115.

[24] Youdim, K. A., Martin, A., Joseph, J. A., *Free Radic. Biol. Med*. 2000, *29*, 51 – 60.

[25] Lazzè, M. C., Savio, M., Pizzala, R., Cazzalini, O. *et al.*, *Carcinogenesis* 2004, *25*, 1427 – 1433.

[26] Brand-Williams, W., Cuvelier, M. E., Berset, C., *Lebensm. Wiss. & Technol*. 1995, *28*, 25 – 30.

[27] Bocco, A., Cuvelier, M. E., Richard, H., Berset, C., *Sciences Des Aliments* 1999, *18*, 13 – 23.

[28] Maines, M. D., *FASEB J.* 1988, *2*, 2557 – 2568.

[29] Dragoni, I., Mariotti, M., Consalez, G. G., Soria, M. R., Maier, J. A. M., *J. Biol. Chem*. 1998, *273*, 31119 – 31124.

[30] Mosmann, T., *J. Immunol.* 1983, *65*, 55 – 63.

[31] Yoshino, K., Matsuura, T., Sano, M., Saito, S., Tomita, I., *Chem. Pharm. Bull*. 1986, *34*, 1694 – 700.

[32] Cabré, A., Girona, J., Vallvé, J.-C., Masana, L., *J. Cell. Physiol*. 2004, *198*, 230 – 236.

[33] Hertog, M. G., Feskens, E. J., Hollman, P. C., Katan, M. B., Kromhout, D., *Lancet* 1993, *342*, 1007 – 1011.

[34] Middleton Jr, E., Kandaswami, C., Theoharides, T. C., *Pharmacol. Rev.* 2001, *52*, 673 – 751.

[35] Corder, R., Douthwaite, J. A., Lees, D. M., Khan, N. Q. *et al.*, *Nature* 2001, *414*, 863 – 864.

[36] Liu, J. C., Chen, J. J., Chan, P., Cheng, C. F., Cheng, T. H., *Hypertension* 2003, *42*, 1198 – 1205.

[37] Wallerath, T., Deckert, G., Ternes, T., Anderson, H. *et al.*, *Circulation* 2002, *106*, 1652 – 1658.

[38] Wallerath, T., Li, H., Gödtel-Ambrust, U., Schwarz, P. M., Forstermann, U., *Nitric Oxide* 2005, *12*, 97 – 104.

[39] Leikart, J. F., Räthel, T. R., Wohlfart, P., Cheynier, V. *et al.*, *Circulation* 2002, *106*, 1614 – 1617.

[40] Xu, J.-W., Ikeda, K., Yamori, Y., *Hypertension* 2004, *44*, 217 – 222.

[41] Stivala, L. A., Savio, M., Carafoli, F., Perucca, P. *et al.*, *J. Biol. Chem*. 2001, *276*, 22586 – 22594.

[42] Maines, M. D., *Annu. Rev. Pharmacol. Toxicol*. 1997, *37*, 517 – 554.

[43] Minetti, M., Mallozzi, C., Di Stasi, A. M. M., Pietraforte, D., *Arch. Biochem. Biophys*. 1998, *352*, 165 – 174.

[44] Raines, E. W., Ross, R., *Br. Heart J.* 1993, *69*, Suppl.1, S30 – 37.

[45] Cao, G., Muccitelli, H. U., Sanchez-Moreno, C., Prior, R. L., *Am. J. Clin. Nutr*. 2001, *73*, 920 – 926.

[46] Nielsen, I. L., Dragsted, L. O., Ravn-Haren, G., Freese, R., Rasmussen, S. E., *J. Agric. Food Chem.* 2003, *51*, 2813 – 2820.

[47] Kuhnau, J., *World Rev. Nutr. Diet* 1976, *24*, 117 – 191.

[48] Matsumoto, H., Inaba, H., Kishi, M., Tominaga, S. *et al.*, *J. Agric. Food Chem.* 2001, *49*, 1546 – 1551.

[49] Kroon, P. A., Clifford, M. N., Crozier, A., Day, A. J. *et al.*, *Am. J. Clin. Nutr*. 2004, *80*, 15 – 21.

