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Research Article

Heme oxygenase induction by cyanidin-3-O-β-glucoside in cultured human endothelial cells

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The aim of the present research was to investigate the effect of cyanidin-3-O-β-glucoside (C3G) on heme oxygenase-1 (HO-1), endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS) and dimethylarginine dimethylamino hydrolase-2 (DDAH-2) expression in cultured endothelial cells. Different concentrations (0.00625–250 μM) of C3G were tested in order to investigate possible beneficial and harmful effects of C3G. Our data demonstrated that C3G increased the induction of eNOS and HO-1 in a dose-dependent manner. Higher concentration (62.5–250 μM) also resulted in increase of isoprostane, cGMP and PGE_2 levels and in induction of iNOS with consequent oxidative stress. In conclusion, our data evidence that C3G may exert various protective effects against endothelial dysfunction, whereas potentially harmful effects of C3G appear to be limited to concentrations very difficult to be reached in physiological conditions unless there is abundant oral supplementation.

Keywords: Cyanidin-3-O-β-glucoside / Dimethylarginine dimethylamino hydrolase-2 / Endothelial cells / Heme oxygenase-1 / Nitric oxide synthase

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1 Introduction

Anthocyanins are a group of polyphenols widely distributed in fruits, vegetables and red wines. Cyanidins are considered the most widespread anthocyanins in the plant kingdom. Cyanidin-3-O-β-glucoside (C3G), also known as kuromanin, is probably the best known and most investigated cyanidin-glycoside. In the Mediterranean diet, one important source of C3G is surely represented by the pigmented oranges, called *Moro*, *Sanguinello* and *Tarocco*, typically grown in Sicily (Italy) [1] as well as in Florida [2]. There are several reports mentioning beneficial effects of C3G, such as free radical-scavenging activity, prevention of LDL oxidation, cardiovascular diseases, inflammation and obesity, vascular failure and myocardium damage [1, 3–9]. Besides, it has been demonstrated that anthocyanins have possible beneficial effects in endothelial dysfunction [5, 7]. The mechanisms involved in endothelial dysfunction are multifactorial. These include decreased nitric oxide (NO) availability [10], altered expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) [11], increased release of the endogenous inhibitor of NOS, asymmetric dimethylarginine (ADMA), dysregulation of dimethylarginine dimethylaminohydrolase-2 (DDAH-2), the enzyme involved, as well as the isoform DDAH-1, in ADMA catabolism [12], overproduction of prostanoid vasocostrictors [13], prostacyclin (PGI_2) deficiency [14], reduced expression and activity of stress protein heme oxygenase-1(HO-1) [15].

Since natural compounds, such as demethylbellidifolin [16] or curcumin [17], are able to limit endothelial dysfunction by increasing DDAH activity or by increasing HO-1 expression and activity, the aim of the present research was to investigate the effect of C3G on NOS/DDAH pathway and HO-1 expression in cultured endothelial cells, in order to identify additional mechanisms of action that might contribute to maintaining endothelial function.

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Abbreviations: **C3G**, cyanidin-3-O-β-glucoside; **DDAH-2,** dimethylarginine dimethylamino hydrolase-2; **eNOS,** endothelial nitric oxide synthase; **HO-1,** heme oxygenase-1; **iNOS,** inducible nitric oxide synthase; **MTT**, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PGE_2**, prostaglandin E_2; **PGI_2**, prostacyclin

Controversial data on the properties of polyphenols are available. It has been reported that high concentrations of polyphenols may produce negative effects [18–20] and studies on different cells and tissues demonstrated that they might induce different and dose-dependent effects [21].

For these reasons, a wide range of C3G concentrations was tested in order to investigate any possible beneficial and harmful effects of this anthocyanin.

2 Materials and methods

2.1 Chemicals

C3G (purity 99%) was obtained from Extrasynthase (Genay, France). All chemical used, were purchased from Sigma (St. Louis, MO), unless otherwise specified. C3G was dissolved in culture medium for all treatments.

2.2 Cell culture conditions

Human iliac artery endothelial cells (HIAE-101, organism: *Homo Sapiens;* tissue: *iliac artery endothelial normal*; age stage: *21 years;* gender: *female*) were purchased from ATCC (ATCC – LGC, Middlesex, UK; Cat. No: CRL-2478) and grown in F12K medium (Kaign's modification of Ham's F12 medium) (ATCC) supplemented with 10% fetal bovine serum (Gibco-BRL-Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere and maintained at subconfluency by passaging with trypsin-EDTA (Gibco).

Subconfluent cells were starved for 24 h in medium containing 0.5% fetal bovine serum. Endothelial cells were then cultured in fresh medium, stimulated with 10% fetal bovine serum and the effect of treatment for 24 h with different concentrations (0.00625–250 μM) of C3G was evaluated.

We verified that after 24 h of treatment there were no interactions of C3G with culture media. In fact, C3G spectrum with max of absorbance at 538 nm after 24 h of treatment was the same as that at time 0 h.

Cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test; oxidative stress was assessed through isoprostane levels, and PGE_2 and PGI_2 levels were measured as markers of endothelial function. NOS activity was determined measuring cGMP levels. Furthermore, Western blotting was performed for HO-1, eNOS, iNOS and for DDAH-2.

2.3 Cell viability

To monitor cell viability, HIAE-101 cells were seeded 2×10^5 cells/well of a 96-well, flat-bottom 200-μL microplate. Cells were incubated at 37°C in a humidified 5% CO_2/95% air mixture and cultured in absence or in presence of different concentrations of C3G (0.00625–250 μM) for 24 h.

Four hours before the end of the treatment, 20 μL of 0.5% MTT in PBS was added to each microwell. After incubation with the reagent, the supernatant was removed and replaced with 100 μL DMSO. The OD was measured with a microplate spectrophotometer reader (Thermo Labsystems Multiskan) at $\lambda = 570$ nm.

2.4 Measurement of isoprostane, cGMP, PGE_2, and 6-Keto-$PGF_{1\alpha}$

The levels of isoprostane, cGMP, PGE_2 and the stable metabolite of PGI_2, 6-keto-$PGF_{1\alpha}$, were determined in the culture medium using appropriate enzyme-linked immunoassay (EIA) kits. HIAE-101 were counted and seeded in T75 flasks ($5–6 \times 10^6$ cells/flask). Cells were cultured in absence or in presence of different concentrations of C3G (0.00625–250 μM) after which the media were removed, stored at -80°C and processed within 1 week. Color development was obtained with EIA isoprostane, cGMP, PGE_2 and 6-keto-$PGF_{1\alpha}$, kits (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. Isoprostane levels, release of PGE_2 and 6-keto-$PGF_{1\alpha}$ are expressed as pg/1×10^6 cells, while cGMP levels are expressed as fmol/1×10^6 cells. All samples were run in triplicate.

2.5 Western blotting

Cells were suspended in 25 mM Tris buffer, pH 8.5, containing 100 mM NaCl, 7 mM mercaptoethanol (Merck, Darmstadt, Germany) and Protease inhibitor cocktail (1:1000) and then sonicated for three cycles of 5 s. The whole lysate was collected for Western blot analysis, to evaluate HO-1, DDAH-2, eNOS and i-NOS expression. Briefly, 50 μg of lysate was loaded in a SDS-PAGE 10% and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules,CA, USA). The membranes were blocked with 3% non-fat dried milk in 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 (TBST) buffer at 4°C for 2 h and then incubated with mouse monoclonal anti-HO-1 (Stressgen Biotechnologies, San Diego, CA, USA), goat polyclonal anti-DDAH-2 (Calbiochem EMD Biosciences, an affiliate of Merck, Darmstadt, Germany), mouse monoclonal anti-eNOS and rabbit polyclonal anti-iNOS (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) antibodies overnight at room temperature, with constant shaking. The filters were then washed and probed with horseradish peroxidase-conjugated donkey secondary anti-mouse, anti-rabbit and anti-goat IgG (Amersham Biosciences, Piscataway, NJ, USA). Chemiluminescence detection was performed with the ECL plus detection kit (Amersham) according to the manufacturer's instructions.

Western blots were quantified by densitometric analysis performed after normalization with actin. Results are expressed as arbitrary units (AU).

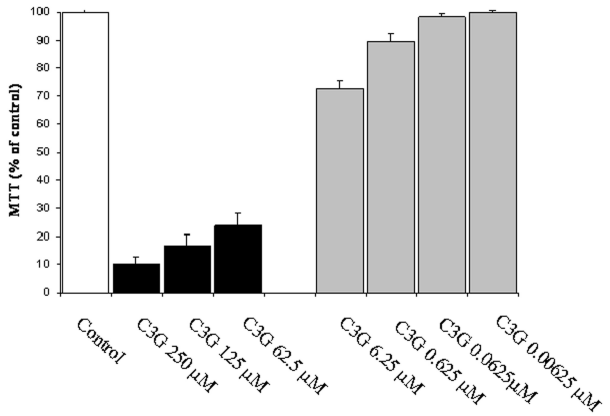

Figure 1. Cell viability in cultured HIAE-101 cells after treatment with different C3G concentrations (0.00625–250 μM) for 24 h. Values are mean ± S.D. of four experiments in triplicate.

2.6 Statistical analyses

The data are presented as means ± SD for four experiments in triplicate. One-way analysis of variance and Student's *t*-test were used where appropriate, and $p < 0.005$ between two experimental groups was regarded as significant.

3 Results

3.1 Endothelial cell viability

MTT assay was performed to monitor cell viability, measuring the conversion of tetrazolium salt to yield colored formazan, the amount of which is proportional to the number of the living cells. Results show a dose-dependent inhibitory effect in succinate dehydrogenase activity, the major mitochondrial enzyme responsible for the MTT formazan conversion, with high (62.5–250 μM) concentrations of C3G, whereas low (6.25–0.00625 μM) C3G concentrations had no significant effect on endothelial cell viability (Fig. 1).

3.2 HO-1, DDAH-2, eNOS, and iNOS expression in cultured endothelial cells

Protein expression was assessed in HIAE-101 cells following 24 h of treatment with different concentrations of C3G. Treatment of endothelial cells with high (62.5–250 μM) C3G concentrations resulted in HO-1, eNOS and iNOS up-regulations, whereas no effect was observed in DDAH-2 expression (Fig. 2). Treatment of endothelial cells with low (6.25–0.00625 μM) C3G concentrations resulted only in HO-1 up-regulation (Fig. 3).

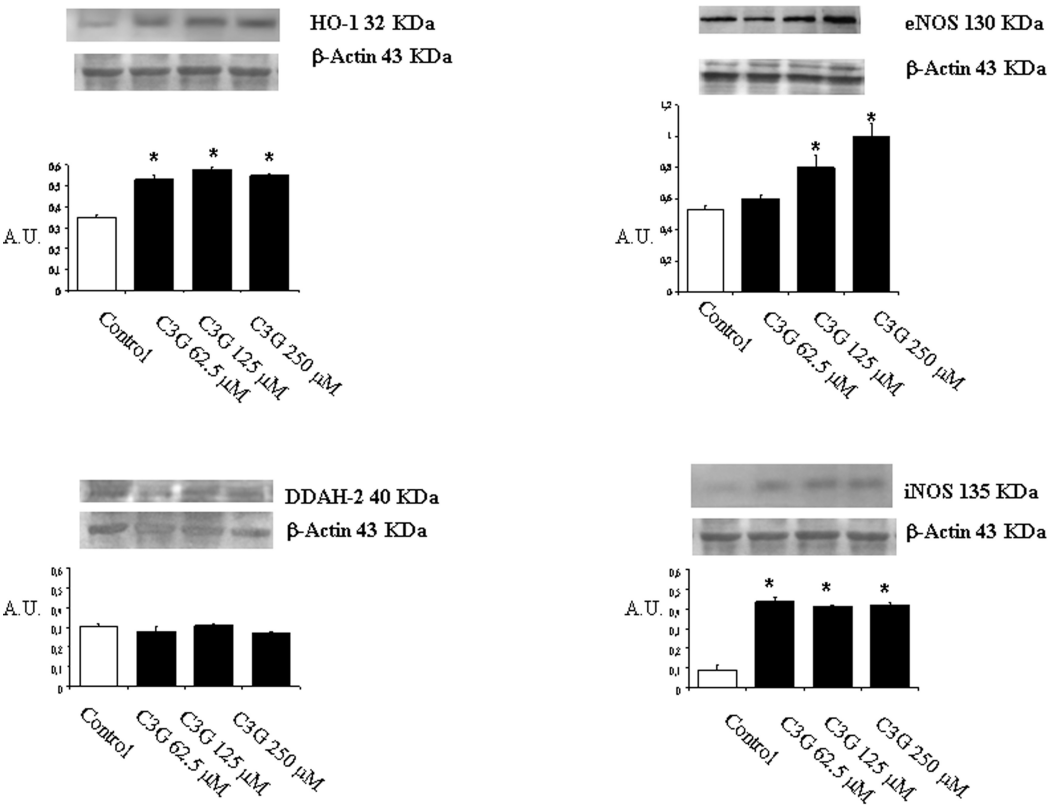

Figure 2. Effect of high C3G concentrations (62.5–250 μM) on DDAH-2, HO-1, iNOS and eNOS expression in cultured HIAE-101 cells. Results, expressed as AU, represent the mean ± SD of four experiments. Significance of C3G *versus* control: * $p < 0.005$.

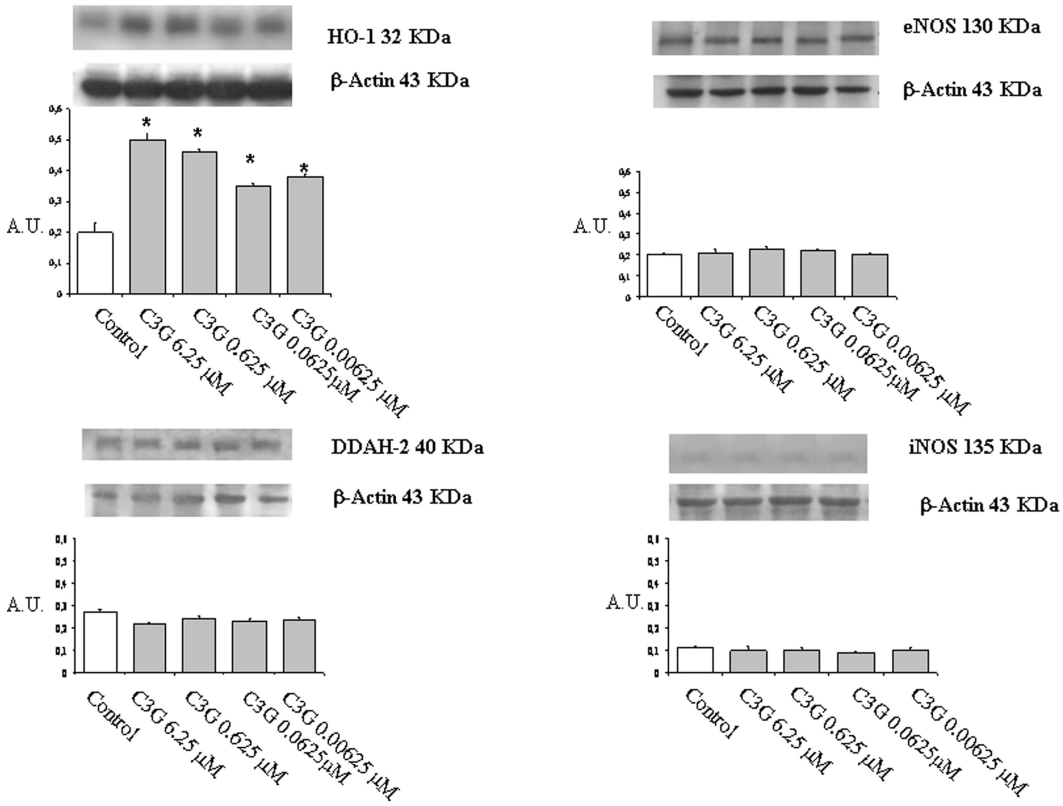

Figure 3. Effect of low C3G concentrations (0.00625–6.25 μM) on DDAH-2, HO-1, iNOS and eNOS expression in cultured HIAE-101 cells. Results, expressed as AU, represent the mean ± SD of four experiments. Significance of C3G *versus* control: * $p < 0.005$.

Table 1. PGI_2, PGE_2, isoprostane and cGMP release in HIAE 101 cells[a)]

	PGI_2 pg/1 × 10^6 cells	PGE_2 pg/1 × 10^6 cells	Isoprostane pg/1 × 10^6 cells	cGMP fmol/1 × 10^6 cells
Control	112.62 ± 3.53	82.49 ± 3.19	44.24 ±1.53	19.7 ± 1.39
C3G 250 μM	114.96 ± 4.68	157.12 ± 4.21*	71.83 ± 3.24*	62 ± 3.71*
C3G 125 μM	103.25 ± 3.51	153.79 ± 3.76*	70.34 ± 2.85*	53.3 ± 2.17*
C3G 62.5 μM	116.22 ± 2.78	134.74 ± 5.15*	60.16 ± 2.42*	36.7 ± 1.84*
C3G 6.25 μM	105.28 ± 2.00	55.25 ± 0.01*	36.22 ± 1.01*	19.2 ± 0.35
C3G 0.625 μM	97.84 ± 4.19	53.80 ± 0.22*	33.37 ± 0.98*	19.5 ± 0.43
C3G 0.0625 μM	109.38 ± 2.87	58.77 ± 2.11*	30.10 ± 1.33*	21.5 ± 0.72
C3G 0.00625 μM	103.19 ± 5.1	69.02 ± 1.87*	28.51 ± 0.54*	20.1 ± 0.84

a) Values represent the mean ± SD of four experiments. Significance of C3G *versus* control: *p <0.005.

3.3 PGE_2 and 6-keto-$PGF_{1\alpha}$ (PGI_2) levels in culture medium of HIAE-101 cells

In order to evaluate the effect of C3G on endothelial cell function, we also evaluated the release of prostaglandins, which have been shown to play a major role in vascular homeostasis.

Treatment of endothelial cells with higher concentrations of C3G resulted in increased release of PGE_2 compared to untreated cells, whereas lower concentrations of C3G significantly reduced PGE_2 release. PGI_2 levels remained unmodified (Table 1).

3.4 cGMP and isoprostane release in culture medium of HIAE-101 cells

Treatment of endothelial cells with higher concentrations of C3G resulted in increased release of isoprostane and cGMP with respect to untreated cells, whereas lower con-

centrations of C3G significantly reduced only isoprostane levels (Table 1).

4 Discussion

Recently, several studies confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases [5, 22, 23], cancer [24, 25] and neurodegenerative diseases [26–30], as well as inflammation [31, 32] and problems caused by cell and cutaneous ageing [33]. A considerable amount of epidemiological evidence has demonstrated an association between diets rich in fruit or vegetables and decreased risk of cardiovascular disease or certain forms of cancer [34]. It was generally assumed that the active dietary constituents contributing to these protective effects are antioxidant nutrients such as alpha-tocopherol and beta-carotene. However, recent investigations highlight an important additional role for polyphenolic compounds of higher plants that may act as antioxidants or via other mechanisms contributing to anticarcinogenic or cardioprotective actions [23, 25].

Anthocyanins are phenolic compounds widespread in food plants. Glycosides of the aglycon cyanidin, represent the most abundant anthocyanins in vegetables. There has been extensive research into the biological properties of C3G, and several *in vitro* and *in vivo* studies noted its antioxidant properties [35]. Furthermore, recent studies demonstrated that C3G might act via other mechanisms contributing to limit endothelial dysfunction. It has been reported that cyanidin and C3G can up-regulate eNOS, with consequent increased release of bioactive NO [36–38]. Moreover, C3G is a scavenger of peroxynitrite and therefore exerts a protective effect against endothelial dysfunction and vascular failure induced by peroxynitrite [5], a pro-oxidant agent that is formed by reaction of NO with superoxide anion (O_2^-). It should be emphasized that an important application of an *in vitro* model is to obtain mechanism-derived information by studying different cells or tissues and target-specific effects. By *in vitro* models, it is possible to assess cytotoxicity and cellular responses, and to perform toxicokinetic modeling. Polyphenols are classified as phytoprotectants due to antioxidant properties [39]. However, controversial results regarding the beneficial properties of phytoprotectants have also been obtained. For example, high doses of some polyphenols may produce free radicals, be cytotoxic and be pro-oxidative [18–20]. Consequently, the determination of toxicity can be used as a tool for defining concentrations at which chemoprotective effects can be further characterized. In view of these considerations, we used a wide range of C3G concentrations (from 0.00625–6.25 μM to 62.5–250 μM).

Data reported in the present study indicate that C3G at high concentrations was able to up-regulate eNOS. These data, according to Lazzè *et al.* [38], indicate that C3G might limit endothelial dysfunction, thus resulting in beneficial effects. An additional cytoprotective effect of high concentrations of C3G may consist in the induction of the stress protein HO-1. This is in agreement with Lazzè *et al.* [38] notwithstanding their study reported the effect of the aglycone cyanidin, which is less active respect to C3G [6, 40] and is not present in human or rat plasma after intake of anthocyanins [41, 42]. HO-1 inducing activity of C3G is also in agreement with results obtained by Motterlini *et al.* [17] in endothelial cells after treatment with curcumin, a natural polyphenol compound. Therefore, C3G might be considered an inducer of HO-1 in vascular endothelial cells. Our data also showed that highest C3G concentrations (62.5–250 μM) reduced cell viability. However, this effect is likely limited to endothelial cells which are particularly vulnerable.

In our study, high C3G concentrations induced isoprostane, cGMP and PGE_2 increases, with a consequent imbalance in the PGE_2/PGI_2 ratio and induction of iNOS. These alterations may contribute to induction of oxidative/nitrosative stresses. In fact, increase in levels of isoprostane, GMPc and iNOS induction may justify a pro-oxidative effect of highest C3G concentrations. Moreover, enhanced formation of PGE_2, that according to Salvemini *et al.* [43] and Sautebin *et al.* [44] is secondary to COX_2 induction, might contribute to impairment of vascular homeostasis.

By contrast, at lower concentrations (0.00625–6.25 μM), C3G had no significant effect on endothelial cell viability. In addition, although at lower concentrations C3G was not able to induce eNOS, it caused HO-1 up-regulation without producing any changes in iNOS expression. These latter data could apparently conflict with the results reported by Wallerath [36] and by Xu [37], but the contrast may be explained by the different method used for evaluating eNOS expression or by different cell type employed. At lower concentrations, C3G also showed the ability to reduce isoprostane and PGE_2 levels, which might be regarded as beneficial effects. Isoprostane reduction may justify an antioxidant activity of low C3G concentrations. In accord with Rossi *et al.* [32], PGE_2 reduction might explain an anti-inflammatory activity of C3G. The ability of C3G to attenuate PGE_2 has also been reported by other authors [45, 46] along with the reduction of NFKB production as well as COX_2 induction.

Results obtained in the present study suggest that C3G may act as scavenger of reactive oxygen species (ROS) or may promote ROS formation depending on its dosage. This is in agreement with controversial results on the beneficial properties reported for other polyphenols and for ascorbate [47, 48]. Reduction of isoprostane levels is in agreement with our previous data reporting a scavenger effect of low C3G concentrations on O_2^- anion [40]. Moreover, as reported by Serraino *et al.* [5], low C3G concentrations exert a scavenger effect on peroxynitrite. Exposure of cells to C3G at any

concentration did not result in modifications in DDAH-2 expression.

It should be noted that the lower C3G concentrations used in the present study are in the nanomolar range, thus close to the plasma concentrations found in physiological condition following the intake of anthocyanins contained in a normal diet [41, 49]. Higher concentrations of C3G should be reached in plasma after anthocyanins-rich meal ingestion [42], whereas it has been reported that C3G may reach plasma concentration up to micromolar range only by substantial oral supplementation [42].

Although our previous research demonstrated beneficial effects of high C3G concentrations in human fibroblasts [50], data obtained in the present study indicate that the same concentrations of C3G reduced the viability of endothelial cells; this confirms the hypothesis that C3G may exert on different cells, different and dose-dependent effects.

It is important to evidence that C3G both at high and low concentrations is able to induce significantly HO-1 protein. Numerous studies have shown that induction of HO-1 protein is an important cellular protective mechanism against oxidative injury [51]. Therefore, we can hypothesize that endothelial protection mechanism of C3G can also be associated with HO-1 induction.

In conclusion, our data evidence that at concentrations easily reachable in physiological conditions C3G may exert various protective effects against endothelial dysfunction. Potentially harmful effects of C3G have to be further elucidated. In any case, they appear to be limited to concentrations which are very difficult to reach under physiological conditions unless there is abundant oral supplementation.

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