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Research Article

Protective Role of Malvidin-3-Glucoside on Peroxynitrite-Induced Damage in Endothelial Cells by Counteracting Reactive Species Formation and Apoptotic Mitochondrial Pathway

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The health-promoted benefits of anthocyanins, including vascular protective effects and antiatherogenic properties, have now been recognized, but the involved molecular mechanisms have not been well elucidated. Following our previous work on cytoprotective mechanisms of some anthocyanins against apoptosis triggered by peroxynitrite in endothelial cells, here we investigated the protective role of malvidin-3-glucoside, a major dietary anthocyanin, on such deleterious process, by exploring the interference on cellular reactive species formation and on apoptotic mitochondrial pathway. Preincubation of cells with 25 μM malvidin-3-glucoside protected efficiently endothelial cells from peroxynitrite-promoted apoptotic death, an effect which may be partially mediated by its ability to decrease the formation of reactive species after cell aggression, as assessed by the dichlorodihydrofluorescein diacetate assay and by carbonyl groups formation. Moreover, malvidin-3-glucoside inhibited mitochondrial apoptotic signaling pathways induced by peroxynitrite, by counteracting mitochondrial membrane depolarization, the activation of caspase-3 and -9, and the increase in the expression of the proapoptotic Bax protein. Altogether, our data expands our knowledge about the molecular mechanisms underlying the vascular protection afforded by malvidin-3-glucoside, and anthocyanins in general, in the context of prevention of endothelial dysfunction and atherosclerosis.

1. Introduction

In the last decade, there has been a remarkable increment in scientific knowledge concerning the beneficial role of anthocyanins, a large group of flavonoids widely distributed in human diet, in several oxidative-stress-related diseases, including atherosclerosis [1, 2]. Atherosclerosis, the main cause of cardiovascular disease, is a multifactorial disorder where endothelial dysfunction and inflammation plays a critical role [3, 4]. Peroxynitrite has been recognized as one relevant intervening factor in atherogenesis, as a strong endogenous oxidizing and nitrating species of several biomolecules. It is formed from the diffusion-limited reaction between nitric oxide (NO) and superoxide anion ($O_2^{\bullet -}$), both produced by immune system cells and vascular cells [5–7]. This reaction has one of the highest rate constants known for reactions with NO, justifying the toxicity of peroxynitrite at certain concentrations [8]. This reactive species can indeed reach high concentrations in vascular endothelium under shear stress and in atherosclerotic vessels, where it has been suggested to promote, in particular, LDL oxidation and extensive protein tyrosine residues nitration [9, 10], contributing to the production of the fatty streaks and subsequent atherosclerotic plaque formation. However, peroxynitrite may be involved in atherogenesis by other pathways, mainly by promoting the impairment of vascular reactivity and the disruption of critical cellular processes leading cells to apoptosis or necrosis [11, 12]. Therefore, counteracting peroxynitrite damaging effects is vital to endothelial integrity, and anthocyanins have already demonstrated their high peroxynitrite-scavenging activity, in several studies [13–15].

Epidemiological studies have evidenced that diets rich in vegetables and fruits are associated with a decreased risk in cardiovascular diseases [16, 17] and some of the major contributors are anthocyanins, whose intake has been

estimated to be up to 9-fold higher than that of other dietary flavonoids [16, 17]. Among dietary anthocyanins, malvidin-3-glucoside (Mv3glc) is one of the major constituents, in particular, in red wine [18]. Despite the controversy about physiologically available concentrations of anthocyanins, there is unquestionable data revealing that they are absorbed in their intact glycoside forms, including Mv3glc, appearing rapidly in the blood plasma and tissues after ingestion [19, 20]. Furthermore, the potential *in vivo* cumulative effects should also be taken into consideration. Therefore, recently, anthocyanins have been considered as promising bioactive molecules [21] in the search for potential functional foods and nutraceuticals, mainly in the context of atherosclerosis prevention [1, 22].

Although in earlier studies the biological activities of anthocyanins were closely related to their antioxidant properties, mainly ascribed to the B-ring hydroxyl groups and to the conjugated double bond system [23], their anti-inflammatory and antiatherogenic effects, among several others, cannot be explained solely on basis of these properties. In this context, there is a plethora of works, which has indicated other action mechanisms beyond such properties, namely, by interfering with crucial signaling pathways and gene regulation [24, 25].

Recently, we have shown that anthocyanins possessing either catecholic or monophenolic structures are able to counteract peroxynitrite-induced endothelial cells apoptosis through the inhibition of crucial signaling cascades, upstream and downstream of mitochondria [14]. Following this work, here we clarified the cytoprotective mechanisms for Mv3glc, an anthocyanin with 3′,5′-dimethoxyl substituents in the B-ring (Figure 1), providing new insights about the potential role of this feature on modulation of apoptotic mitochondrial signaling pathway. In fact, different patterns of hydroxylation and methoxylation, mainly on the B-ring, are known to modulate the antioxidant properties of polyphenols [26, 27] and thus could also account for their protective effects against endothelial cells under peroxynitrite injury, a process that involves the production of reactive species, which may be either directly or indirectly mediators of cellular signaling cascades. Thus, besides the antioxidant activity of Mv3glc, we assessed its capacity to counteract peroxynitrite-induced apoptotic effects by interfering in mitochondrial apoptotic signaling cascades, in primary cultures of bovine arterial endothelial cells (BAECs) as a typical endothelial cell model. This work indicates that preincubation of cells with 25 μM Mv3glc prevented several apoptotic events, such as the loss of mitochondrial membrane potential, the caspases-9 and -3 activation, and the increase in cytoplasmatic Bax levels, inhibiting peroxynitrite cell injury, an effect partially mediated by its ability to scavenge reactive species resulting from cellular oxidative aggression.

2. Materials and Methods

2.1. Reagents. Malvidin 3-*o*-β-glucoside chloride, purified from natural sources, was obtained from Extrasynthése (Genay, France). Mv3glc had purity above 97% as measured by HPLC and was used as solutions (5 mM) in DMSO and stored in the dark, under nitrogen atmosphere, at −80°C; the final solvent concentration did not exceed 1% by volume. Quercetin was from Sigma-Aldrich Co.

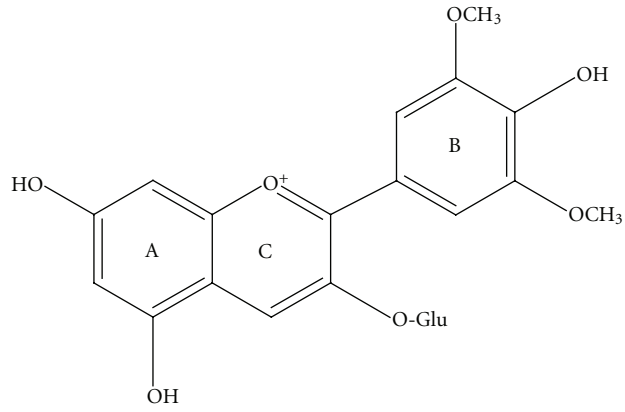

Figure 1: Malvidin-3-glucoside chemical structure.

Laboratory chemicals, namely, dihydrorhodamine 123 (DHR 123), dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT), collagenase, gelatin, Hoescht 33258, substrates for caspase-3 (Ac-DEVD-AMC), caspase-9 (Ac-LEDH-AFC), 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA), and streptomycin/penicillin were purchased from Sigma-Aldrich Co as well as other chemical reagents used.

For cell culture, Dulbecco's modified Eagle's medium (DMEM), trypsin 0.25%, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) pH 7.4 were obtained from Gibco-Invitrogen.

Primary specific mouse monoclonal antibody to Bax and anti-mouse IgG secondary antibody were obtained from Abcam (Cambridge, UK); mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich Co.

2.2. Primary Cultures of Bovine Aortic Endothelial Cells. Bovine aortic endothelial cells (BAECs) were obtained from thoracic aorta by treatment with collagenase (2 mg/mL). Cells were cultivated on gelatin-coated (0.2%) tissue culture plastic (bovine origin, Sigma-Aldrich Co), in DMEM supplemented with 10% heat inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5%CO_2. Endothelial cells, identified by their cobblestone morphology, were subcultured at confluence and used between the fourth and the tenth passages. Before each experiment, cells at 80% confluence were starved in serum-free medium for at least 6 h.

2.3. Peroxynitrite Synthesis and Peroxynitrite Treatment of Cells. Peroxynitrite was synthesized using a quenched flow reactor, as previously described [9]. The obtained peroxynitrite was then stored at −80°C under N_2 atmosphere.

Immediately before use, it was always quantified from its absorbance at 302 nm in 1 M NaOH ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$).

Peroxynitrite treatment of BAECs was performed as we have previously described [14, 28]. Briefly, $ONOO^-$, diluted in recently prepared NaOH 10 mM, was delivered (to give a final concentration of 500 μM) as a single bolus, against one side of the culture plate containing cells previously washed and equilibrated with PBS with calcium and magnesium, pH 7.4, while rapidly swirling the medium to ensure uniform exposure to $ONOO^-$ before decomposition. After 10 min, cells were gently washed with PBS, which was replaced with DMEM without serum for the required times. PBS was used to avoid interfering reactions of $ONOO^-$ with the medium constituents. No pH shift was observed during this treatment. The same volumes of either 10 mM NaOH (vehicle control) or decomposed peroxynitrite ($ONOO^-$ was decomposed in PBS or in 10 mM NaOH overnight) were used as controls. In experiments with Mv3glc, cells were preincubated with this compound for 14 h, and after this time the incubation medium was removed and the cells were submitted to $ONOO^-$ aggression, as described above. Thus, in these assays, Mv3glc was not present in the culture medium during the experiments with $ONOO^-$.

2.4. Cell Viability Test. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT) to formazan, as reported [29]. After incubation with the indicated concentrations of Mv3glc, for 14 h, cells in 6-well plates (0.5×10^6 cells/well) were washed with PBS and incubated with MTT (0.5 mg/mL) for 1 h, at 37°C, in the dark, in new serum-free culture medium. Then, after removing the medium, the formazan crystals were dissolved in DMSO (300 μL), and the extent of MTT reduction was quantified by the measurement of absorbance at 550 nm in a Synergy HT plate reader. Results were expressed as a percentage of control cells.

2.5. Morphology of Cells Nuclei. The morphology of cells nuclei was observed by fluorescence microscopy using the cell-permeable DNA-binding fluorescent dye Hoechst 33258. Briefly, peroxynitrite-treated cells were fixed with 4% (w/v) paraformaldehyde for 10 min, at room temperature, and subsequently stained with Hoechst 33258 (5 μg/mL) for 10 min, at same temperature, washed with PBS, and mounted using PBS : glycerol (3 : 1, v/v). Nuclear morphological changes were visualized in an inverted fluorescence microscope (Zeiss Axiovert 40) with a Zeiss DAPI filter. Cells with chromatin condensation as well as nuclear fragmentation were considered apoptotic, in contrast with the normal cells presenting homogeneously stained nuclei. Cells from at least nine randomly chosen microscope fields (400x) per sample were counted and the number of apoptotic cells was expressed as a percentage of the total number of cells.

2.6. Dihydrorhodamine Fluorescence Assay. The *in vitro* specific ability of Mv3glc to scavenge peroxynitrite was evaluated by the inhibition of peroxynitrite-mediated oxidation of dihydrorhodamine 123 (DHR123) to rhodamine 123, followed by the decrease in fluorescence, as previously described by Kooy et al. [30] with minor modifications. Mv3glc (5 mM stock solution) and DHR123 (5.78 mM stock solution) were dissolved in DMSO and stored at −80°C under nitrogen atmosphere. The working solutions were prepared daily in saline phosphate buffer pH 7.4 (50 mM Na_2HPO_4, 5 mM KCl, 90 mM NaCl, and 100 μM DTPA). Briefly, 25 μM DHR123 were added to saline phosphate buffer with 100 μM DTPA, at 37°C, containing Mv3glc (2–10 μM), and after adding peroxynitrite (1.2 μM in NaOH 0.1 M), the fluorescence intensity of oxidized DHR123 was measured in a Perkin-Elmer LS 50B spectrofluorometer, at 37°C in a thermostatized cuvette with magnetic stirring, at the excitation and emission wavelengths of 500 and 536 nm (slit widths of 2.5 and 3.0), respectively. The results were expressed in terms of % of control (DHR123 plus $ONOO^-$). Decomposed peroxynitrite in buffer did not oxidize DHR123. Quercetin was used as a reference compound.

2.7. Evaluation of Intracellular Reactive Species and of Carbonyl Group Formation. Intracellular reactive species were assessed by using the nonfluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA), which permeates cell membranes and may be oxidized by reactive species, yielding the fluorescent 2′,7′-dichlorofluorescein (DCF), essentially as previously described [31, 32]. Briefly, cells in 24-well plates (1×10^5 cells/well) were previously incubated with or without 25 μM Mv3glc and further submitted to above-referred treatment with peroxynitrite and incubated up to 6 h. Then, 20 min before the end of each hour, cells were incubated with 2 μM DCFHDA prepared in serum-free DMEM, at 37°C, in the dark; after washing of cells with PBS, they were maintained in serum-free DMEM and then observed by fluorescence microscopy (Zeiss Axiovert 40). Also, the fluorescence intensity measurements were obtained in a Synergy HT plate reader (Bio-Tek Instruments) (excitation and emission wavelengths at 485 and 530 nm, resp.). Results were expressed as fluorescence intensities (arbitrary units).

The peroxynitrite-promoted cellular oxidations were also followed in terms of the carbonyl group formation. The treated cells were lysed in HBS buffer supplemented with 1% protease inhibitor cocktail, pH 7.5 on ice. After 5 cycles of liquid N_2/37°C water bath, lysates were centrifuged at 15000 g for 20 min at 4°C, and the supernatants (total extracts) were collected and stored at −20°C. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, USA), based on the Bradford reaction.

The carbonyl content was determined in duplicates using an already established colorimetric assay [33]. Briefly, 800 μg of protein were incubated with 500 μL of 0.2% 2,4-dinitrophenylhydrazine (DNPH) (in 2 N HCl) for 1 h in the dark, and blanks were prepared using 2 N HCl alone. Samples and blanks were precipitated by the addition of an equal volume of 20% trichloroacetic acid and centrifugation at 10000 g for 5 min, 4°C. The supernatants were discarded and the pellets were submitted three times to 10 minutes

wash with 1 mL of ethanol : ethyl acetate (1 : 1 v/v), vortexing each 3 minutes during the process. The samples were then centrifuged at 10000 g for 3 min, 4°C. The final pellets were resuspended in 0.5 mL of 6 M guanidine-HCl, and the carbonyl contents were determined by measuring the absorbance of the hydrazones at 370 nm. Results were calculated as μmol carbonyl/mg protein using an extinction coefficient of 22000 M^{-1} cm^{-1}.

2.8. Measurement of Mitochondrial Membrane Potential. Measurement of the mitochondrial membrane potential ($\Delta\Psi m$) was performed by loading cells with the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), a membrane-permeable cationic fluorophore that accumulates inside mitochondria in a potential-dependent manner, which is visualized by a shift in its fluorescence emission from green (525 nm) to red (590 nm) [34]. Cells were preincubated with Mv3glc and treated with peroxynitrite as described above; at the indicated times, the supernatants were removed and cells were labeled with JC-1 (2 μg/mL) at 37°C, for 20 minutes. Further, after washing with PBS, cells were visualized in an inverted fluorescence microscope (Zeiss Axiovert 40) with an FITC filter set. In the different experiments, the ratios between the fluorescence intensities of the J aggregates, in mitochondria (excitation 550 nm, emission 600 nm), and of the monomers remaining in cytoplasm (excitation 485 nm, emission 535 nm) were used to obtain the red/green fluorescence ratios. Fluorescence intensities were measured in a Synergy HT microplate reader (BioTek Instruments, Inc, USA).

2.9. Measurement of Caspases Activities. Caspase activation was assessed, essentially, as previously described by Brito et al. [12], in cytosolic protein extracts. After cells incubation with peroxynitrite, with or without the anthocyanin, as above described, cells were washed with PBS and then scrapped on ice in a lysis buffer containing 1 mM sodium ethylenediaminetetracetic acid (Na-EDTA), 1 mM Na-EGTA, 2 mM $MgCl_2$-$6H_2O$, 25 mM HEPES (pH 7.5) supplemented with 0.1% CHAPS, 100 mM PMSF, 2 mM DTT, and 1% (v/v) protease inhibitor cocktail. After 5 cycles of freezing and thawing with liquid N_2 and 37°C water bath, lysated cells were centrifuged at 14000 g for 10 min at 4°C and the collected supernatants were stored at −80°C.

The enzymatic activities were determined in 25 μg of lysates protein in a reaction buffer solution (25 mM HEPES, pH 7.5 supplemented with 0.1% CHAPS, 5 mM DTT, and 100 mM PMSF). The reactions were initiated by addition of adequate fluorogenic substrates (100 μM) containing specific cleavage sites, linked to a fluorochrome: Ac-DEVD-AMC for caspase-3-like activity and Ac-LEDH-AFC for caspase-9-like activity. After incubation for 150 min at 37°C, the release of AMC or AFC was determined by fluorescence measurements at 380/460 nm or 400/505 nm (excitation/emission), respectively, in a Synergy HT microplate reader. Caspases activities were expressed as a percentage of the control, that is, cells incubated without neither peroxynitrite nor anthocyanin.

2.10. Western-Blot Analysis. Protein expression was evaluated by Western-blot, as essentially described [14]. Cytoplasmic protein extracts were obtained from lysed cells prepared in a solution containing 300 μL of 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and 1% protease inhibitor cocktail, pH 7.5, for 10 min on ice. After centrifugation at 5000 rpm for 5 min at 4°C, the supernatants were collected and stored at −20°C. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, USA), based on the Bradford reaction.

Thirty micrograms of reduced and denaturated total protein from each extract were separated by SDS/PAGE [10% (v/v)] and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, UK) by electroblotting. The membranes were blocked with skimmed milk in TBS buffer supplemented with 0.1% (v/v) Tween 20 (TBS-T: 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween) and then probed with antibodies against Bax for 2 h, at room temperature. After extensive washings with TBS-T, the blots were incubated with an alkaline phosphatase-conjugated secondary antibody for 1 h, at room temperature. Immunoreactive complexes were detected by chemifluorescence after blots exposition to enhanced chemifluorescent reagent (Amersham Biosciences) in a Typhoon 9000 scanner (Amersham Biosciences). β-Actin was used as a control for protein loading in the cytoplasmic extracts. Bands were analyzed using the ImageQuant TM software from Amersham Biosciences.

2.11. Statistical Analysis. All data were expressed as means ± SEM of at least 3 independent assays, each one in duplicate. Differences between groups were analyzed by one-way analysis of variance (ANOVA), Bonferroni's, Tukey's, or Student tests were used as appropriate. A value of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Protection of Endothelial Cells against Peroxynitrite-Mediated Apoptosis. In agreement with our previous reports [12, 14], peroxynitrite under this experimental conditions, induced apoptotic death in bovine aortic endothelial cells (Figure 2(a)), a process significantly reduced in cells previously incubated with Mv3glc. In fact, in that figure, it is evidenced that peroxynitrite cell treatment, after 6 h of cell incubation, as described in Section 2, led to about 30% of apoptotic cells, as assessed by nuclear condensation or fragmentation visualized by Hoechst staining, whereas in untreated cells these features were not detected (less than 2%). A reduction in about 70% of apoptotic cells was observed in preincubated cells with 25 μM Mv3glc, before $ONOO^-$ aggression, a result similar to those obtained with other anthocyanins. At this concentration, and during all the experience time, Mv3glc *per se* did not interfere with endothelial cells viability and prevented the peroxynitrite-induced decrease in cell viability, as evaluated by the MTT test (Figure 2(b)). Of note is that the anthocyanin was not present in the medium during peroxynitrite treatment and

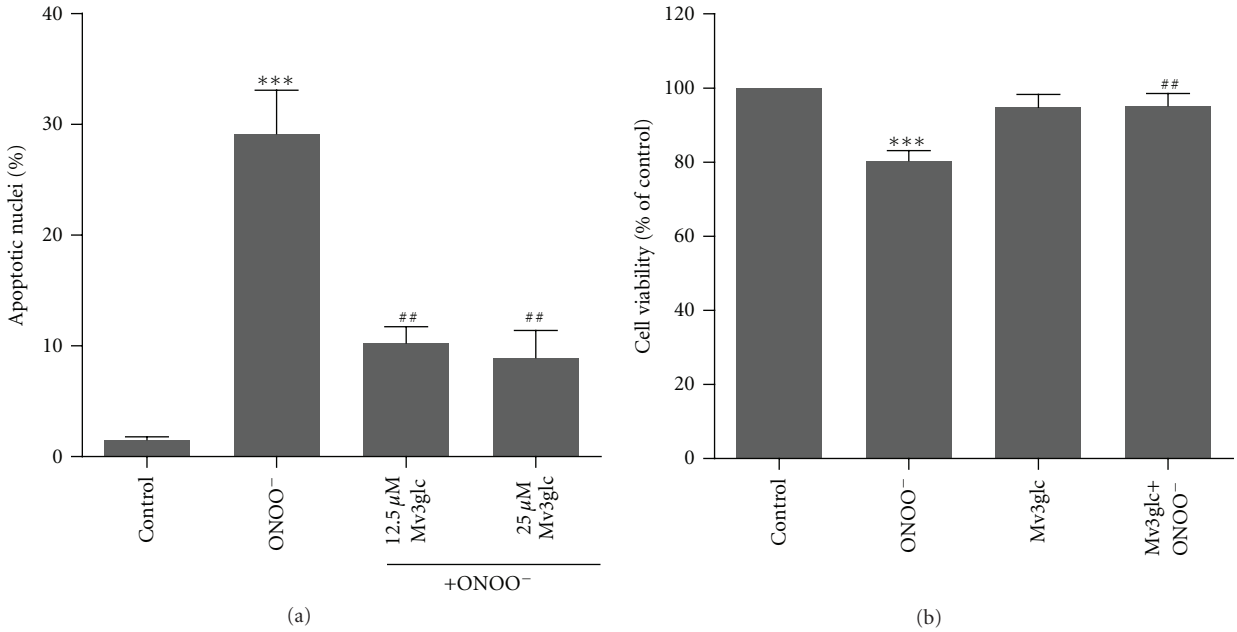

Figure 2: Mv3glc prevented peroxynitrite-mediated apoptotic and viability changes in endothelial cells. (a) Confluent BAECs were preincubated with either 12.5 or 25 μM Mv3glc for 14 h. Subsequently, cells were washed with PBS and treated with peroxynitrite, as described in Section 2. Morphological apoptotic changes were assessed after nuclei staining with Hoechst 33258. (b) Cellular viability of BAEC incubated for 14 h with 25 μM Mv3glc without further treatment or treated with peroxynitrite, as assessed by MTT reduction, and expressed as percentage of control cells. Control refers to the experiment in similar conditions without peroxynitrite and anthocyanin; $ONOO^-$ refers to the experiment with 500 μM peroxynitrite without anthocyanin. Values are mean ± SEM of at least five different experiments, each one assayed in duplicate. $^{***}P < 0.001$ versus control; $^{\#\#}P < 0.01$ versus $ONOO^-$.

afterwards, and after the incubation period, intact Mv3glc was detected in endothelial cells (3 nmoles/mg protein), as evaluated by HPLC (results not shown).

3.2. Peroxynitrite Scavenging Activity of Malvidin-3-Glucoside. It is known that the methoxy and hydroxyl substituents in the B-ring of malvidin-3-glucoside make this anthocyanin highly reactive toward radical species, although its role as physiological antioxidant has been questionable due to a probable instability under neutral conditions and to unknown reachable cellular concentrations [22]. The capacity of this compound to scavenge peroxynitrite, as compared with a reference antioxidant, quercetin, was previously assessed in a cell-free model system, in terms of inhibition of dihydrorhodamine oxidation promoted by this reactive species.

As shown in Figure 3, Mv3glc at very low concentrations (2–10 μM) strongly decreases the percentage of peroxynitrite-mediated DHR123 oxidation, revealing a high capacity of this flavonoid to scavenge peroxynitrite in a concentration-dependent way. It presented a relative low IC_{50} value (2.9 μM), as compared to quercetin (IC_{50} value of 1.12 μM), a well-known reference compound with a high peroxynitrite scavenging activity. The use of bicarbonate in the assay buffer did not alter significantly the IC_{50} values (results not shown).

3.3. Inhibition of Intracellular Formation of Reactive Species and of Carbonyl Groups during Peroxynitrite Aggression. The

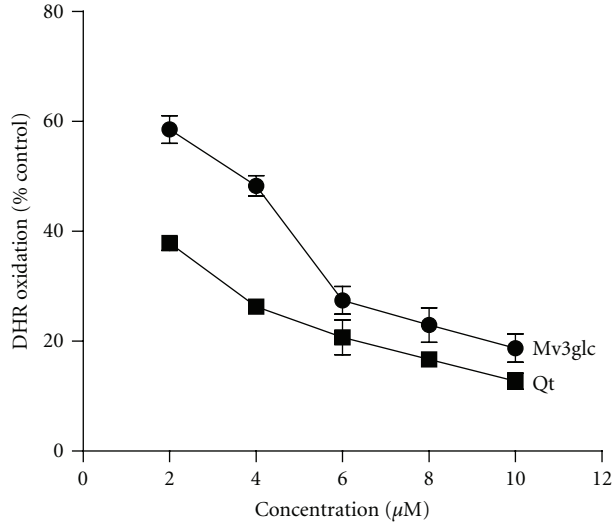

Figure 3: Mv3glc, at low concentrations, efficiently inhibit peroxynitrite-induced dihydrorhodamine 123 oxidation. Briefly, 25 μM DHR 123 was incubated with either Mv3glc or quercetin (Qt), used as a reference compound, at 37°C, and after addition of 1.2 μM peroxynitrite the fluorescence was immediately assessed, as described in Section 2. The measured fluorescence intensities were expressed in percentage relative to control (DHR123 plus $ONOO^-$ without Mv3glc or quercetin). Values are results of five independent experiments. $P < 0.001$ for all concentrations of Mv3glc or of quercetin versus control.

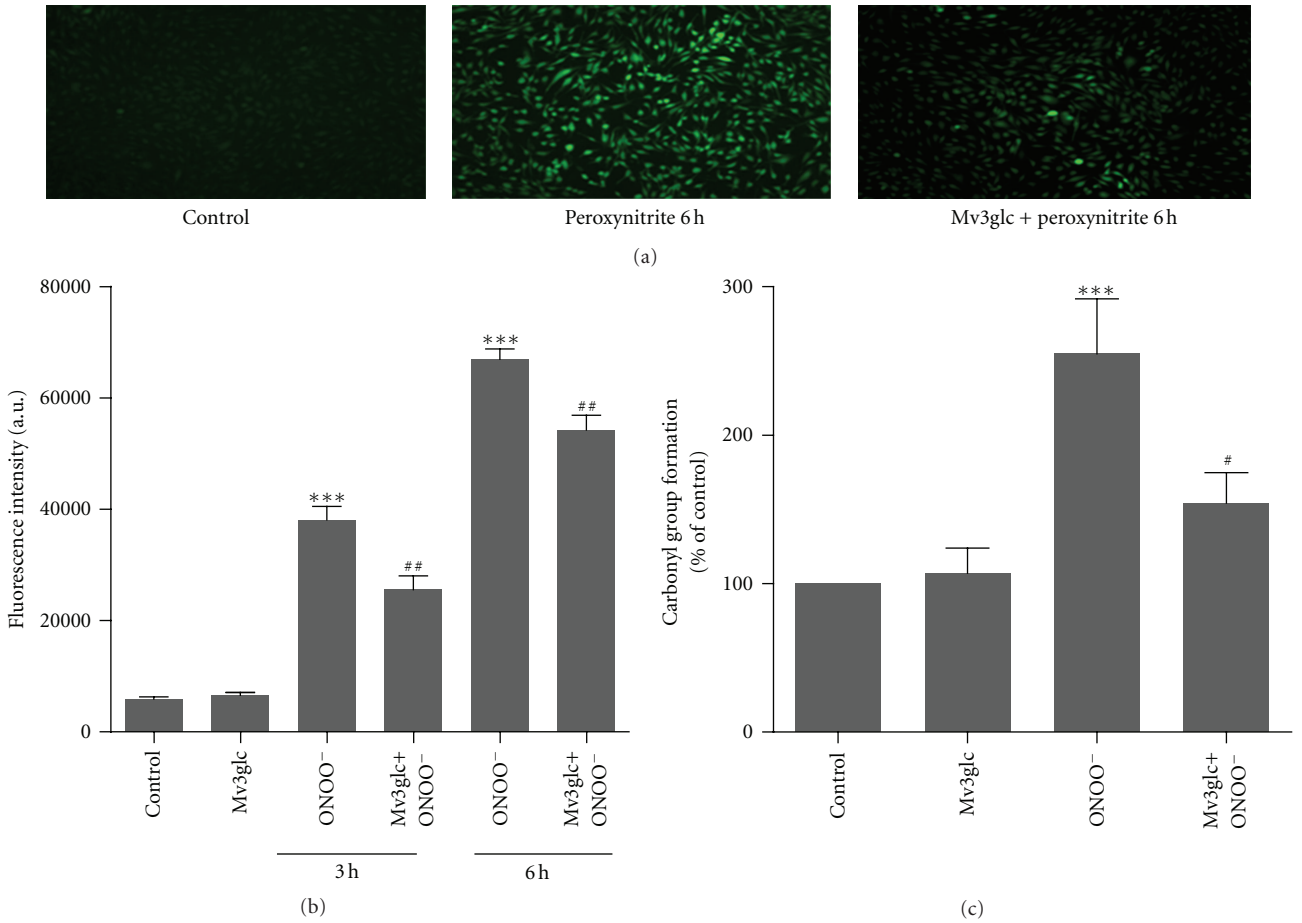

Figure 4: Mv3glc decreases peroxynitrite-mediated reactive species production (a, b) and carbonyl groups formation (c) in endothelial cells. BAECs were preincubated with 25 μM Mv3glc for 14 h and then submitted to peroxynitrite aggression, which was carefully removed after 10 min of incubation and maintained during the indicated times, in a serum-free medium, at 37°C, as described in Section 2. (a) Representative images of endothelial cells, at 6 h of incubation after $ONOO^-$ treatment, in the absence or presence of 25 μM Mv3glc, obtained by fluorescence microscopy (400 x). (b) Reactive species production was measured, at 3 h and 6 h of incubation, in terms of fluorescence intensity of DCF. (c) Carbonyl groups formation was assessed in total protein cell extracts obtained, as indicated in Section 2. Results are expressed as % of control, that is, cells incubated in similar conditions without either peroxynitrite or Mv3glc. Values are mean ± SEM of five experiments. $^{***}P < 0.001$ versus Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus $ONOO^-$.

effect of Mv3glc on peroxynitrite-induced oxidative stress on endothelial cells was also assessed by the detection of the dichlorofluorescein (DCF) form by fluorescence microscopy. This assay has been commonly used to assess cellular production of reactive oxidant species as a result of the oxidation of the nonfluorescent polar molecule dichlorodihydrofluorescein diacetate (DCFH-DA). This compound is passively loaded into whole cells, and once inside them, it is cleaved by esterases to yield DCFH, which may be oxidized to DCF by a variety of reactive species [35]; thus, an increase in DCF fluorescence is an index of cellular reactive oxidant species formation, as shown typically in Figure 4(a) at 6 h of incubation after peroxynitrite treatment. As expected, such aggressive treatment induced an increase in intracellular oxidative stress, as indicated by the increase in cell mean fluorescence, as early as 3 h of incubation, which continues to increase up to 6 h, according to the kinetic analysis previously reported [14]. This fluorescence was significantly reduced in cells preincubated with 25 μM Mv3glc before peroxynitrite addition, as shown typically in Figure 4(a) and presented in Figure 4(b) at 3 and 6 h of incubation after peroxynitrite treatment; at these times, the decreases in fluorescence were about 37 and 24%, respectively, relative to the assay with peroxynitrite stimulus without the anthocyanin.

On the other hand, the protein carbonyl formation in endothelial cells treated with peroxynitrite was also evaluated, as a marker for protein oxidation and recognized to be useful to estimate the extent of peroxynitrite oxidative damage to proteins [36]. In peroxynitrite-mediated endothelial cells injury, an increase in about 150% of carbonyl groups formation was observed, as evaluated by the general assay with DNPH [37] (Figure 4(c)). When preincubated with

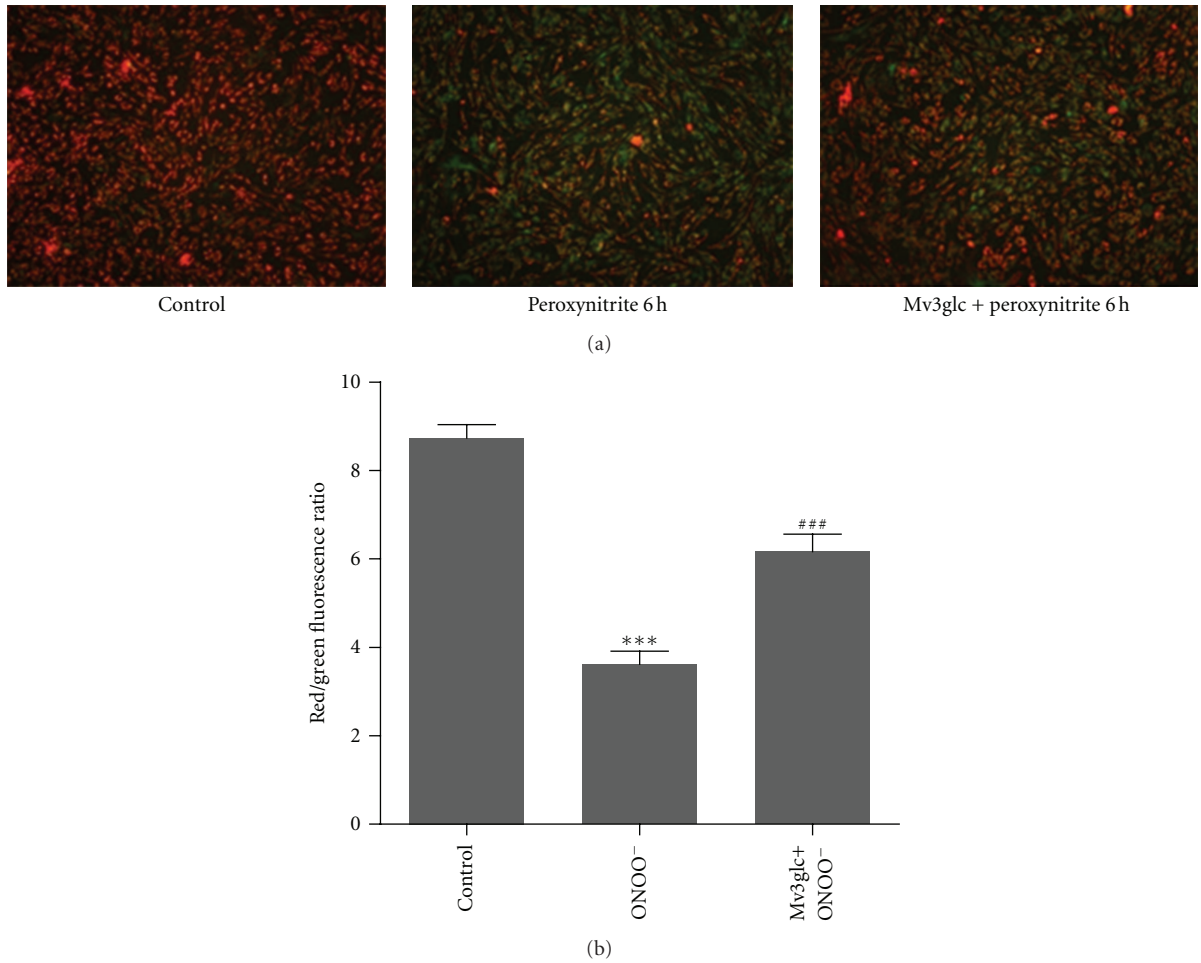

Figure 5: Mv3glc prevents peroxynitrite-induced depolarization of mitochondria. Endothelial cells, preincubated or not with 25 μM Mv3glc, were loaded with JC-1 fluorophore 20 min prior to the end of the 6 h of incubation with peroxynitrite and incubated for 20 min at 37°C, in the dark, as described in Section 2. Further, cells were visualized in a fluorescence microscope (a) and the fluorescence intensities of either the monomers (green) or J-aggregates (red) were assessed in a microplate reader (b). In (a), representative images of endothelial cells labeled with JC-1, treated with peroxynitrite in the absence or presence of Mv3glc, obtained by fluorescence microscopy (400x). In (b), quantification of mitochondrial membrane potential in terms of red/green fluorescence ratio, as measured in the same cell cultures. A decrease in red/green fluorescence intensity ratio indicates mitochondria depolarization. Values are mean ± SEM of five experiments, each one assayed in duplicate. $^{***}P < 0.001$ versus Control; $^{###}P < 0.001$ versus $ONOO^-$.

Mv3glc, a reduction in the carbonyl groups formation by about 70% occurred, pointing to a significant decrease in oxidative damage to cell proteins.

3.4. Inhibition of Peroxynitrite-Induced Depolarization of Mitochondria. It is well known that the loss of the mitochondrial membrane potential is a hallmark of cellular apoptosis. In order to evaluate whether inhibition of this event accounts for the antiapoptotic effects of Mv3glc, changes in the mitochondrial membrane potential were assessed after loading the cells with the fluorescent probe JC-1 previously submitted to peroxynitrite challenge. In cells with high mitochondrial membrane potential, the monomeric JC-1 dye accumulates in mitochondria and aggregates, emitting red fluorescence, but in cells undergoing apoptosis, the mitochondria depolarization prevents the entrance of JC-1 within the mitochondria. Thus, in these cells, JC-1 remains in the cytoplasm in a green fluorescent monomeric form (Figure 5(a)). Results in this figure show that in endothelial cells submitted to peroxynitrite injury, a strong decrease occurred in the cell population emitting red fluorescence accompanied by an increase in the population with green fluorescence, reflected in a decrease in the red/green fluorescence ratio of about 60% relative to the control, without peroxynitrite (Figure 5(b)).

The preincubation of cells with 25 μM Mv3glc before peroxynitrite aggression led to an increase in this ratio by 30% of the control with peroxynitrite, indicating that this anthocyanin prevented significantly the loss of peroxynitrite-mediated mitochondrial membrane potential.

3.5. Inhibition of Peroxynitrite-Induced Activation of Caspases-9 and -3. The mitochondrial depolarization is commonly related with the sequential activation of caspases-9 and -3

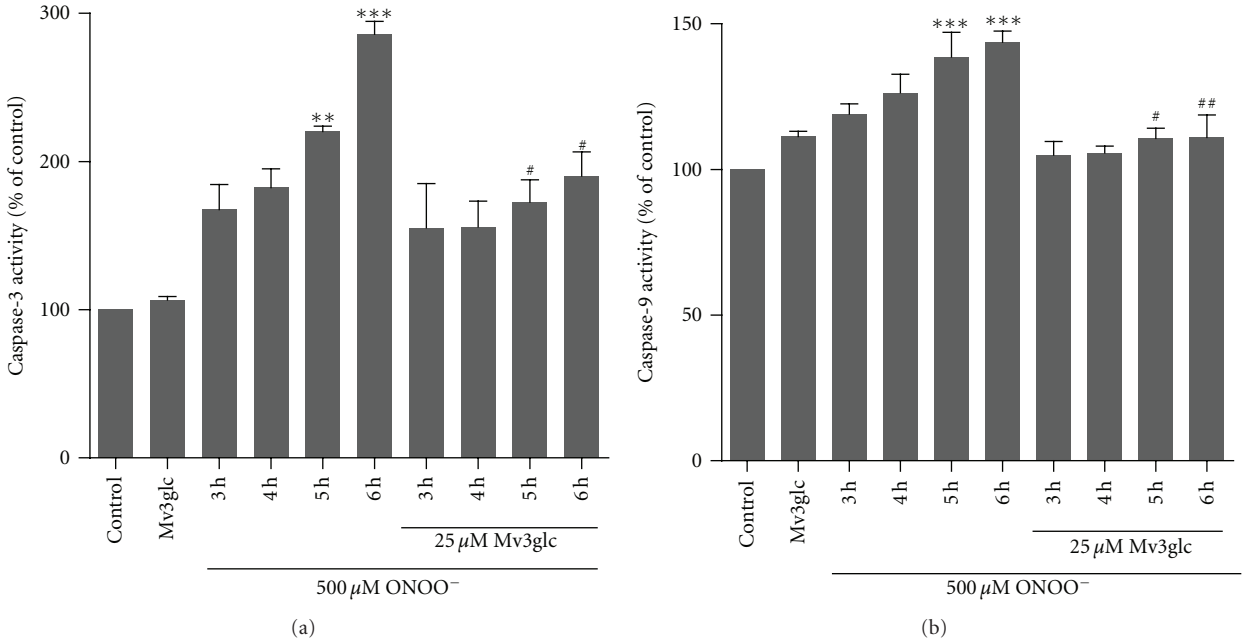

Figure 6: The activation of caspases-3 and -9 in endothelial cells submitted to peroxynitrite injury is significantly inhibited by the preincubation of cells with 25 μM Mv3glc, for 14 h. BAECs were treated with 500 μM peroxynitrite after this preincubation step, as described in Section 2, and graphs show the kinetic activities of cellular caspase-3 (a) and caspase-9 (b) induced by peroxynitrite treatment ($ONOO^-$) in the absence and presence of anthocyanin. Values are mean ± SEM of five different experiments, each one assayed in duplicate. For caspase-3, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Control; $^{\#}P < 0.05$ versus $ONOO^-$. For caspase-9: $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus $ONOO^-$.

[38], and previous data from our laboratory have already reported that peroxynitrite-induced endothelial cells apoptosis is mediated by activation of both caspases [12]. Figure 6 shows that preincubation of cells with 25 μM Mv3glc prevented almost completely the caspases-9 activity and led to a decrease in caspase-3 activity by about 60% at 6 h of incubation after peroxynitrite aggression.

Therefore, these results are in accordance with others previously reported indicating that in our experimental conditions, peroxynitrite triggers apoptosis through the mitochondrial or intrinsic pathway, as caspases-3 and -9 are the effector and the initiator caspases, respectively, of this cell death cascade and indicate that Mv3glc was able to disrupt this pathway, inhibiting cell apoptosis.

3.6. Decrease in Peroxynitrite-Induced Bax Levels. The Bcl-2 family of proteins has been established to play a crucial role in mitochondrial-mediated apoptosis [39, 40]. Data from our laboratory have already shown that peroxynitrite *per se* disrupts the intracellular balance between proapoptotic and antiapoptotic proteins by increasing Bax intracellular levels without affecting those of Bcl-2. We confirmed that peroxynitrite did not modify significantly the level of Bcl-2, even in the presence of Mv3glc (results not shown).

On the contrary, Bax expression level suffered a strong increase during the time course after peroxynitrite treatment, reaching about 2-fold the control at the 4th hour of incubation (Figure 7). Such increase was significantly reduced by about 70% in cells preincubated with Mv3glc, at the end of the third and fourth hours, after peroxynitrite injury.

4. Discussion

During the last years, there has been an increasing interest in dietary anthocyanins mainly due to the increasing knowledge of their broad pharmacological activities, in particular, in the context of prevention of atherosclerosis [41], and among these compounds, Mv3glc is one of the major constituents. Despite that the exact mechanisms, by which anthocyanins exert health beneficial effects, are still not well understood, it has been shown that they inhibit different proatherogenic pathways, namely, increasing resistance of LDL to peroxidation, modulating platelet aggregation, and promoting nitric oxide-mediated vasorelaxation [16, 42]. Concerned with the atherogenic process, peroxynitrite is a potent physiological oxidant and a crucial intervenient in its development, as above referred. The ability of anthocyanins to scavenge peroxynitrite *in vitro* is well known, mainly due to their electrodonating properties and as alternative substrates for nitration [43]. However, although research over the last decades has been focused on the correlation between biological activities and antioxidant properties, their role as critical cellular mediators with interference in signaling pathways and gene regulation is an important research field [44, 45]. Thus, following our previous work [14], here

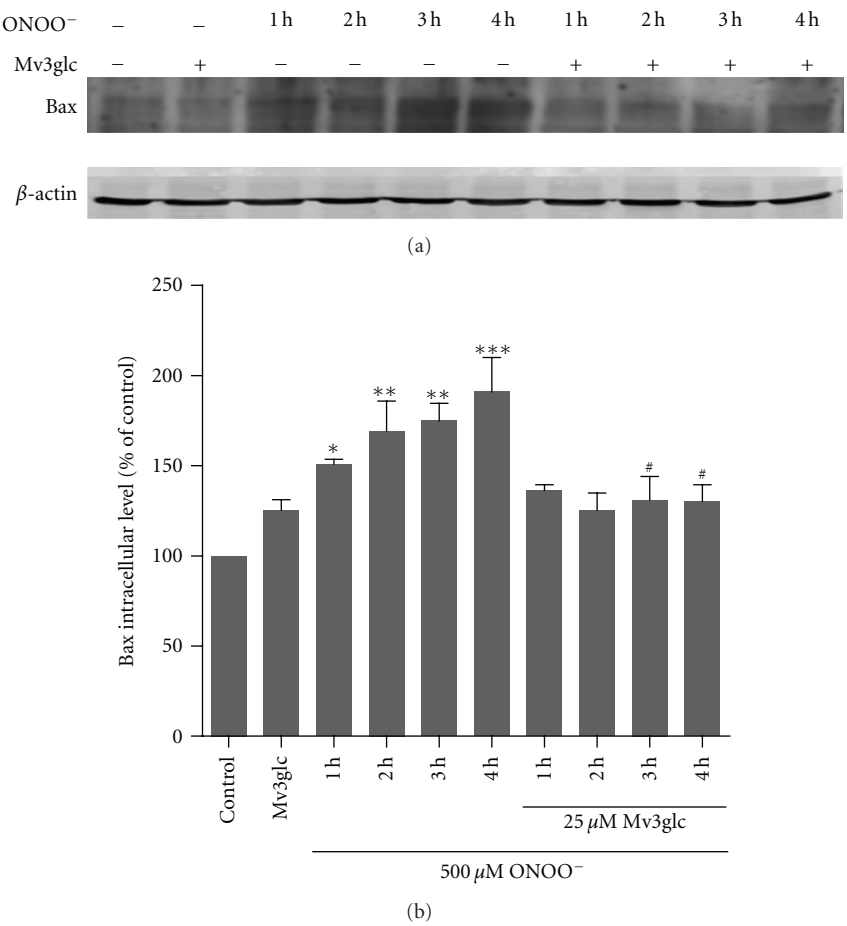

Figure 7: Mv3glc reduced the increase in Bax cytoplasmatic levels induced by peroxynitrite. BAEC were preincubated with 25 μM Mv3glc with no further treatment or post-treated with peroxynitrite for 10 min, as described in Section 2 and maintained during 6 h in a serum-free medium, at 37°C. Cytoplasmatic total protein extracts were then analyzed by immunoblot with specific antibody against Bax. Representative blots and densitometric quantifications of three independent experiments are shown. Results were normalized to endogenous β-actin and expressed as mean ± SEM of percentage of control. Values are mean ± SEM of four experiments, each one assayed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Control; $^{\#}P < 0.05$ versus $ONOO^{-}$.

we explored the cytoprotective mechanisms for Mv3glc in endothelial cells under peroxynitrite aggression.

Specifically, we demonstrated that Mv3glc, when previously incubated with endothelial cells, was able to protect them from peroxynitrite-mediated apoptotic cell death (Figure 2(a)) revealing that the potential steric hindrance due to its hydroxylation pattern and methoxy groups is not an impediment for its cellular uptake, allowing degrees of protection similar to other studied anthocyanins [14]. In fact, Mv3glc incorporates into endothelial cells under our experimental conditions, as verified by HPLC analysis (results not shown), in agreement with results previously reported [20].

In endothelial cells, Mv3glc counteracted the intracellular formation of reactive species after peroxynitrite-mediated insult, as evaluated by the DCF assay (Figure 4), in accordance with its high ability to scavenge peroxynitrite, as observed in the dihydrorhodamine oxidation assay (Figure 3). The observed reactive species were formed as a consequence of the primary insult to the cells, considering that the added peroxynitrite was removed after the incubation period, by washing cells carefully. Moreover, the ability of Mv3glc to neutralize the oxidative injury to cells triggered by such species is evidenced by a reduction in peroxynitrite-mediated carbonyl groups formation, the most commonly measured products of protein oxidation in biological samples [46], by about 70% relative to the assay without the anthocyanin (Figure 4). Proteins are major targets for damage due to their abundance and rapid rates of reaction with a wide range of reactive species, which are well recognized as relevant intervenients in atherogenic process, and the protein carbonyls have been reported as useful markers of protein oxidative events either *in vitro* or *in vivo* assays.

Therefore, the protection afforded by Mv3glc against peroxynitrite-mediated injury in endothelial cells may be partially mediated by its ability to scavenge reactive species formed as a consequence of cellular oxidative aggression.

Such cellular reactive species are thought to be responsible for triggering several deleterious events including mitochondrial-associated apoptosis. In accordance with previous studies from our laboratory and others, peroxynitrite, at lower concentrations, induces apoptosis by triggering the mitochondrial pathway, while at higher concentrations it leads mainly to cellular necrosis [12]. Although the used concentration of peroxynitrite (500 μM) is apparently high, the net exposure of cells to this species is much lower considering its short half life (less than 1 s, 37°C, pH 7.4), reaching levels more closed to those which have been estimated to be produced *in vivo* [47].

Actually, in our experimental conditions, peroxynitrite insult into endothelial cells induced apoptotic cell death, and Mv3glc, in a similar way to other anthocyanins with a different B-ring structure, was able to counteract this deleterious effect through the inhibition of crucial mitochondrial signaling cascades. In fact, the loss of mitochondrial potential due to peroxynitrite, as indicated by the green fluorescence resulting from the monomeric form of JC-1 outside the mitochondria, was prevented efficiently by cellular pretreatment with Mv3glc, as evidenced by the increase in JC-1 aggregated red fluorescence inside the mitochondria (Figure 5). This result is relevant, given the importance of mitochondria in regulating cell death pathways. Moreover, Mv3glc proved its effectiveness in preventing the activation of both caspases-3 and -9 (Figure 6), an event involved in mitochondrial-mediated apoptotic death pathway. Thus, Mv3glc ability to interfere with the apoptotic intracellular signaling cascade may be closely related to cell protection from mitochondrial disruption.

Recently, we have shown that peroxynitrite induces mitochondrial membrane permeabilization by interfering in the balance between pro- and antiapoptotic levels of the Bcl-2 family members, by increasing Bax levels without changes in Bcl-2, leading to a raise in Bax/Bcl-2 ratio [12, 14]. Bax is a proapoptotic protein that forms cytoplasmatic heterodimers with antiapoptotic Bcl-2 family members but when cells are submitted to an apoptotic insult it may suffer mitochondrial translocation, promoting outer membrane permeabilization and the subsequent mitochondrial-mediated apoptotic pathway. Similarly to other anthocyanins previously studied, namely, cyanidin-, delphinidin- and pelargonidin-3-glucoside [14], Mv3glc was capable of decreasing Bax intracellular levels induced by peroxynitrite stimulus (Figure 7), indicating that its protective effect may precede mitochondria depolarization and caspases activation. Besides, as Mv3glc counteracted the raise in Bax levels without affecting Bcl-2 levels, a decrease in the Bax/Bcl-2 ratio occurred, restoring the balance between pro- and antiapoptotic proteins.

In conclusion, our results support the potential benefits of Mv3glc, one of the most prevalent anthocyanins in diet, as a vascular protectant. Clearly, it protects endothelial cells against peroxynitrite-induced damage by counteracting reactive species formation and interfering in the regulation of apoptotic intracellular signaling mechanisms mediated by mitochondria. The potential steric hindrance due to its B-ring substitution pattern is not an impediment to its cellular action, allowing a protection profile similar to that reported for structurally different anthocyanins. Indeed, although the o-dihydroxy (catechol) structure of the B-ring, associated to a hydroxyl group in the 3 position, seems to confer to anthocyanins significant higher general antioxidant and peroxynitrite-scavenging capacities [14], the protection afforded by all the studied compounds, including the monophenolic pelargonidin and Mv3glc, against peroxynitrite-induced mitochondrial apoptotic effects in endothelial cells is not significantly different. All the tested anthocyanins showed ability to counteract such effects in a similar way, through the inhibition of crucial signaling cascades, suggesting that their cellular actions are much beyond their antioxidant activities. Despite the need for further *in vivo* studies to confirm these findings, our data together with other previously reported by us support mechanistically the health benefits of this class of dietary nutrients. Moreover, it reinforces the view that anthocyanins, including Mv3glc, can be promising molecules for development of functional foods and nutraceuticals to improve endothelial function, in the context of prevention of atherosclerosis.

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