**Malvidin 3-glucoside, a dietary anthocyanin, contributes to the balance between pro- and anti-inflammatory mediators in human endothelial cells: a potential role in the atherogenic process**

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**Key words** – Inflammation; Endothelial dysfunction; Malvidin-3-glucoside; Anthocyanins; Peroxynitrite; Nrf2; NF-κB.

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## *Abstract*

Malvidin 3-glucoside (Mv3glc) is one of the main natural anthocyanins, widely distributed in berries, grapes and vegetables. These bioflavonoids have shown protective effects against a myriad of human diseases including cardioprotective activity. Beyond their recognized radical scavenging activity, recent attention has been focused on anthocyanins as vascular protectants, in particular, as modulators of vascular signalling pathways.

The endothelium seems to have a central role in atherogenesis, which is now viewed rather as a chronic inflammatory process associated with an overproduction of oxidant species and a disruption in the balance between pro- and anti-inflammatory mediators, both emerging as crucial events in this disease progression.

Following our previous research about the cytoprotective role of Mv3glc and other anthocyanins in several aspects of endothelial dysfunction, in this study we ought to continue the study of the potential effects of Mv3glc in signaling pathways known to be involved in the atherogenic process. Specifically, we assessed, by immunoblotting, Mv3glc ability to inhibit the activation of NF-κB induced by peroxynitrite, through the suppression of important mediators of inflammation, such as COX-2 and iNOS, known to be dependent of that transcription factor activation. We have also investigated the role of Mv3glc in proteasome activity induced by peroxynitrite. On the other hand, we analysed the effect of this anthocyanin on the production of the protective enzyme heme oxygenase-1 (HO-1), resulting from the activation of the transcription factor Nrf2, triggered by peroxynitrite.

The set of results presented here were not only able to clarify the mechanisms that underpin Mv3glc non-toxic proteasome inhibition, but also to confirm the inhibition of NF-κB activation and the exacerbation of nuclear Nrf2 levels with the resulting increase in HO-1 expression, which was associated with a notorious increase in cell viability, as compared to cells submitted to peroxynitrite and untreated with Mv3glc.

Concluding, Mv3glc benefits in endothelial cells against oxidative aggression may be potentially assigned to the concerted action on the modulation of proteasome's activity associated to the effect on the balance between NF-κB/Nrf2 transcription factors. Since the pathogenesis of atherosclerosis involves multiple events, including endothelial dysfunction, increased oxidative stress and inflammation, Mv3glc antiatherogenic potential at all of these levels and cellular pathways, is undoubtedly worthy of further investigation, aiming to the development of dietary or therapeutic strategies capable of preventing or delaying the progression of the atherosclerotic process.

## *1. Introduction*

In the last decade, we have witnessed a remarkable increase in scientific knowledge about the beneficial role of anthocyanins, a group of pigments belonging to flavonoids, which are widely distributed in the human diet and with several described effects against various diseases related to oxidative stress, including atherosclerosis (Wang and Stoner 2008; Wallace 2011). Atherosclerosis, the common denominator of cardiovascular disease, is a multifactorial disorder in which endothelial dysfunction and inflammation play a critical role (Lusis 2000; Tousoulis *et al.* 2011).

Peroxynitrite has been recognized as an important player in atherogenesis, since it is a strong oxidant and nitrating species of several endogenous biomolecules. It results from the diffusion-limited reaction between nitric oxide ($^{\bullet}NO$) and superoxide anion ($O_2^{\bullet -}$), both produced by vascular cells and immune system (Darley-Usmar *et al.* 1992; Ischiropoulos *et al*. 1992; Cooke 2003). This reaction has one of the highest rate constants described for reactions with $^{\bullet}NO$, justifying the toxicity of peroxynitrite under certain conditions (Szabo 2003). This reactive species can reach high concentrations in vascular endothelium submitted to shear stress, particularly, in the atherosclerotic vessels, where peroxynitrite seems to promote the oxidation of LDL and the extensive nitration of tyrosine residues (Denis *et al.* 2002; Peluffo and Radi 2007), contributing to the production of fatty streaks and subsequent formation of atherosclerotic plaques. However, peroxynitrite may be involved in atherogenesis by other pathways, namely causing the disruption of vascular reactivity and critical cellular processes that lead to apoptosis or necrosis, in this way exacerbating inflammatory vascular status (Pacher *et al.* 2007; Paixão *et al.* 2011).

Several investigators have shown that most of the genes overexpressed in inflammatory diseases, namely, those encoding pro-inflammatory cytokines,

chemokines, adhesion molecules and inflammatory enzymes, are predominantly controlled by the nuclear factor kappa B (NF-κB) (Blackwell *et al.* 2000;. Sharma *et al.* 2006). In fact, in endothelial cells, NF-κB is involved in transcriptional and post-transcriptional regulation of several inflammatory mediators important in the development of atherosclerotic process, including iNOS and COX-2 among many others (Denk *et al*. 2001; Cooke and Davidge 2002). The NF-κB inhibitor, IκBα, prevents the activation of this transcription factor, while proteasome allows its activation by IκBα degradation (Rajkumar *et al.* 2005; Milano *et al.* 2007). Proteasome is a multicatalytic complex responsible for the elimination of altered or expendable cellular proteins after their ubiquitination and whose activity is highly affected by oxidants, including peroxynitrite (Grune *et al.* 1998; Cascio *et al.* 2001; Grune *et al.* 2001).

There are several evidences suggesting that the ubiquitin-proteasome system is not only capable of modulating the activity of nitric oxide synthases, the key enzymes in the maintenance of vascular homeostasis, but also to interact with vasoactive mediators involved in the regulation of endothelial function and in the vasculature stress response, thereby contributing to regulate endothelial function (Stangl and Stangl 2010). Furthermore, it is also assigned to this pathway the responsibility for the "quality control" of proteins, cell cycle control, regulation of transcription factors, gene expression, cell differentiation and immune response (Jentsch and Jesenberger 2002; Goldberg 2003; Tansey Muratani 2003). However, their increasing relevance in the context of atherosclerosis raises the question whether the ubiquitin-proteasome pathway could be involved in the initial phase of vascular damage, that is, in the process of endothelial dysfunction (Marfella *et al*. 2007). The increase in the expression of endogenous antioxidants, such as HO-1, glutathione S- transferases and glutathione, by

inhibition of the proteasome and the subsequent activation of the nuclear erythroid 2 p45-related factor (Nrf2$_)$ has been demonstrated in different types of cells (Yamamoto *et al.* 2007; Sahni *et al*. 2008; Dreger *et al.* 2009).

Endogenous defense mechanisms against oxidative stress, either resulting from reactive species overproduction or antioxidants depletion, consist mainly on the increase of cellular enzymes defenses, such as glutathione (GSH) and phase II detoxification enzymes, whose encoding are controlled by Nrf2. In response to oxidative insult, Nrf2 is translocated into the nucleus from the cytosol, where it binds sequentially to antioxidant response elements located in the promoter region of many antioxidant and phase II genes, such as γ-glutamylcysteine synthetase (GCS γ), NAD(P)H:quinone oxidoreductase 1 (NQO1) and HO-1 (Prestera, *et al*. 1995, Lee and Surh 2005), resulting in a cytoprotective response characterized by the increased production of the above cited proteins and the consequent decreased susceptibility to oxidative damage (Jaiswal 2004).

Heme oxygenase-1 is an important antioxidant enzyme that plays a key role in cellular protection. It is the limiting enzyme of heme degradation, generating equimolar amounts of carbon monoxide (CO), biliverdin and free iron. Accumulating evidences suggest that the inducible HO-1 and its reaction products function as adaptive molecules to oxidative insult (Morita 2005). Although the mechanisms underlying the anti-inflammatory activity of HO-1 are not known in detail, cytoprotection appears to be due to its ability to degrade the pro-oxidant free heme, yielding biliverdin, which is subsequently converted to bilirubin, both with antioxidant properties (Paine *et al.* 2010). However, it is the simultaneous production of CO that has received particular attention, since this molecule, in particular circumstances, exhibits anti-apoptotic and anti-inflammatory properties, acting as an important vasodilator when NO bioavailability is

limited (Allen *et al.* 2012). Accordingly, any agent capable of modulating the genes above mentioned will be beneficial in the pathophysiology of atherosclerosis, a condition in which oxidative and inflammatory damage, direct or indirect, resulting from the action of peroxynitrite, may be responsible for the origin and disease progression. Thus, preventing the deleterious effects of peroxynitrite is crucial for endothelial integrity and anthocyanins have shown, in several studies, their high scavenger capacity of this reactive species (Serraino *et al.* 2003; Rahman *et al*. 2006; Paixão *et al.* 2011, 2012a).

Epidemiological investigations revealed that diets rich in fruits and vegetables are associated with a decrease in cardiovascular risk, being anthocyanins major contributors to this reality with an intake estimated to be up to nine times higher than that of other dietary flavonoids (Chong *et al*. 2010; Mulvihill and Huff 2010). Among consumed anthocyanins, malvidin 3-glucoside (Mv3glc) (Fig.1) is one of the most prevalent, particularly in red wine (Mazza 1995) and red fruits such as raspberries, strawberries and blackcurrants (Zamora-Ros *et al.* 2011). Despite all the controversy about the physiological availability of these polyphenols, there are numerous studies describing their intact absorption in a glycosylated form, appearing rapidly in the bloodstream and tissues after ingestion (Youdim *et al*. 2000; McGhie and Walton 2007). In addition, the potential *in vivo* cumulative effects must also be taken into account. Thus, anthocyanins have been considered as promising health-promoting bioactive molecules to incorporate new functional foods and nutraceuticals, particularly, in the context of atherosclerosis prevention (He and Giusti 2010).

Although on early studies the biological activity of anthocyanins had been primarily attributed to their antioxidant properties due mainly to the existence of hydroxyl groups on the B ring and to the conjugated double bonds system (Wu and

Prior 2006), their anti-inflammatory and anti-atherogenic effects in addition to many others, could not be solely explained based on such properties. In this context, there are numerous works that have revealed additional mechanisms of action including the interference with crucial signaling cascades and gene regulation (Nothlings *et al.* 2008; Pascual-Teresa *et al*.2010).

Recently, we examined the cytoprotective mechanisms of Mv3glc, highlighting new insights about the potential role of its 3',5-'metoxyl groups in the B-ring on the modulation of mitochondrial apoptotic pathway (Paixão *et al.* 2012a). Following this study we ought to study the effects of Mv3glc on other signaling pathways known to be involved in the atherogenic process (Paixão *et al.* 2012b).

The main goal of the present work was to confirm in human endothelial cells some of the above mentioned results previously obtained in bovine cells and continue to explore other molecular mechanisms underlying the role of Mv3glc as an endothelial protective factor. Specifically, we assessed Mv3glc ability to inhibit the peroxynitrite-mediated NF-κB activation through the suppression of important mediators of inflammation, such as COX-2 and iNOS, known to be dependent on that transcription factor activation. We have also investigated the role of Mv3glc in proteasome activation induced by peroxynitrite. On the other hand, we analysed the anthocyanin effect on the production of the protective enzyme HO-1, resulting from the activation of the transcription factor Nrf2.

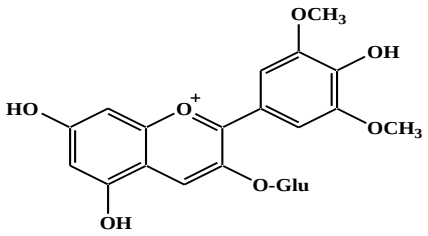


**Fig.1** Chemical structure of Mv3glc.

## 2. *Materials and Methods*

### *2.1.Reagents*

Malvidin 3-*o*-β-glucoside chloride, purified from natural sources was obtained from Extrasynthése (Genay, France). Mv3glc purity was above 97%, as measured by HPLC, and was used from stock solutions (5 mM) in dimethylsulfoxide (DMSO) stored in the dark, under nitrogen atmosphere, at -80$^{o}$C; the final solvent concentration did not exceed 1% by volume. For cell culture, MCDB131 medium, trypsin 0.25%, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) pH 7.4, were obtained from Gibco-Invitrogen.

Laboratory chemicals, namely, epidermal growth factor (EGF), hydrocortisone, protease inhibitor cocktail, phosphatase inhibitors, DMSO, and 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma-Aldrich Co as well as other chemical reagents used.

Primary specific mouse monoclonal antibody to heme-oxygenase-1 and iNOS were obtained from Abcam (Cambridge, UK); primary specific mouse monoclonal antibody to lamin A was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal antibody to COX-2 was obtained from Millipore and rabbit polyclonal antibody to IκBα was from Cell Signalling Technology, Inc; mouse monoclonal antibody to β-actin as well as anti-mouse and anti-rabit IgG secondary antibodies were purchased from Sigma-Aldrich Co.

### *2.2. Peroxynitrite synthesis*

Peroxynitrite was synthesized using a quenched flow reactor, as previously described (Brito *et al.* 2006). The obtained peroxynitrite was then stored at -80ºC under $N_2$ atmosphere. Immediately before use, it was always quantified from its absorbance at 302 nm in 1 M NaOH ($\varepsilon$= 1670 $M^{-1}$ $cm^{-1}$).

### *2.3. Cell culture and treatment with peroxynitrite*

Human microvascular endothelial cells (HMEC) (ATCC, USA) were cultured in T-75 culture flasks in MCDB 131 supplemented with 10% fetal bovine serum, 10 ng/ml EGF and 1 µg/ml hydrocortisone at 37ºC in a humidified atmosphere of 5% $CO_2$. For the majority of experiments, cells were subcultured at about 80% of confluence in 60 mm culture plates (1-2x$10^6$cells/plate) and starved in serum-free medium for at least 6 h.

In all the experiments, the peroxynitrite used was authentic, i.e, synthesized by us and not from a donor compound. In experiments with Mv3glc, cells were pre-incubated with 25 µM of this compound for 14 h. At the end of this time, the incubation medium was removed and cells were submitted to $ONOO^-$ aggression, as previously described (Paixão *et al.* 2011). After 10 min of exposure to $ONOO^-$, cells were gently washed with PBS and then maintained in MCDB 131 medium without serum for the required times. Thus, in these assays, neither the anthocyanin nor any degradation product of Mv3glc or from its reaction with the culture medium was present in the medium during the experiments with $ONOO^-$. No pH shift was observed during this treatment. The same volumes of either 10 mM NaOH (vehicle control) or decomposed peroxynitrite (in 10 mM NaOH or PBS, overnight) were used as controls.

### *2.4. Cell viability and nuclear morphology*

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT) to formazan (Denizot and Lang 1986) and nuclear morphology was analyzed by fluorescence microscopy, with a cell-permeable DNA dye Hoechst 33258 (5µg/ml), as previously described (Paixão *et al.* 2011).

### *2.5. Western-blot analysis*

Protein expression was evaluated by Western-blot analysis. Cells were lysed 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 and phosphatase inhibitors, pH 7.5, for 10 min on ice. Lysates were centrifuged at 5000 rpm for 5 min at 4ºC and the supernatants (cytoplasmic extracts) were collected and stored at -20ºC. The pellet was dissolved in 40 µl of a solution containing 20 mM Hepes, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% glycerol and 1 % protease inhibitor cocktail, pH 7.5 and further centrifuged at 14 000 rpm for 20 min at 4ºC, to obtain nuclear extracts. To obtain total cellular extracts, cells were scrapped in a solution containing ice-cold lysis buffer [50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1 mM PMSF, 1% (v/v) protease inhibitor cocktail] for 20 min and then centrifuged at 16000 g for 20 min at 4ºC. Supernatants were then collected and stored at -20ºC. 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 proteins were separated by SDS/PAGE [10% (v/v)] and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, UK) by electroblotting. To avoid non-specific binding, 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 IκBα, HO-1, iNOS and COX-2 (overnight, 4ºC). After extensive washings with TBS-T, the blots were incubated with alkaline phosphatase-conjugated secondary antibodies (1 h, room temperature). Immunoreactive complexes were detected by chemifluorescence after blots exposition to enhanced chemifluorescent reagent (ECF) (Amersham Biosciences) in a Typhoon 9000 scanner

(Amersham Biosciences). β-Actin and lamin A were used as controls for protein loading in the total and nuclear extracts, respectively. Bands were analyzed using the ImageQuant TM software from Amersham Biosciences.

### *2.6. Proteasome activity*

Proteasome activity (26S complex) was determined, essentially, as previously described (Coux *et al.* 1996; Thomas *et al.* 2007). Briefley, cells were washed with buffer I (50 mM Tris, pH 7.4; 2 mM DTT; 5 mM MgCl2; 2 mM ATP) and further homogeneized with buffer I supplemented with 250 mM sucrose. After cellular extract centrifugation at 10000 g, 20 µg supernatant were diluted with buffer I until 900 µl of total volume. The proteasome fluorogenic substract, SucLLVY-AMC (*chymotrypsin-like*), was added at a final concentration of 80 µM. Proteolytic activity was measured trough the monitorization of the release of the fluorescent group 7-amide-4-metilcoumarin (excitation 380 nm; emission 460 nm).

### *2.7. Statistical analysis*

All data were expressed as means ± SEM of the number of experiments indicated in the figures, each one assayed at least in duplicate. Differences between groups were analysed by one-way analysis of variance (ANOVA), Dunnet, Bonferroni´s or Tukey´s tests as appropriate. A value of $P<0.05$ was accepted as statistically significant.

## *3. Results*

### *3.1. Mv3glc protects endothelial cells against peroxynitrite mediated apoptosis*

According to our previous studies in bovine endothelial cells (BAEC) (Brito *et al.* 2008; Paixão *et al.* 2011; 2012a,b), peroxynitrite induced apoptotic death in a human endothelial cell line (HMEC) used as a model, a process significantly reduced when these cells were pre-incubated with Mv3glc (Fig. 2A).

In fact, as shown in figure 2A, cell insult with peroxynitrite after 6 h of incubation, as described in "Materials and Methods", led to a percentage of apoptosis of about 40%, according to results obtained by fluorescence microscopy using a DNA probe, Hoechst 33258. Nuclear condensation and fragmentation were used as apoptosis indicators which were not significantly detected in control cells (just about 3%).

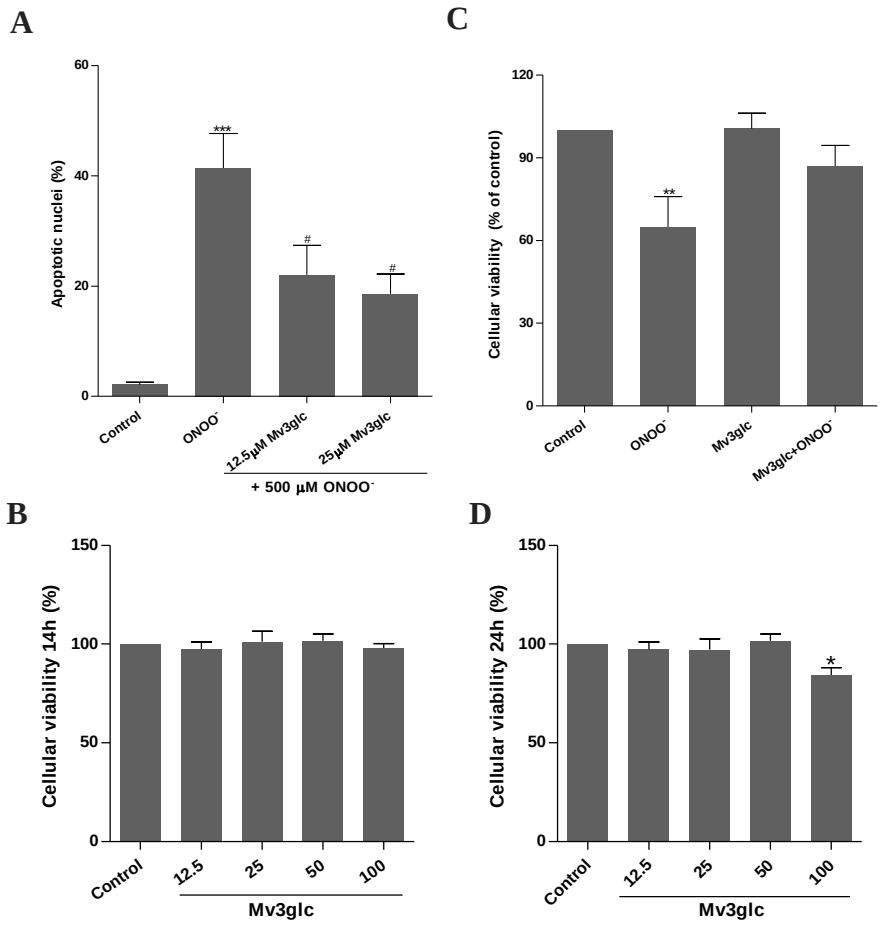


**Fig.2 Mv3glc effects on peroxynitrite mediated apoptosis (A) and on cellular viability (B, C, D)** (A) Mv3glc prevented apoptotic damage mediated by peroxynitrite on endothelial cells. Confluent HMEC maintained in medium without serum were pre-incubated with two subtoxic concentrations of Mv3glc (12.5 and 25 µM) for 14 hours. Then, cells were washed with PBS and treated with 500 µM peroxynitrite. Apoptotic morphological alterations were assessed by nuclear staining with Hoechst 33258 (5µg/ml) 6 h after the oxidant addition. Control refers to the experience in similar conditions without peroxynitrite and anthocyanin. (B) Cell viability of HMEC incubated with 25 µM Mv3glc with or without peroxynitrite treatment and incubated for 14 h ( C) and 24 h (D) with 4 different concentrations of Mv3glc, in the absence of peroxynitrite, was assessed by MTT reduction and expressed as percentage of control. The results represent the mean value ± SEM of at least 6 independent experiments, each performed in duplicate. $*P$ <0.05,$**P$ <0.01 , $***P$ <0.001 *vs.* control, $^{\#}P$ <0.05 $ONOO^-$.

A reduction of about 60% and 70% of apoptotic cells was observed in those pre-incubated with Mv3glc 12.5 µM and 25 µM respectively, before peroxynitrite addition, a result similar to that obtained for this anthocyanin in bovine endothelial cells model (Paixão *et al.* 2012a). At the highest concentration used (25 µM), Mv3glc alone did not interfere with the viability of endothelial cells even in the longest incubation periods (Fig. 2) and also prevented the decrease in cell viability in cells treated with peroxynitrite, according to MTT test results (Figure 2B). Of notice is that anthocyanin was not present in the culture medium during the insult with peroxynitrite or thereafter.

### *3.2. Mv3glc prevents peroxynitrite mediated NF-κB activation*

In order to evaluate if NF-κB pathway could be activated by peroxynitrite in HMEC, in our experimental conditions, cells were exposed to 500 µM of authentic peroxynitrite for 10 minutes.

It was observed that peroxynitrite induced a transient activation of NF-κB, shown by the decrease of about 70% in IκBα values relative to control, 1h after peroxynitrite aggression (Fig. 3 A and B). However, in cells pre-incubated with 25 µM Mv3glc no degradation was observed, suggesting that at this concentration, anthocyanin completely neutralized the deleterious effects of peroxynitrite. As noted during the reported experiments with bovine endothelial cells (Paixão *et al.* 2012b), we did not detect any phosphorylation of IκBα, whereby it was assumed that the nitration process can also here be responsible for the inactivation of the endogenous inhibitor of NF-κB.

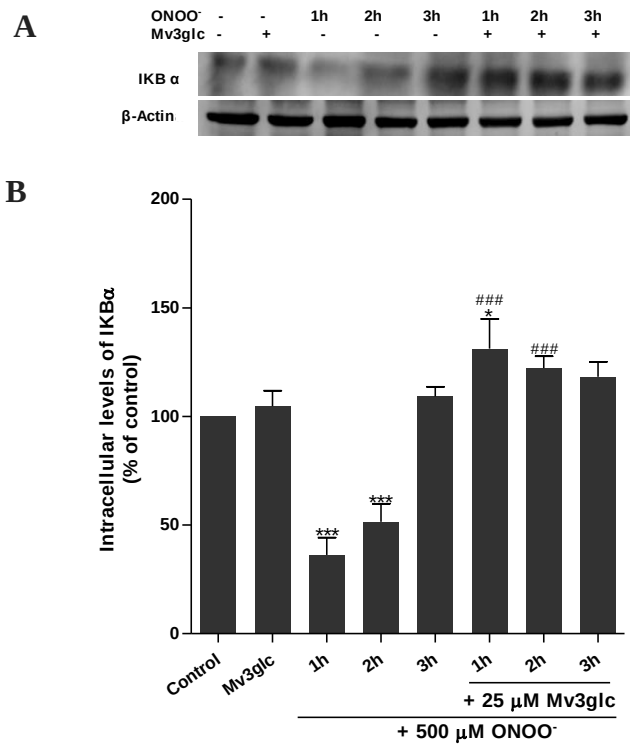


**Fig.3 Mv3glc prevents degradation of IκBα mediated by peroxynitrite on endothelial cells.** Confluent HMEC maintained in medium without serum were pre-incubated with 25 µM Mv3glc and then aggressed with 500 µM $ONOO^-$ for 10 min as described in "Materials and Methods". The cytoplasmatic protein extracts were then analyzed by immunoblotting with a specific antibody against IκBα. Representative blots (A) and quantification by densitometry are presented from three independent experiments (B). The results were normalized to endogenous β-actin and expressed as mean values ± SEM of the percentage of control or $ONOO^-$. $^*P$ <0.05, $^{***}$ $P$ <0.001 *vs.* control, $^{\#\#}P$ <0.001 *vs.* $ONOO^-$for the same incubation time.

### *3.3. Mv3glc prevents NF-κB activation and the production of iNOS and COX-2 mediated by peroxynitrite*

The nuclear translocation of the transcription factor NF-κB is responsible for the activation of many genes involved in the endothelial cells inflammatory response such as of iNOS and COX-2. As shown in Figure 4, peroxynitrite was able to significantly increase the levels of intracellular iNOS, similarly increasing the expression of COX-2, between 1 h and 6 h after addition of peroxynitrite, reaching 2 to 2.5x the control values. These effects were effectively inhibited by Mv3glc, particularly for the last two evaluated periods of time (5 and 6 h).

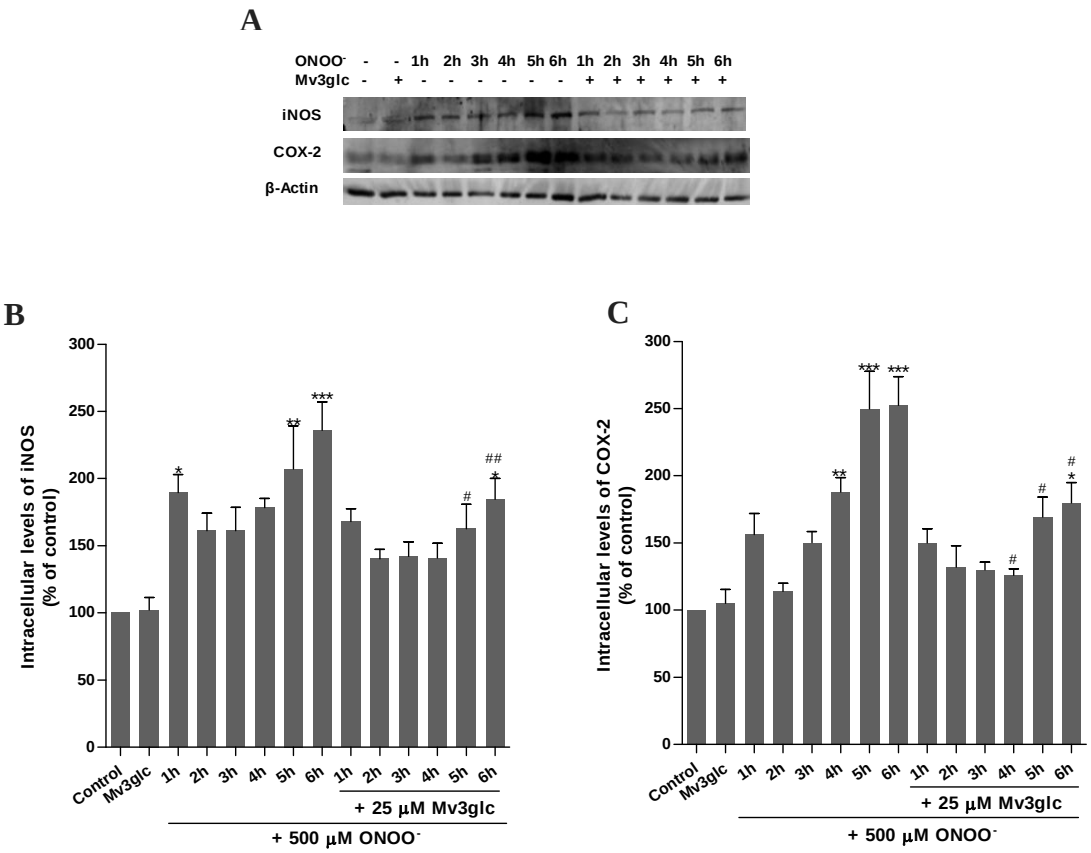


**Fig.4 Mv3glc significantly reduced the increased of peroxynitrite-induced levels of iNOS and COX-2 in HMEC**. Cytoplasmatic total proteins were extracted from cells pre-incubated with 25 µM Mv3glc with no further treatment or post-treated with peroxynitrite for 10 min, as described in "Materials and Methods", and maintained for the indicated times in serum-free medium at 37ºC. These protein extracts were then analyzed by immunoblot with specific antibodies against COX-2 and iNOS. Representative blots normalized to endogenous β-actin (A) and densitometric quantifications of three independent experiments for iNOS (B) and COX-2 (C) are shown**,** expressed as mean ± SEM of percentage of control, obtained without peroxynitrite. The results were expressed as mean ± SEM. **P* <0.05, ***P* <0.01 and ****P* <0.001 *vs.* control, and $^{\#}$P <0.05 and $^{\#\#}$*P* <0.01 *vs.* $ONOO^-$ for the same incubation time.

### *3.4. Mv3glc prevents peroxynitrite-mediated increase in proteasome activity*

The proteasome pathway is a crucial route of elimination of damaged proteins preventing their cellular accumulation. Proteasome activity was assessed by determining the chymotrypsin-*like* activity, one of the most important enzymatic proteasomic functions.

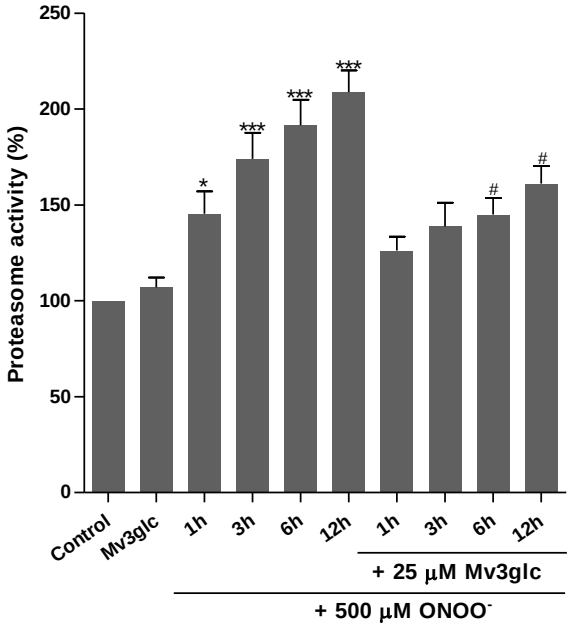


**Fig.5 Mv3glc significantly reduced the increased in proteasome activity induced by peroxynitrite in HMEC**. Cell extract supernatants obtained by centrifugation as described in "Materials and Methods" were added to the proteasome fluorogenic substrate SucLLVY-AMC (chymotrypsin-like) at a final concentration of 80 µM. The proteolytic activity was measured by monitoring the release of fluorescent 7-amide-4-methylcoumarin group (excitation 380 nm, emission 460 nm). The results were expressed as the mean ± SEM of four independent experiments.**P* <0.05 and ****P* <0.001 *vs.* control, and $^{\#}P$ <0.05 *vs.* $ONOO^-$ for the same incubation time.

This function of the 26S proteasomic complex was evaluated in peroxynitrite treated HMEC, with and without previous incubation with Mv3glc. Treatment of HMEC with 500 µM peroxynitrite, according to the procedure described in "Materials and Methods", led to a significant increase in proteasomal activity after the first hour following the insult with the oxidant, being more significant at t=6 and t =12 h (1.5 to 2 x control) (Fig.5). This activation was inhibited by about 25 % by Mv3glc pre-incubation of the cells in the last determined period of time. The activity of the proteasome was significantly decreased, for all conditions in the presence of its inhibitor MG132 (10 µM), added simultaneously with the fluorogenic substrate (results not shown).

### *3.5. Mv3glc extends the Nrf2 activation triggered by peroxynitrite leading to the increase in HO-1expression*

In order to assess whether Nrf2 was involved in Mv3glc cellular protection, we analyzed this nuclear transcription factor by Western-blot, using nuclear extracts from HMEC incubated with peroxynitrite, with or without Mv3glc pre-treatment (Fig.6). As can be observed, pre-incubation with 25 μM Mv3glc allowed that the increase in Nrf2 nuclear translocation, although not significant, found in cells submitted to peroxynitrite in the first three hours of incubation after oxidant addition, was significantly prolonged in time, in contrast with that observed in cells not protected by the anthocyanin.

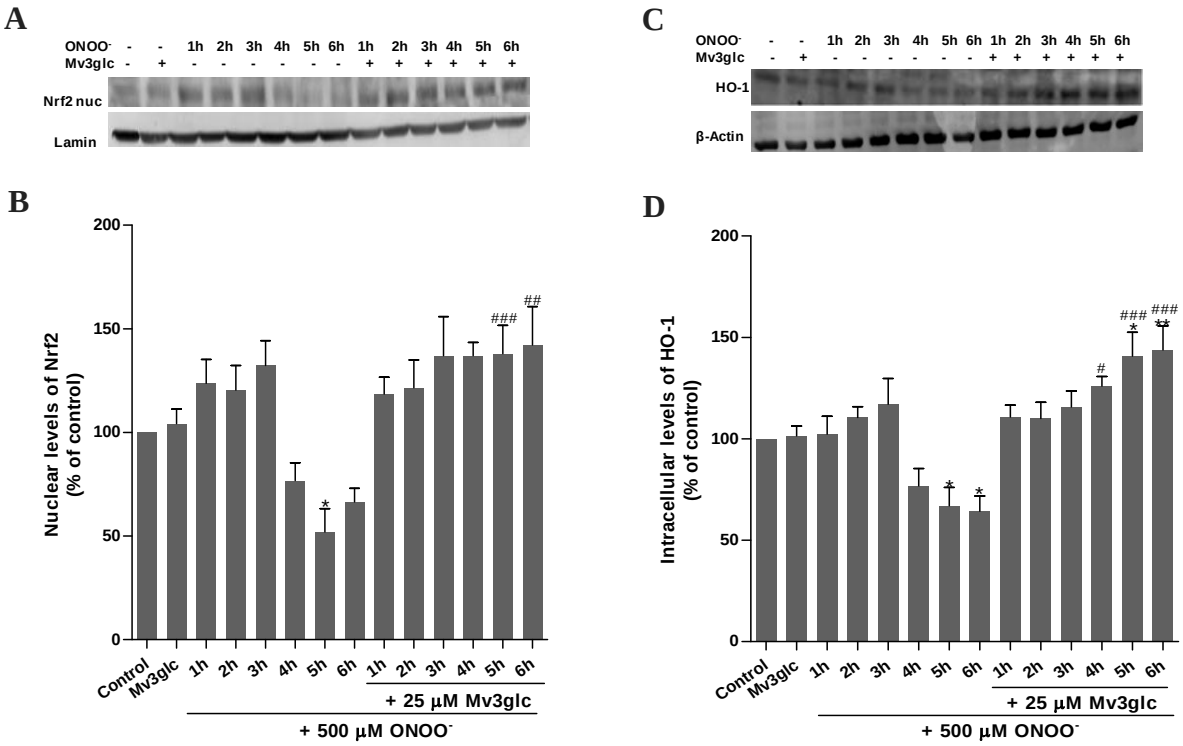


**Fig.6** Mv3glc increased and prolonged significantly the translocation of transcription factor Nrf2 to the nucleus (A,B) induced by peroxynitrite in HMEC and the consequent production of HO-1(C,D). The nuclear and total extracts obtained as described in "Materials and Methods" were analyzed by immunoblot with a specific antibodies to Nrf2 (A) and HO-1 (C), followed by densitometric quantification of at least three independent experiments (B, D). The results were normalized to β-actin in the case of total extracts and to endogenous lamin A for the nuclear ones and expressed as mean value ± SEM. **P* <0.05 *vs.* control, $^{\#\#}P$ <0.01 and $^{\#\#}P$ <0.001 *vs.* $ONOO^-$ for the same incubation time.

In these cells, there was a marked decrease of about 50% of baseline levels of nuclear Nrf2 (Fig.6A, B). Specifically, HMEC pre-treatment with Mv3glc led to nuclear Nrf2 increases of about one and half times as compared to cells not treated with this anthocyanin, 5-6 h after the addition of peroxynitrite.

Based on the above results, we investigated the involvement of Nrf2 translocation enhanced by Mv3glc on the increase of HO-1 levels, an anti- inflammatory enzyme that may result from Nrf2 activation. Thus, results indicated that cells incubation with Mv3glc allowed them to recover from the decrease in HO-1 levels resulting from peroxynitrite insult (Fig. 6 C, D). This recovery has become particularly evident at 5 and 6 h after the addition of the oxidizing agent, where it was noticed a doubling of the values of HO-1, as compared to those obtained in the absence of pre-treatment with the anthocyanin.

### *4. Discussion and Conclusions*

In recent years, we have assisted to a growing interest in anthocyanins primarily due to deeper knowledge about the broad spectrum of their pharmacological activity, a particularly in the prevention of atherosclerosis (Tsuda 2012). Among these compounds, Mv3glc is one of the most prevalent (Zamora -Ros *et al.* 2011). Although the exact mechanisms by which anthocyanins carry out their activities in the context of cardiovascular diseases prevention remain to be fully elucidated, it has been demonstrated that they are able to inhibit various pro-atherogenic pathways, namely, by increasing the resistance of LDL to peroxidation, modulating platelet aggregation and promoting nitric oxide mediated vascular relaxation (Grassi *et al.* 2009; Chong *et al.* 2010).

Regarding the atherogenic process, peroxynitrite is a potent oxidant with a crucial pathophysiological role in the development of atherogenesis. The ability of

various anthocyanins to neutralize peroxynitrite *in vitro* is well recognized, mainly due to its electrodonatting properties and because they constitute alternative nitration substrates (Pannala *et al.* 1999). However, despite research in recent years has been focused on the correlation between the biological activity and antioxidant properties of this class of flavonoids, their interference with critical cellular signaling pathways and gene regulation goes far beyond that capacity, becoming an important and challenging area of study (Marzocchella *et al.* 2011; Mauray *et al.* 2012). .

In this context, and following our previous studies with endothelial cells of bovine origin (Paixão *et al.* 2011; 2012a,b), in this work we intended to consolidate the cytoprotective mechanisms due to Mv3glc on endothelial cells of human origin submitted to peroxynitrite aggression. Specifically, we demonstrated that Mv3glc, when pre-incubated with endothelial cells, was able to protect them from apoptotic death due to peroxynitrite, confirming the results obtained in BAEC (Paixão *et at.* 2012a,b). Given the similar degree of protection afforded by Mv3glc in human endothelial cells against peroxynitrite and considering the high probability that the mechanisms of apoptosis/protection here involved overlap those previously reported in BAEC, the study was directed to the inflammatory process that also characterizes the peroxynitrite aggression, assessing the ability of Mv3glc to counteract this process.
In this context, the main goal was to assess in what extent the cytoprotective properties of Mv3glc against cellular injury due to peroxynitrite were mediated by pathways dependent on NF-κB. To accomplish this intent we analyzed the interference of the anthocyanin on the degradation of IκBα, the cytoplasmatic inhibitor of that transcription factor, and on the production of inflammatory mediators resulting from the activation of NF-κB, particularly COX-2 and iNOS. In fact, it was observed that the production of these mediators of inflammation, mediated by IκBα degradation, was stimulated by cell

treatment with peroxynitrite, a process significantly inhibited when cells were pre-incubated with Mv3glc. There are several properties recognized to NF-κB, namely the transcriptional activation of adhesion molecules, growth factors and inflammatory molecules. The NF-κB inhibitor, IκBα, prevents its activation while the proteasome increases such activation, for example through IκBα degradation (Rajkumar *et al.* 2005; Milano *et al.* 2007).

There are several studies suggesting that proteasome's activity is likely to be modified by several mediators and processes involved in endothelial dysfunction, such as oxidative stress and peroxynitrite (Lalu *et al.* 2012; Liu *et al.* 2012). Oxidative stress is implicated in several pathologies, including the inflammatory ones, as exemplified by atherosclerosis.

The oxidation of proteins and enzymes leads to the modification of their functions and these changes may be a key factor in the progression of the disease process. By forming adducts with proteins, oxidizing agents may change their structure and functional properties (Ducrocq *et al.* 1999; Stadtman 2006). In its turn, peroxynitrite is able to nitrate tyrosine residues forming a stable product, 3-nitrotyrosine, apart from interacting with other amino acids such as cysteine, tryptophan and methionine (Ischiropoulos and al-Mehdi 1995, Alvarez *et al.* 1996; Alvarez and Radi 2003).

However, although the sensitivity of the proteasome to peroxynitrite is not completely understood, it is known that it is tissue-specific and depends on the relative intracellular proteasome levels and of that oxidant concentration (Amici *et al.* 2003). It is described that peroxynitrite increases the accessibility of the substrates to proteasome active sites, thereby increasing its activity. This hypothesis is supported by recent studies depicting that peroxynitrite induces changes in proteasomal activity, which are distinct from other oxidants (Amici *et al*. 2003; Osna *et al*. 2004). In moderate

conditions of oxidative damage at relatively low concentrations of peroxynitrite, proteasome appears to be activated stimulating protein degradation. Conversely, in more severe oxidative environments, the high amounts of peroxynitrite can suppress the activity of the proteasome, allowing intracellular accumulation of modified proteins (Osna *et al.* 2004). Summarizing, peroxynitrite appears to act on NF-κB in two different levels, on one hand through stimulation of IκBα tyrosine residues nitration (Paixão *et al.* 2012), and secondly, by increasing the degradation of this inhibitor molecule, in this way exacerbating proteasome activity, both culminating in the activation of NF-κB and in the production of inflammatory mediators (Liu *et al*. 2012). Mv3glc interferes with these two mechanisms, preventing either peroxynitrite-mediated IκBα degradation or proteasome activation. It should be considered, as seen in Figure 5, that Mv3glc did not totally inhibit the increase in proteasomal activity triggered by peroxynitrite. For this reason, and taking into account that pre-incubation of cells with the anthocyanin increased cell survival in the presence of peroxynitrite, one can speculate that cell clearance mechanisms, particularly of proteins affected by this oxidant are in some way preserved by Mv3glc, allowing the cell to spare IκBα, one of the major proteasome substrates (Magnani *et al.* 2000). Although the pro-apoptotic effects of a marked decrease in proteasome activity should be considered, its milder inhibition has shown to mediate beneficial cellular responses in nontoxic conditions (Meiners *et al.* 2008). These protective effects arise mostly through the decrease in NF-κB activation (Pye *et al.* 2003; Williams *et al.* 2006). Also, in such milder conditions, it has been described improvements in blood flow and endothelial function, resulting from a positive balance between vasodilators and vasoconstrictors (Meiners *et al.* 2006). Specifically, it has been reported a decrease in endothelin-1 concentrations and an increase in eNOS expression and activity, a key regulator in vascular homeostasis, with

protective effects in atherosclerosis (Stangl *et al.* 2004.; Meiners *et al.* 2006) and whose activity is significantly increased in the presence of Mv3glc, as we have already shown (Paixão *et al.* 2012).

On the other hand, a key regulator of the cellular defenses against oxidative insult or to xenobiotics is the transcription factor Nrf2 which, after nuclear translocation, forms heterodimers with small Maf nuclear proteins, with subsequent binding to antioxidant response elements/electrophilic (ARE / EpRE) in the promoter region of genes encoding various antioxidant and detoxification Phase II enzymes, among which stands out HO-1. The Nrf2 repressor protein, Keap1, appears to be an important cytoplasmatic sensor of oxidative stress, due to its high content in cysteine residues (Baird and Dinkova - Kostova 2011). Disruption of the interaction Nrf2/Keap1, allowing Nrf2 to translocate to the nucleus, can be triggered by numerous stimuli, including polyphenols, which are recognized as important inducers of HO-1, a redox-inducible enzyme that provides protection against various forms of insult (Balogun *et al.*2003; Hwang *et al.* 2011) and also plays a key role in cardiovascular protection (Siow *et al.* 1999; Ryter *et al.* 2006). Our data indicate that although Mv3glc *per se* did not alter significantly HO-1 levels, probably due to the extended pre-incubation period, its influence is notoriously observed during cellular injury with peroxynitrite, not only preventing the depletion of HO-1 levels induced by this oxidant but mostly leading to a significant increase in the levels of this protective enzyme, in particular for 5-6 h after the addition of the oxidant (Fig. 6). These effects are in accordance to the significant increase in Nrf2 nuclear levels, promoted by Mv3glc, observed at the same incubation periods, confirming the interdependence between this transcription factor and HO-1 expression, in our experimental conditions.

In conclusion, the set of results presented here allowed at first, to enhance the cytoprotective role of anthocyanins, specifically Mv3glc, in an inflammatory scenario such as that underlying atherogenesis. Furthermore, we were able to clarify the mechanisms that underpin non-toxic proteasome inhibition, inducing a defense response in vascular endothelial cells. Actually, in our experimental conditions, in cells previously treated with Mv3glc, it was not only confirmed the inhibition of peroxynitrite-induced NF-κB activation, but also the presence of exacerbated nuclear Nrf2 levels and the resulting increase in HO-1 expression, associated with a notorious increase in cell viability as compared to cells submitted to peroxynitrite and untreated with Mv3glc. Therefore, the inhibitory action of Mv3glc against peroxynitrite aggression may be potentially assigned to the concerted action on the modulation of proteasome's activity associated to the effect on the balance between NF-kB/Nrf2 transcription factors. Since the pathogenesis of atherosclerosis involves multiple events, including endothelial dysfunction, increased oxidative stress and inflammation, Mv3glc antiatherogenic potential at all of these levels and its cellular pathways, are undoubtedly worthy of further investigation, aiming the development of dietary or therapeutic strategies capable of preventing or delaying the progression of the atherosclerotic process.

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