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Anthocyanins block ischemia-induced apoptosis in the perfused heart and support mitochondrial respiration potentially by reducing cytosolic cytochrome c☆

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ABSTRACT

Anthocynanins, found in fruits and vegetables, have a variety of protective properties, which have generally been attributed to their antioxidant capacity. However, antioxidants are generally strong reductants, and some reductants have been found to block apoptosis by reducing cytosolic cytochrome c, which prevents caspase activation. We tested the ability of various anthocyanins: to reduce cytochrome c, to support cytochrome c-induced mitochondrial respiration and to inhibit apoptosis induced by heart ischemia. Anthocyanins such as delphinidin-3-glucoside (Dp3G) and cyanidin-3-glucoside (Cy3G) were able to reduce cytochrome c directly and rapidly, whereas pelargonidin-3-glucoside (Pg3G), malvinidin-3-glucoside (Mv3G) and peonidin-3-glucoside (Pn3G) had relatively low cytochrome c reducing activities. Dp3G and Cy3G but not Pg3G supported mitochondrial state 4 respiration in the presence of exogenous cytochrome c. Pre-perfusion of hearts with 20 μM Cy3G but not Pg3G prevented ischemia-induced caspase activation. This suggests that the ability of anthocyanins to block caspase activation may be due to their ability to reduce cytosolic cytochrome c. This article is part of a Directed Issue entitled: Bioenergetic dysfunction, adaptation and therapy.

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1. Introduction

Mitochondria are organelles that produce ATP by oxidative phosphorylation and also control cell survival and death. To accomplish their energetic function they have to be structurally and functionally intact. However, in many pathological situations including heart ischemia or ischemia plus reperfusion mitochondrial injury occurs, which is manifested as inhibition of oxidative phosphorylation, loss of mitochondrial membrane integrity, and loss of cytochrome *c* from mitochondria (Rouslin, 1983; Regitz et al., 1984; Toleikis et al., 1989; Borutaite et al., 1996; Chen et al., 2008; Lesnefsky and Hoppel, 2008; Pasdois et al., 2011). The release of cytochrome c from mitochondria was found to be one of the earliest events in ischemic heart damage (Borutaite et al., 1996) which may lead to apoptosis of cardiomyocytes (Bialik et al., 1999; De Moissac et al., 2000) by the mechanism involving binding of cytosolic cytochrome c to adaptor protein Apaf-1. This results in formation of the apoptosome and subsequent activation of caspase-9 leading to apoptotic cell death (Yang et al., 1997; Li et al., 1997). The process of caspase activation may be regulated by the redox state of cytochrome *c* as several investigators have shown that reduced cytochrome *c* is less potent in caspase activation than its oxidized form (Pan et al., 1999; Suto et al., 2005; Borutaite and Brown, 2007; Barauskaite et al., 2011). Cytochrome *c* can be reduced by several factors such as intracellular glutathione or added N,N,N′,N′-tetramethylphenylene-1,4-diamine (TMPD) (Vaughn and Deshmukh, 2008; Borutaite and Brown, 2007; Barauskaite et al., 2011). Interestingly, there are various plant flavonoids (epicatechin, quercetin, kaempferol) known as

Abbreviations: Cy3G, Cyanidin 3-O-glucoside; DEVD, Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin, caspase -3 substrate; Dp3G, Delphinidin 3-O-glucoside; EGTA, ethylene glycol tetraacetic acid; eNOS, endothelial nitric oxide synthase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mv3G, Malvinidin 3-O-glucoside; Pg3G, Pelargonidin 3-O-glucoside; Pn3G, Peonidin 3-O-glucoside; TMPD, N,N,N′,N′-tetramethylphenylene-1,4-diamine.

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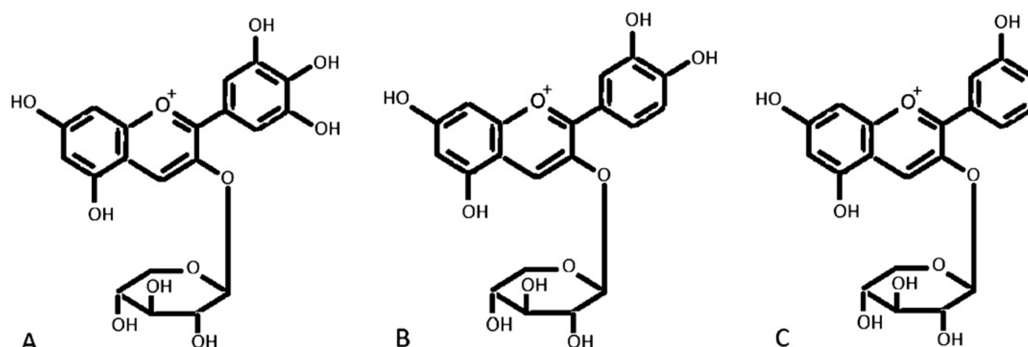

Fig. 1. Chemical structure of anthocyanins: A – Dp3G, B – Cy3G, and C – Pg 3G.

strong natural antioxidants, and other natural dihydroxy conjugated compounds (like catechols), which have been reported to reduce cytochrome *c* (Saleem and Wilson, 1982; Lagoa et al., 2011). In principle, such compounds could block caspase activation by reduction of cytosolic cytochrome *c*, however, this has not been investigated yet.

Among different groups of flavonoids, anthocyanins, which are widely distributed amongst colored fruits and vegetables, might also inhibit apoptosis by a similar mechanism. It has been proposed that the consumption of anthocyanin-rich dietary plants and fruits can provide strong neuro- and hepato-protective effects (Shin et al., 2006; Dani et al., 2008; Lu et al., 2010), and reduce the risk of cardiovascular diseases (Amorini et al., 2003; Toufektsian et al., 2008; Akhlaghi and Bandy, 2009; De Pascual-Teresa et al., 2010) in humans and animals. It is generally considered that these protective activities are related to the antioxidant properties of anthocyanins which in turn are determined by the number and position of hydroxyl groups as substituents (Fig. 1), and by the extent of glycosylation (Galvano et al., 2004; Prior and Wu, 2006; Muselik et al., 2007; Yao and Vieira, 2007) However, the exact molecular mechanism(s) of the protective effects of anthocyanins has not been fully elucidated yet, and there are recent reports that anthocyanins such as delphinidin and some of other flavonoids may protect the heart against ischemia-reperfusion injury by mechanisms not related to their antioxidant activities (Scarabelli et al., 2009).

In this study we have examined the effect of five common anthocyanidin glucosides: delphinidin (Dp3G), cyanidin (Cy3G), pelargonidin (Pg3G), peonidin (Pn3G), and malvidin (Mv3G), on the redox state of extramitochondrial cytochrome *c* in vitro. To the best of our knowledge, this is the first report that Dp3G and Cy3G (but not Pg3G, Pn3G or Mv3G) are potent and effective reducers of cytochrome *c*. Moreover, we show that Cy3G prevents the activation of caspases in ischemic rat hearts possibly by reduction of cytosolic cytochrome *c*, whereas Pg3G was uneffective. Our results suggest new therapeutic applications of certain anthocyanins.

2. Materials and methods

2.1. Chemicals

Delphinidin-3-O-, cyanidin-3-O-, pelargonidin-3-O-, peonidin-3-O-, and malvidin-3-O-glucosides were from Extrasynthese (Genay, France). All other reagents were obtained from Sigma, unless specified otherwise.

2.2. Isolation of rat heart mitochondria

All experiments were performed on hearts from 2 to 4 months old female Wistar rats. Rats were killed by increasing concentration of CO_2 in the air followed by cervical dislocation. The hearts, control or after ischemia, were cooled in 0.9% KCl, then cut into small pieces and homogenized with a Teflon-glass homogenizer in the isolation buffer (10 ml/g of tissue) containing 250 mM sucrose, 5 mM HEPES, 2 mM EGTA, pH 7.3. Cytosolic and mitochondrial fractions were separated by differential centrifugation (5 min at 750 × *g*, then 10 min at 6800 × *g*). Total cytosolic and mitochondrial protein was measured by the Biuret method.

2.3. Ischemia model

The hearts were perfused on a Langendorff perfusion system with Krebs-Henseleit solution (11 mM glucose, 118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 0.5 M $CaCl_2$, 1.6 mM $MgSO_4$ and 0.7 mM Na pyruvate, saturated with 95% O_2 – 5% CO_2, pH 7.4 at 37 °C) at a pressure of 80 cm H_2O. After a 10 min equilibration period, 20 μM anthocyanins Cy3G or Pg3G were added to the perfusate and hearts were perfused for another 15 min. Control hearts were perfused for the same time but without anthocyanins. After perfusion with/without anthocyanins, hearts were subjected to 45 min total ischemia at 37 °C.

2.4. Measurement of mitochondrial respiratory rates

Mitochondrial respiration was measured using an Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria) in the buffer containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose (pH 7.1 at 37 °C), and 6 mM pyruvate plus 6 mM malate were used as respiratory substrates. Mitochondrial State 3 respiration rate was achieved by adding 1 mM ADP. The effect of cytochrome c on respiration rate was determined by adding 32 μM cytochrome *c* to mitochondria inhibited with 140 μM atractyloside (see Fig. 2). Mitochondrial respiration rates were expressed as pmol/s/0.125 mg protein. The

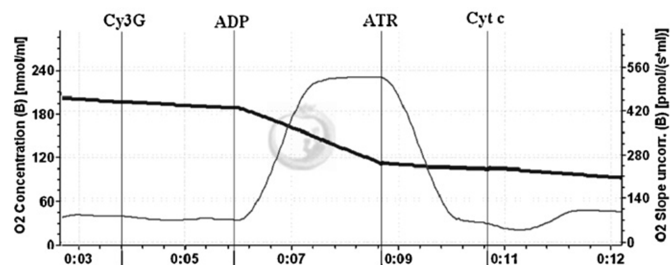

Fig. 2. Typical traces of respirometric measurements of mitochondria isolated from rat heart. At the beginning the routine respiration rate with 0.125 mg of mitochondria and 6 mM pyruvate + 6 mM malate was measured. Further additions: ADP – 1 mM ADP; ATR – 140 μM atractyloside; Cyt c – 32 μM cytochrome c; Cy3G – 40 μM Cy3G. The upper trace indicates oxygen concentration, the lower trace – oxygen flux.

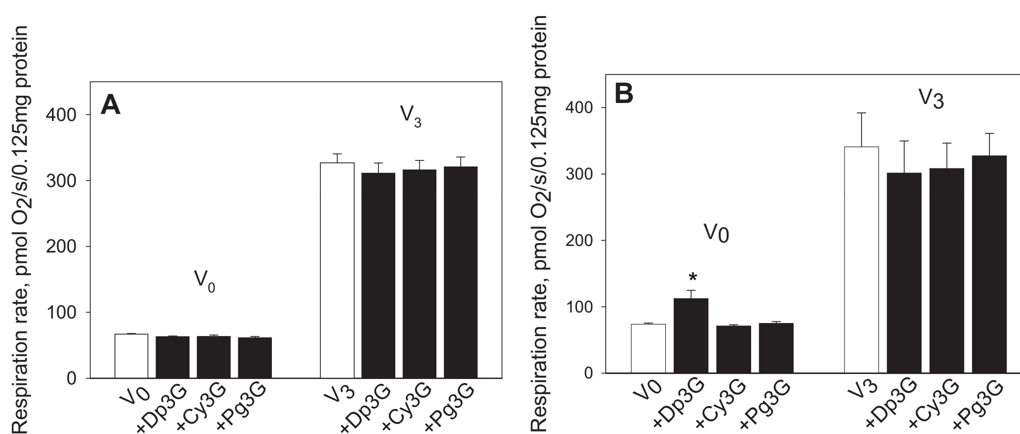

Fig. 3. Effects of anthocyanins on the routine and State 3 respiration rate. Mitochondrial respiration rate was measured as described in Methods using 6 mM pyruvate plus 6 mM malate as substrates. +Dp3G, +Cy3G or +Pg3G – respiration rates in the presence of 20 μM (A) and 40 μM (B) anthocyanins. V_0 – routine respiration rate, V_3 – State 3 respiration rate, *statistically significant effect compared to V_0 in the absence of anthocyanins. Means ± standard errors of 3–5 separate experiments are presented.

final mitochondrial protein concentration in all experiments was 0.125 mg/ml.

2.5. Measurement of cytochrome c reduction level

The cytochrome c reduction level was recorded with a Helios λ spectrophotometer. Absorbance was scanned over 500–600 nm wavelenght range and the cytochrome c reduction maximum was take as 550 nm. The spectra of various anthocyanins in 1 ml of buffer containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium lactobionate, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose (pH 7.1) were recorded and taken as the experimental blanks. Then 32 μM cytochrome c from horse heart (from Sigma–Aldrich, purity ≥95%) was added to the same buffer and the cytochrome c spectrum over the range 500–600 nm was recordered every minute. The absorption peak height at 550 nm was taken and compared to the absorption peak of completely reduced cytochrome c at the end of the test when all added cytochrome c was completely reduced by adding few crystals of dithionite. The absorption peak of dithionite-reduced cytochrome *c* was equated to 100% of reduced cytochrome *c*. All measurements were done at room temperature.

2.6. Measurement of caspase activity

For measurement of caspase 3-like activity, 1 mg/ml of total cytosolic protein was incubated for 60 min in buffer containing 250 mM sucrose, 5 mM HEPES, 2 mM EGTA (pH 7.3 at 37 °C) and 0.1 mM Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (DEVD). The hydrolysis of caspase substrate was followed fluorimetrically, excitation set at 380 nm, emission – 460 nm. Substrate cleaving activity was completely suppressed by 0.02 mM N-acetyl-Asp-Glu-Val-Asp-aldehide, a reversible inhibitor of caspase 3.

2.7. Measurement of mitochondrial and cytosolic cytochrome c content

Cytochrome c content in mitochondria and cytosolic fractions was detected using Quantikine rat/mouse Immunoassay ELISA kit (R&D System). For measurement of cytochrome *c* content, mitochondria were solubilized with 1% Triton X-100 and further procedures were performed according to the manufacturer's instructions.

2.8. Statistical analysis

Data are expressed as means ± standard errors of at least three separate experiments. Statistical comparison between experimental groups was performed by a paired *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of anthocyanins on heart mitochondrial respiration

In order to investigate the direct effect of anthocyanins on mitochondrial oxidative phosphorylation, the mitochondrial respiration rate in various metabolic states was measured. According to the experimental protocol shown in Fig. 2, at the beginning mitochondrial routine respiration (V_0) was measured in the presence of the respiratory substrates. When Dp3G, Cy3G and Pg3G were used, they were added into mitochondrial incubation medium during the phase of routine respiration. Mitochondrial State 3 respiration was achieved by adding ADP. State 4 respiration rate was measured in the presence of atractyloside to inhibit ATP/ADP transport. Finally, the exogenous cytochromes *c* was added in order to check the intactness of the mitochondrial outer membrane.

As can be seen in Fig. 3A, all tested anthocyanins (Dp3G, Cy3G and Pg3G) had no effect on mitochondrial routine or phosphorylating (State 3) respiration rate at 20 μM concentrations. However, 40 μM Dp3G induced an increase (by 53%) in the routine respiration rate indicating that at higher concentrations Dp3G may exert an uncoupling effect (Fig. 3B). State 3 respiration was not affected by anthocyanins at both 20 μM and 40 μM concentrations (Fig. 3A and B). The non-phosphorylating respiration rate, which was measured after addition of atractyloside, was also not affected by all anthocyanins used at 20 μM, and by Cy3G and Pg3G at 40 μM, except for Dp3G, which at higher (40 μM) concentrations caused a significant increase (by 54%) of the non-phosphorylating respiration rate (data not shown).

Interestingly, when Dp3G and Cy3G were added to mitochondria and their state 4 respiration was measured in the presence of external cytochrome *c*, a powerful stimulation of the respiratory rate (by 71% and 30%, respectively) was observed as compared to cytochrome-*c*-stimulated non-phosphorylating respiration of control mitochondria not treated with anthocyanins (Fig. 4). In contrast, Pg3G had no effect on this parameter (Fig. 4).

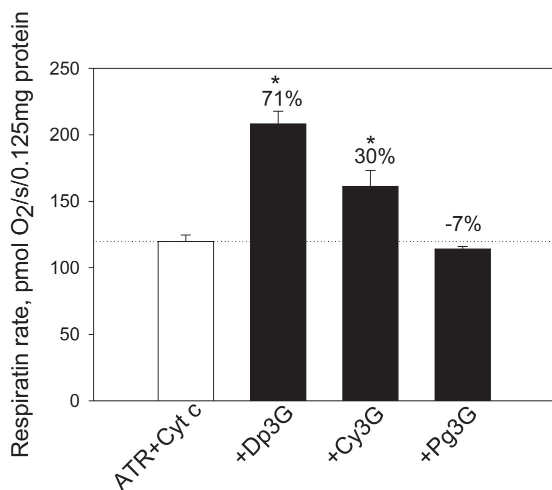

Fig. 4. The effects of anthocyanins on cytochrome c-activated State 4 respiration rate. +Dp3G, +Cy3G or +Pg3G–respiration rates in the presence of 40 μM anthocyanins indicated. Numbers above the bars indicate the effects of anthocyanins on cytochrome c – stimulated respiration expressed in %. Means ± standard errors of 3–5 separate experiments are presented.

3.2. *Effect of anthocyanins on cytochrome c redox state*

We hypothesized that the observed increase of state 4 respiration rate induced by some anthocyanins in the presence of added cytochrome *c* may be caused by the ability of anthocyanins to reduce cytochrome *c*. Therefore, next we determined the capacity of various anthocyanins to reduce cytochrome c directly in solutions. For that we recorded the absorbance spectrum in the range of 500–600 nm of cytochrome *c* over time in the presence of several anthocyanins. Initially the added 32 μM cytochrome c was in the oxidized form and did not change over the time of the experiment (data not shown). In the presence of Dp3G there was fast and almost complete reduction of cytochrome c within 6 min of incubation: during the first minute in the presence of 40 μM Dp3G, cytochrome c was reduced to 67 ± 1%, and reached 78 ± 2% at 6 min (Fig. 5). 40 μM Cy3G was also able to reduce cytochrome c though this was less rapid: within the first minute Cy3G reduced 19 ± 2% of cytochrome c reaching 42 ± 4% and 50 ± 4% after 3 min and

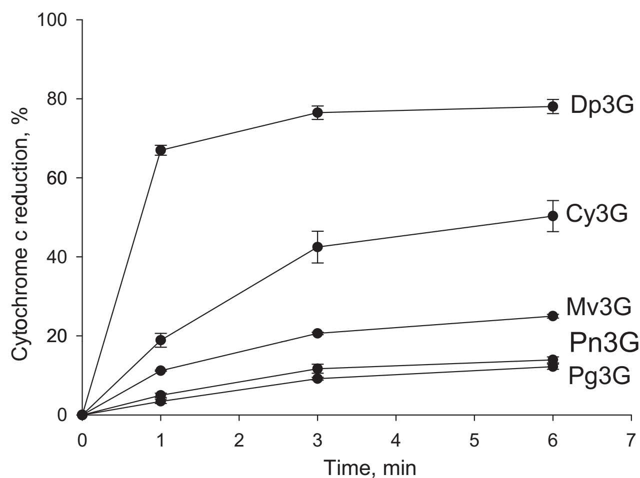

Fig. 5. Effect of anthocyanins on cytochrome c reduction level. The cytochrome c reduction level was measured spectrophotometrically as described in Methods in the presence of 40 μM of anthocyanins (Dp3G, Cy3G, Mv3G, Pn3G, Pg3G) and 32 μM cytochrome c. The same sample was measured over the time and the peak absorbance at 550 nm wavelength was estimated. Dithionite-reduced cytochrome c level was taken as 100%.

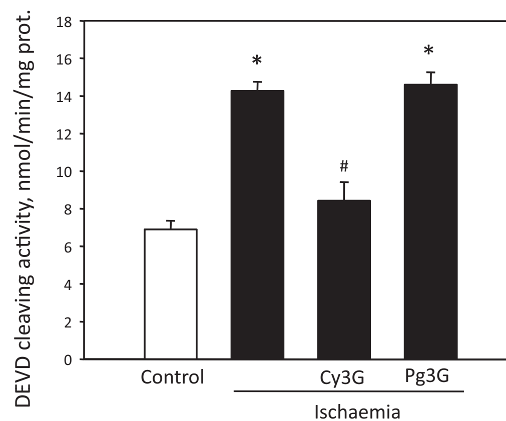

Fig. 6. Perfusion of hearts with Cy3G but not Pg3G prevents ischemia-induced caspase-3 activation. Hearts were pre-perfused for 15 min with 20 μM Cy3G or 20 μM Pg3G prior to induction of 45 min total ischemia and the caspase-3- activity was measured as described in Methods. *Statistically significant effect compared to control group. #Statistically significant effect of Cy3G compared to ischemic group. Means ± standard error of 4–9 separate experiments are presented.

6 min, respectively. Mv3G and Pn3G were much less able to reduce cytochrome c causing about 11–21% and 5–14% reduction over the 1–6 min interval, respectively (Fig. 5). Pg3G had the lowest reducing capacity among all the investigated anthocyanins so that only 3.4 ± 0.6%, 9.2 ± 0.4% and 12 ± 0.7% of cytochrome c was reduced in the presence of 40 μM Pg3G after 1, 3 and 6 min, respectively (Fig. 5). The pattern of cytochrome c reduction by various anthocyanins in the presence of mitochondria was in principle the same as described above in the absence of mitochondria, only reduction levels were lower (data not shown). When experiments with mitochondria were done in the presence of azide to inhibit cytochrome oxidase, the pattern of reduction of cytochrome *c* was essentially the same as in the absence of mitochondria: 40 μM Dp3G reduced cytochrome *c* within 6 min to 84 ± 6%, 40 μM Cy3G – to 56 ± 4%, and 40 μM Pg3G – to 22 ± 6%.

These results show that Dp3G and Cy3G are the most potent cytochrome c reducing anthocyanins whereas Pg3G has the lowest capacity to reduce cytochrome *c*.

3.3. *Effect of anthocyanins on ischemia induced caspase activation in perfused heart*

It has been previously reported that cytochrome c reducing chemicals such as TMPD can protect against caspase activation and apoptosis in several experimental models of cell death including ischemia-induced cardiomyocyte death in perfused heart (Borutaite and Brown, 2007; Barauskaite et al., 2011). In this study we were interested whether a similar protective effect may be exerted by another class of cytochrome *c* reducing compounds – the anthocyanins. For this purpose we have chose Cy3G, which had a high capacity to reduce cytochrome c but did not uncouple mitochondrial oxidative phosphorylation (as Dp3G did, Fig. 3B) and compared its effect on ischemia-induced caspase activation to the effect of Pg3G, which was found to have relatively little cytochrome c reducing activity. In this series of experiments, hearts were pre-perfused with 20 μM Cy3G or 20 μM Pg3G for 15 min and then subjected to 45 min total ischemia. DEVD-cleaving, caspase-3-like protease activity was taken as a measure of apoptosis induced by heart ischemia. We found that after ischemia caspase-3-like, DEVD-cleaving activity was increased by two-folds compared to the non-ischemic control group (Fig. 6). When hearts were pre-perfused with 20 μM Cy3G, ischemia-induced activation of caspases was almost completely blocked: the DEVD-cleaving activity in the ischemia plus Cy3G group was significantly reduced

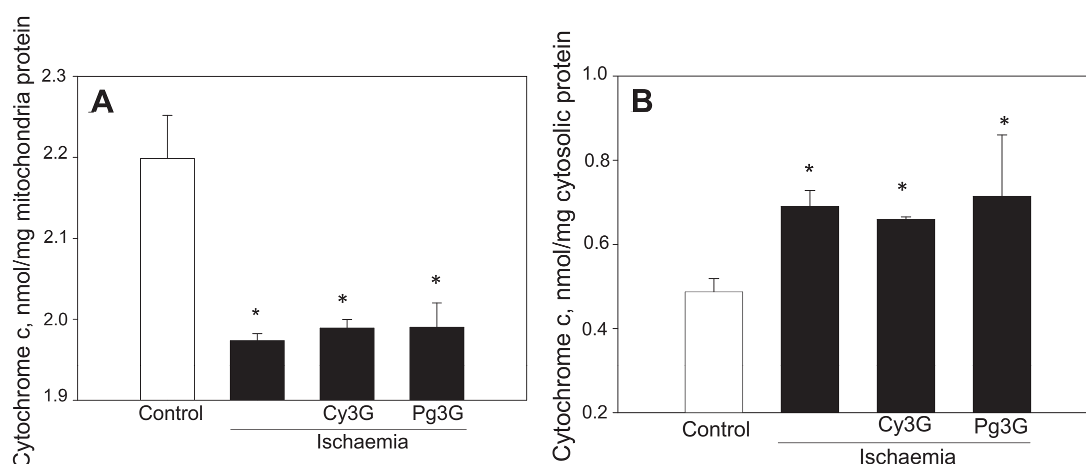

Fig. 7. Perfusion of hearts with Cy3G and Pg3G did not prevent ischemia-induced cytochrome c release from mitochondria into cytosol. (A) – cytochrome c concentration in mitochondria isolated from control and ishemic hearts; (B) – cytochrome c concentration in cytosolic fractions of control and ischemic hearts. Hearts were pre-perfused for 15 min with 20 μM Cy3G or 20 μM Pg3G prior to induction of 45 min total ischemia. The cytochrome c concentration in mitochondria and cytosolic fractions were measured as described in Methods. *Statistically significant effect if compared with control group. Means ± standard error of 4–9 separate experiments in each group are presented.

and practically was not different from non-ischemic control (Fig. 6). In contrast, pre-perfusion of the hearts with 20 μM Pg3G did not prevent ischemia-induced caspase activation: the DEVD-cleaving activity in the ischemia plus Pg3G group was not statistically different from the ischemic group (Fig. 6). Pre-perfusion of normal (non-ischemic) hearts with 20 μM Cy3G or Pg3G had no significant effect on caspase activity (data not shown). This suggests that Cy3G but not Pg3G prevents activation of caspases in perfused heart during ischemia.

3.4. Anthocyanins did not prevent re-distribution of cytochrome c in ischemic hearts

Though Cy3G protected against ischemia-induced caspase activation it had no effect on the release of cytochrome *c* from mitochondria during heart ischemia. As can be seen in Fig. 7A, after 45 min ischemia the mitochondrial content of cytochrome c was significantly decreased, and the level of cytochrome c in the cytosolic fractions of ischemic hearts significantly increased by 41% compared to the control group (Fig. 7B). The levels of mitochondrial and cytosolic cytochrome c in ischemic hearts pre-perfused with 20 μM Cy3G were not significantly different from the respective levels in ischemic group (Fig. 7). Pre-perfusion of the hearts with 20 μM Pg3G also had no effect on the mitochondrial and cytosolic cytochrome c content compared to ischemic group levels. This indicates that both Cy3G and Pg3G did not prevent ischemia-induced cytochrome *c* release from mitochondria into cytosol.

3.5. Anthocyanins did not prevent ischemia-induced mitochondrial dysfunction in perfused hearts

As can be seen from Fig. 8, 45 min of ischemia caused a statistically significant decrease in mitochondrial state 3 respiration rate by 52% without any effect on the routine (V_0) respiration rate. Pre-perfusion of the hearts with 20 μM Cy3G or 20 μM Pg3G did not protect mitochondria against ischemia-induced damage: in these treated groups, the state 3 respiratory rates were decreased to a similar extent as in the untreated ischemic group.

4. Discussion

The main findings of our study are that (i) anthocyanins such as Dp3G and Cy3G are able to reduce cytochrome c directly and rapidly whereas the cytochrome c reducing activities of Pg3G, Mv3G and Pn3G are relatively low; (ii) Dp3G and Cy3G but not Pg3G support mitochondrial state 4 respiration in the presence of exogenous cytochrome *c*; and (iii) pre-perfusion of the hearts with Cy3G but not Pg3G at 20 μM concentrations prevents ischemia-induced caspase activation which is a hallmark of apoptotic cell death. This suggests that the cytochrome c reducing capacity of anthocyanins may correlate with their ability to prevent caspase activation. As both Cy3G and Pg3G had no effect on the ischemia-induced loss of cytochrome c from mitochondria and the related inhibition of mitochondrial respiration but only Cy3G was able to suppress caspase activation, it is evident that Cy3G should act downstream of cytochrome c release but upstream of caspase activation. It has been previously reported that reduced cytochrome *c* is less capable of caspase activation in the apoptosome than its oxidized form (Pan et al., 1999; Hancock et al., 2001; Suto et al., 2005; Borutaite and Brown, 2007). Also it has been shown that TMPD – a powerful reductant of cytochrome c, prevents caspase activation and apoptosis in several models of mitochondria-mediated cell death including staurosporine-induced cell death in HeLa cell cultures (Borutaite

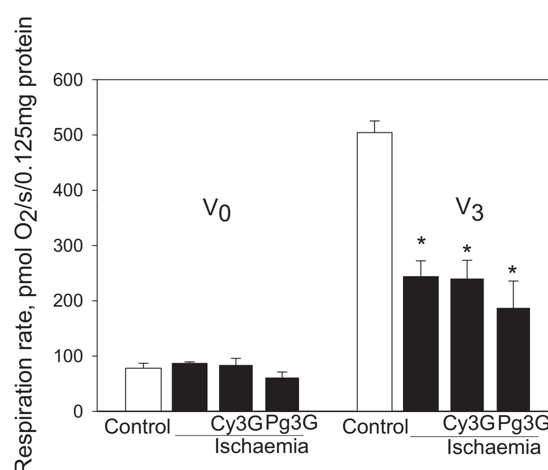

Fig. 8. Perfusion of hearts with Cy3G and Pg3G did not prevent ischemia-induced mitochondrial dysfunction. Hearts were pre-perfused for 15 min with 20 μM Cy3G or 20 μM Pg3G prior to induction of 45 min total ischemia. Substrates 6 mM pyruvate plus 6 mM malate. *Statistically significant effect compared to control group. Means ± standard error of 4–9 separate experiments are presented.

and Brown, 2007) and ischemia- or ischemia-reperfusion-induced apoptosis and necrosis in perfused rat heart by a mechanism involving reduction of cytosolic cytochrome *c* (Barauskaite et al., 2011). Therefore, on the basis of results obtained in this study and previous reports in the literature we conclude that the observed Cy3G-mediated inhibition of caspase activation in ischemic heart may be due to the reduction of cytosolic cytochrome c by Cy3G.

On the other hand, we cannot rule out the possibility that Cy3G may protect against ischemia-induced caspase activation via other mechanisms such as direct inhibition of apoptosome function or support of mitochondrial respiration after release of cytochrome *c*. Our results showed that neither of the investigated anthocyanins at 20 μM concentrations had an effect on routine or phosphorylating respiration of mitochondria except Dp3G which exhibited a slight uncoupling effect at 40 μM concentration. However, Cy3G, as well as Dp3G but not Pg3G, stimulated atractyloside-inhibited (corresponding to state 4) mitochondrial respiration in the presence of exogenous cytochrome c. This effect may be particularly important at the reperfusion phase after heart ischemia supporting mitochondrial respiration that becomes inhibited due to the loss of cytochrome c from mitochondria (Borutaite et al., 1996).

A possibly related neuroprotective effect of Cy3G without inhibition of cytochrome c release from mitochondria has been demonstrated in mice fed with 2 mg/kg Cy3G and subjected to brain ischemia induced by permanent cerebral artery occlusion (Min et al., 2011). In general, the cardio- or neuro-protective effects of anthocyanins are usually attributed to their antioxidant, free radical scavenging properties (Galvano et al., 2004; Prior and Wu, 2006; Muselik et al., 2007). However, there is accumulating evidence that not all beneficial effects of anthocyanins are due to their antioxidant properties and that other protective mechanisms can be involved. For instance, long, 1 h pre-perfusion of hearts with the anthocyanin delphinidin as well as with the flavonoid myricetin has been shown to protect against ischemia/reperfusion-induced heart injury possibly by a mechanism involving inhibition of signal transducer and activator of transcription 1 (Scarabelli et al., 2009). Another study has reported that 8 h exposure of cultured bovine artery endothelial cells to 0.1 μM Cy3G caused upregulation of eNOS expression and production of NO (Xu et al., 2004), which, in turn, may exert a protective effect during heart ischemia/reperfusion (Bolli et al., 1997; Schulz et al., 2004; Jones and Bolli, 2006). Note that protective effects mentioned above were observed after prolonged treatments with anthocyanins whereas in our study a short 15 min pre-perfusion of the hearts with Cy3G was enough to provide the cardioprotective effect. This points to the acute action of Cy3G in the ischemic heart that may provide some additional time for the development of the late mechanisms of protection related to the induction of gene expression. It is possible that various anthocyanins exert different cardioprotective effects depending on their structures (Fig. 1) and chemical activities, concentrations, duration of treatment, types of cells affected and other factors.

In our study, 20 μM Cy3G was used to protect the ischemic heart against caspase activation. Such a concentration is somewhat higher than the reported post-absorption levels of anthocyanins in plasma: about 0.14–3 mg/l (Tsuda et al., 1999; Ziberna et al., 2010) which would correspond to 0.2–0.6 μM Cy3G, suggesting that concentrations of dietary anthocyanins might be too low for therapeutic purposes. However, the bioavailability of anthocyanins, their accumulation in various tissues and metabolism are still controversial questions that need further investigations.

It should be mentioned that at higher, 40 μM concentrations Cy3G was found to be cardiotoxic (unpublished data). It has been reported that bilberry anthocyanins at concentrations corresponding to 50–100 μM Cy3G caused a marked decrease of coronary flow and an increase in lactate dehydrogenase release in perfused hearts (Ziberna et al., 2010). The reasons for such toxic effect are not entirely clear but may be related to the pro-oxidant activity of anthocyanins leading to the formation of reactive oxygen species, particularly H_2O_2 which besides other damaging effects may cause oxidation of cytochrome *c*. Then if the production of H_2O_2 would exceed the reductive capacity of anthocyanins, the redox state of cytochrome *c* may shift toward the oxidized form, which promotes caspase activation.

One of the novel findings of our study was the observation that Dp3G and Cy3G are potent and powerful reductants of cytochrome c whereas Pg3G, Pn3G and Mv3G were much less effective. While our paper was in preparation, Lagoa and colleagues have reported that representatives of another class of polyphenolic compounds – flavonoids epicatechin, quercetin and kaempferol exhibit cytochrome *c* reducing activities (Lagoa et al., 2011) which are comparable to that of Dp3G and Cy3G. Further studies are required to determine which chemical structures of polyphenolic compounds provide most powerful cytochrome *c* reducing activities as such compounds may have a potential application in inhibiting apoptotic cell death in various pathologies associated with detrimental apoptosis. It is worth mentioning also that, due to the same mechanism, anthocyanins may exert an unwanted effect during chemotherapy when medicines (such as cisplatin) inducing apoptosis via the mitochondrial pathway in tumor cells are used.

Acknowledgements

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References

Akhlaghi M, Bandy B. Mechanisms of flavonoid protection against myocardial ischemia-reperfusion injury. Journal of Molecular and Cellular Cardiology 2009;46:309–17.

Amorini AM, Lazzarino G, Galvano F, Fazzina G, Tavazzi B, Galvano G. Cyanidin-3-O-β-glucopyranoside protects myocardium and erythrocytes from oxygen radical-mediated damages. Free Radical Research 2003;37:453–60.

Barauskaite J, Grybauskiene R, Morkuniene R, Borutaite V, Brown CG. Tetramethylphenylenediamine protects the isolated heart against ischaemia-induced apoptosis and reperfusion-induced necrosis. British Journal of Pharmacology 2011;162:1136–42.

Bialik S, Cryns VL, Drincic A, Miyata S, Wollowick AL, Srinivasan A, et al. The mitochondrial apoptotic pathway is activated by serum and glucose deprivation in cardiac myocytes. Circulation Research 1999;85:403–14.

Bolli R, Manchikalapudi S, Tang XL, Takano H, Qiu Y, Guo Y. The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase. Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. Circulation Research 1997;81:1094–107.

Borutaite V, Brown GC. Mitochondrial regulation of caspase activation by cytochrome oxidase and tetramethylphenylenediamine via cytosolic cytochrome c redox state. Journal of Biological Chemistry 2007;282:31124–30.

Borutaite V, Morkuniene R, Budriunaite A, Krasauskaite D, Ryselis S, Toleikis A, et al. Kinetic analysis of changes in activity of heart mitochondrial oxidative phosphorylation system induced by ischemia. Journal of Molecular and Cellular Cardiology 1996;28:2195–201.

Chen Q, Moghaddas S, Hoppel CL, Lesnefsky EJ. Ischemic defects in the electron transport chain increase the production of reactive oxygen species from isolated rat heart mitochondria. American Journal of Physiology Cell Physiology 2008;294:C460–6.

Dani C, Oliboni LS, Pasquali MAB, Oliveira MR, Umezu FM, Salvador M, et al. Intake of purple grape juice as a hepatoprotective agent in wistar rats. Journal of Medicinal Food 2008;11:127–32.

De Moissac D, Gurevich RM, Zheng H, Singal PK, Kirshenbaum LA. Caspase activation and mitochondrial cytochrome c release during hypoxia-mediated apoptosis of adult ventricular myocytes. Journal of Molecular and Cellular Cardiology 2000;32:53–63.

De Pascual-Teresa S, Moreno DA, Garcia-Viguera C. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. International Journal of Molecular Sciences 2010;11:1679–703.

Galvano F, La Fauci L, Lazzarino G, Fogliano V, Ritieni A, Ciappellano S, et al. Cyanidins: metabolism and biological properties. The Journal of Nutritional Biochemistry 2004;15:2–11.

Hancock JT, Desikan R, Neill SJ. Does the redox status of cytochrome c act as a fail-safe mechanism in the regulation of programmed cell death. Free Radical Biology and Medicine 2001;31:697–703.

Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. Journal of Molecular and Cellular Cardiology 2006;40:16–23.

Lagoa R, Graziani I, Lopez-Sanchez C, Garcia-Martinez V, Gutierrez-Merino C. Complex I and cytochrome *c* are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. Biochimica et Biophysica Acta 2011;1807:1562–72.

Lesnefsky EJ, Hoppel CL. Cardiolipin as an oxidative target in cardiac mitochondria in the aged rat. Biochimica et Biophysica Acta 2008;1777:1020–7.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997;91:479–99.

Lu J, Wu DM, heng YLuB, hang ZF. Purple sweet potato color alleviates D-galactose-induced brain aging in old mice by promoting survival of neurons via PI3K pathway and inhibiting cytochrome c-mediated apoptosis. Brain Pathology 2010;20:598–612.

Min J, Yu SW, Baek SH, Nair KM, Bae ON, Bhatt A, et al. Neuroprotective effect of cyaniding-3-O-glucoside anthocyanin in mice with focal cerebral ischemia. Neuroscience Letters 2011;500:157–61.

Muselik J, Garcia-Alonso M, Martin-Lopez MP, Zemlicka M, Rivas-Gonzalo JC. Measurement of antioxidant activity of wine catechins, procyanidins, anthocyanins and pyranoanthocyanins. International Journal of Molecular Sciences 2007;8:97–809.

Pan Z, Voehringer DW, Meyn RE. Analysis of redox regulation of cytochrome c-induced apoptosis in a cell-free system. Cell Death and Differentiation 1999;6:683–8.

Pasdois P, Parker JE, Griffiths EJ, Halestrap AP. The role of oxidized cytochrome c in regulating mitochondrial reactive oxygen species production and its perturbation in ischaemia. Biochemical Journal 2011;436:493–505.

Prior RL, Wu X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. Free Radical Research 2006;40:1014–28.

Regitz V, Paulson DJ, Hodach RJ, Little SE, Scharpe W, Shug A. Mitochondrial damage during myocardial ischemia. Basic Research in Cardiology 1984;79:207–17.

Rouslin W. Mitochondrial complexes I, II, III, IV and V in myocardial ischemia and autolysis. American Journal of Physiology 1983;244:H743–8.

Saleem MMM, Wilson MT. Kinetic studies on the reduction of cytochrome c. Biochemical Journal 1982;201:433–44.

Scarabelli TM, Marotto S, Abdel-Azeim S, Shoji K, Darra E, Stephanou A, et al. Targeting STAT1 by myricetin and delphinidin provides effcicient protection of the heart from ischemia/reperfusion-induced injury. FEBS Letters 2009;583:531–41.

Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischemia/reperfusion injury. Cardiovascular Research 2004;61:402–13.

Shin WH, Park SJ, Kim EJ. Protective effect of anthocyanins in middle cerebral artery occlusion and reperfusion model of cerebral ischemia in rats. Life Sciences 2006;79:130–7.

Suto D, Sato K, Ohba Y, Yoshimura T, Fuji J. Suppression of the pro-apoptotic function of cytochrome c by singlet oxygen via a heme redox state-independent mechanism. Biochemical Journal 2005;392:399–406.

Toleikis A, Dzeja P, Prashkiavichus A. Functional state and the mechanism of mitochondrial injury of ischemic heart. Soviet Medical Reviews A Cardiology 1989;2:95–132.

Toufektsian MC, de Lorgeril M, Nagy N, Salen P, Donati MB, Giordano L, et al. Chronic dietary intake of plant-derived anthocyanins protect the rat heart against ischemia-reperfusion injury. Journal of Nutrition 2008;138:747–52.

Tsuda T, Horio F, Osawa T. Absorption and metabolism of cyaniding 3-O-beta-D-glucoside in rats. FEBS Letters 1999;449:179–82.

Vaughn AE, Deshmukh M. Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. Nature Cell Biology 2008;10:1477–83.

Xu JW, Ikeda K, Yamori Y. Upregulation of endothelial nitric oxide synthase by cyanidin-3-glucoside, a typical anthocyanin pigment. Hypertension 2004;44:217–22.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997;275:1129–32.

Yao Y, Vieira A. Protective activities of *Vaccinium* antioxidants with potential relevance to mitochondrial dysfunction and neurotoxicity. Neurotoxicology 2007;28:93–100.

Ziberna L, Lunder M, Moze S, Vanzo A, Tramer F, Passasmonti S, et al. Acute cardioprotective and cardiotoxic effect of bilberry anthocyanins in ischemia-reperfusion injury: beyond concentration-dependent antioxidant activity. Cardiovascular Toxicology 2010;10:283–94.