Protecting the Heart Against Ischemia/Reperfusion-Induced Necrosis and Apoptosis: the Effect of Anthocyanins

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Key Words: *heart ischemia and reperfusion; anthocyanins; apoptosis; necrosis; cardioprotection.*

Summary. *Background and Objective. It is well known that cardiomyocyte apoptosis contributes to ischemic heart damage. There is also increasing evidence that the polyphenolic compounds of natural origin, such as anthocyanins, may attenuate ischemia/reperfusion injury though the mechanisms of such protection are not clear. Following our previous studies showing the effect of certain anthocyanins on cytochrome c redox state, mitochondrial functions, and ischemia-induced caspase activation in the heart, here we investigated whether these anthocyanins can rescue cardiac cells from death by the mechanism involving the reduction of cytosolic cytochrome c.*

Material and Methods. Before global ischemia and reperfusion, isolated rat hearts were preloaded with cyanidin-3-O-glucoside (Cy3G) that has high cytochrome c-reducing capacity or pelargonidin-3-O-glucoside (Pg3G) that possesses low reducing activity. Cell death was evaluated assessing apoptosis by the TUNEL method or necrosis measuring the release of lactate dehydrogenase into perfusate.

Results. The perfusion of hearts with 20-μM Cy3G prevented ischemia/reperfusion-induced apoptosis of cardiomyocytes: the number of TUNEL-positive myocytes was decreased by 73% if compared with the untreated ischemic group. The same effect was observed measuring the activity of lactate dehydrogenase as the measure of necrosis: perfusion with 20-μM Cy3G reduced the level of LDH release into the perfusate to the control level. The perfusion of hearts with 20-μM Pg3G did not prevent ischemia/reperfusion-induced apoptosis as well as necrosis.

Conclusions. Cy3G protected the rat heart from ischemia/reperfusion-induced apoptosis and necrosis; meanwhile, Pg3G did not exert any protective effect. The protective effect of Cy3G may be related due to its high capacity to reduce cytosolic cytochrome c.

Introduction

Heart diseases, such as myocardial infarction or ischemic heart failure, are the major causes of death in the developed countries, and unfortunately, the mortality rate from these diseases is still increasing. Heart ischemia or heart ischemia with reperfusion can initiate both apoptosis and necrosis of cardiomyocytes (1, 2). It has been shown that ischemia induces rapid cytochrome c release from mitochondria in the perfused heart (3–5). There is evidence that the reduced form of cytochrome c has little or no ability to activate caspases, and that factors maintaining cytosolic cytochrome c in the reduced form may prevent caspase activation and death of cells in cultures (6–8) or isolated perfused hearts (9). Some compounds of natural origin – anthocyanins, which are widely distributed among colored fruits and vegetables – can be considered as such reducing factors. There is evidence that the consumption of fruits and vegetables, rich in anthocyanins, may play an important role in the prevention of cardiovascular diseases (10–12). Usually, the beneficial effects of anthocyanins are considered to be related to their antioxidant properties (13, 14), though some other mechanisms of action of these compounds may be also involved. We have recently shown that anthocyanins may protect the heart by the mechanism that is not related to their antioxidant activities: cyanidin-3-*O*-glucoside (Cy3G), anthocyanin exhibiting high cytochrome c-reducing activity, was found to prevent ischemia-induced caspase activation without blocking cytochrome c release from mitochondria, while another anthocyanin, pelargonidin-3-*O*-glucoside (Pg3G), possesing low cytochrome c reducing activity, did not exert any protective effect (15). Caspase activation may lead to nuclear apoptosis and cell death; however, in many model systems, the inhibition of caspases may also end up in necrosis. Therefore, in this study, we sought to determine whether the anthocyanin-induced blockage of caspase activation during ischemia may prevent cell death during reperfusion of the heart and whether this effect can be related to the capacity of anthocyanins to reduce cytochrome c.

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Material and Methods

Animal Model. All experimental procedures were performed according to the European Convention

for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (license No. 0006).

All experiments were performed on hearts from 2–4-month-old male Wistar rats. Rats were killed by an increasing concentration of CO_2 in the air followed by cervical dislocation.

The hearts were perfused on a Langendorff perfusion system with the 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, anthocyanins Cy3G and Pg3G at a concentration of 20 μM were added to the perfusate, and the hearts were perfused for another 15 minutes. The control hearts were perfused for the same time but without anthocyanins. We have measured the rate of heart contractions and noticed that the perfusion of the heart with 20-μM Cy3G significantly increased the heart rate during normoxic perfusion (control hearts, 103±7 beats/min; hearts perfused with 20-μM Cy3G, 152±11 beats/min; and hearts perfused with 20-μM Pg3G, 92±6 beats/min). After perfusion with/without anthocyanins, the hearts were subjected to 45-min stop-flow global ischemia at 37°C followed by 30-min reperfusion with/without anthocyanins.

Determination of Lactate Dehydrogenase Activity in Reperfusates. For the evaluation of necrosis, the perfusates from the hearts were collected during reperfusion, and the activity of lactate dehydrogenase (LDH) was measured spectrophotometrically recording a NADH (pH 7.5) oxidation rate at a wavelength of 340 nm (16). LDH activity was measured in the medium containing 0.1-M TRIS-HCl, 1-mM pyruvic acid potassium salt, 0.1-mM NADH (pH 7.5) and was expressed in relative units, showing the amount of the enzyme that is used to oxidize 1-μM NADH during 1 minute.

Measurement of Apoptosis. The tissues of heart ventricles were cut and fixed in 3.7% formalin for 24 hours, then immersed into 30% sucrose solution for 24 hours and frozen at –80°C. Then the samples were cryosectioned (Microm Cryo-Star HM 560 Cryostat) and mounted on microscopic slides. The thickness of criosections was 12 nm. The number of apoptotic cells was detected by the terminal transferase-mediated dUTP nick end-labeling (TUNEL) assay using a CardioTACS in situ apoptosis detection kit (R&D Systems, Minneapolis, MN, USA). All staining procedures were performed according to the manufacturer's instructions. The number of TUNEL-positive cardiomyocytes was counted in at least 10 different microscopic fields on each section containing approximately 100 cardiomyocytes in each. Only cells with clear myocytic morphology were counted.

Statistical Analysis. Data are expressed as mean±SE of at least 3 separate experiments. Statistical comparison between experimental groups was performed by a paired *t* test. A *P* value of <0.05 was considered statistically significant.

Results

In the cardiac sections prepared from control, nonischemic hearts, several myocytes with DNA strand breaks were observed (Fig. 1A). However, the number of TUNEL-positive cardiomyocytes greatly increased after ischemia/reperfusion: from 0.31%±0.06% in control hearts to 5.69%±0.46% (of all cardyomyocytes) after 45-min ischemia and 30-min reperfusion (Fig. 1B and Fig. 2). When the hearts were perfused with 20-μM Cy3G before ischemia/reperfusion, there was a 73% decrease in the number of TUNEL-positive cardiomyocytes if compared with the untreated ischemia/reperfusion group (Fig. 1C and Fig. 2). In contrast, the number of TUNEL-positive cells in the ischemia/reperfusion group treated with Pg3G was not statistically different from that in the ischemia/reperfusion group (Fig. 1D and Fig. 2). This indicates that Cy3G but not Pg3G can prevent ischemia/reperfusion-induced apoptosis in the heart.

Next we investigated whether these anthocyanins have any effect on ischemia/reperfusion-induced necrosis. In these experiments, necrosis was determined by measuring LDH activity in the reperfusate. For this purpose, buffers from control, nonischemic perfused hearts were collected within the last 10 minutes during perfusion and every 10 minutes during the reperfusion period after ischemia. As shown in Fig. 3, 45-min ischemia plus 30-min reperfusion significantly increased LDH activity by 6 times in the perfusion buffer compared with the control group. Perfusion with 20-μM Cy3G reduced the level of LDH released into perfusate to the control levels (control, 0.098±0.031 U/L; 45-min ischemia/30-min reperfusion with 20-μM Cy3G, 0.076±0.003 U/L). Again, the perfusion of the hearts with 20-μM Pg3G had no significant effect on ischemia/reperfusion-induced LDH release: in this group, LDH activity was significantly higher than in the control group and similar to the ischemia/reperfusion group. These data suggest that Cy3G but not Pg3G can protect the heart against ischemia/reperfusion-induced necrosis.

We also tested whether higher concentrations of Cy3G and Pg3G can exert a protective effect. However, when the hearts were preperfused with 40-μM Cy3G or Pg3G before ischemia/reperfusion, the necrosis level was similar as in the ischemia/reperfusion group (0.680±0.115 and

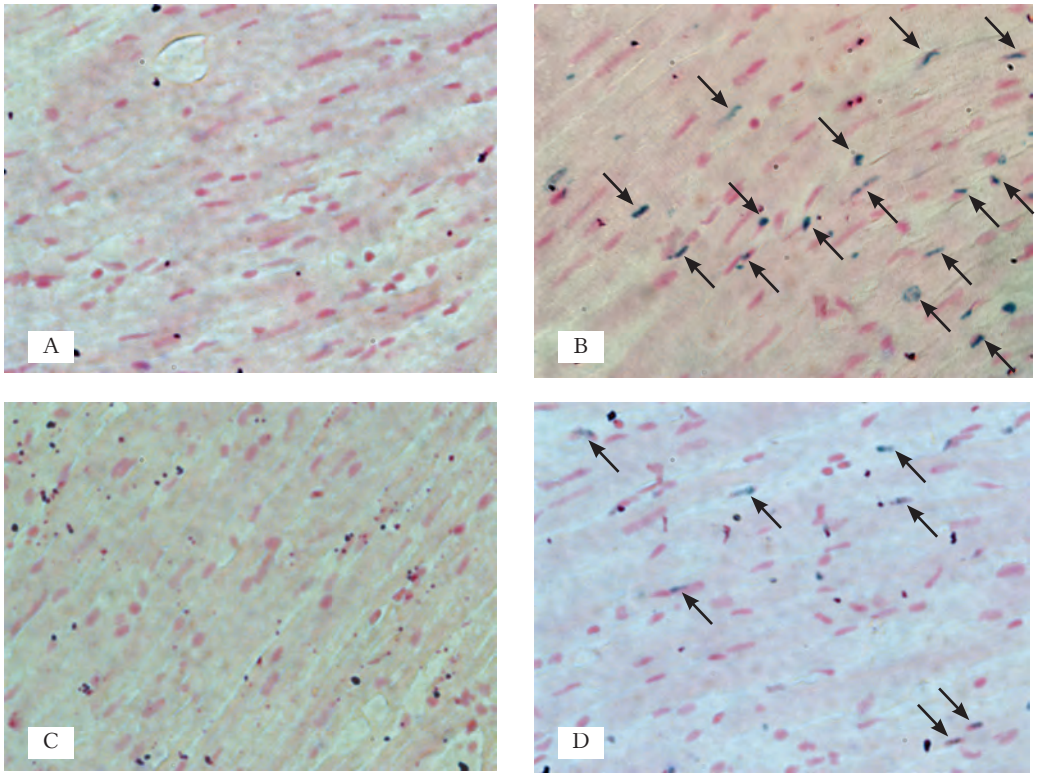

Fig. 1. Strand breaks in nuclear DNA caused by ischemia/reperfusion
Cardiomyocytes with a TUNEL-positive nucleus (dark stains) were determined in the sections of control heart tissue (A); tissue after 45-min ischemia/30-min reperfusion without anthocyanins (B) or with investigated anthocyanins – Cy3G (C) and Pg3G (D) – as described in Methods. Magnification ×40 000.

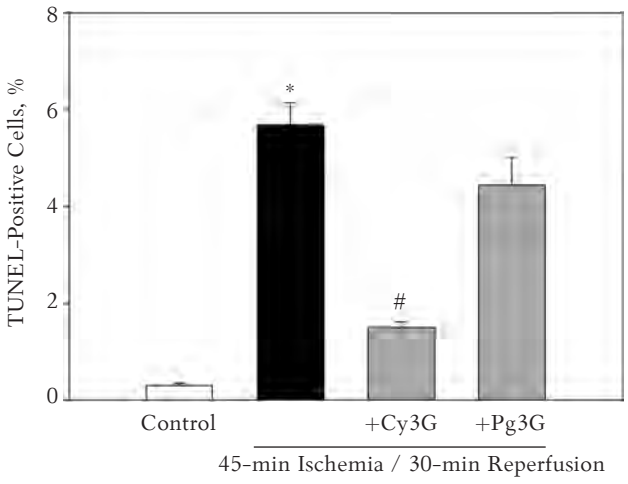

Fig. 2. The effect of Cy3G and Pg3G ischemia/reperfusion induced apoptosis
Perfusion of the hearts with 20-μM Cy3G – an anthocyanin with high reducing capacity – prevented ischemia/reperfusion-induced apoptosis; meanwhile, 20-μM Pg3G – an anthocyanin with low reducing activity – had no effect on ischemia/reperfusion-induced apoptosis. After ischemia/reperfusion, hearts were fixed and stained using the Cardiotacs kit for the determination of cardiomyocytes with fragmented DNA. The number of TUNEL-positive cells is expressed as the percentage of total number of cardiomyocytes in tissue sections investigated. *Statistically significant effect of ischemia/reperfusion if compared with control (P<0.05). #Statistically significant effect of added anthocyanin (P<0.05). Data are expressed as means±SE (3–5 separate experiments).

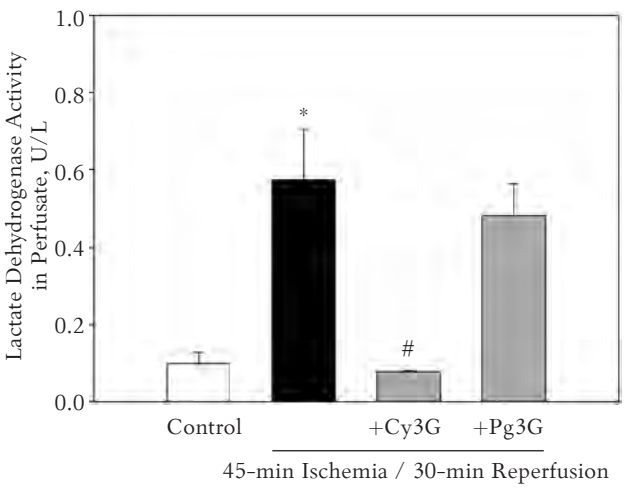

Fig. 3. The effect of Cy3G and Pg3G on ischemia/reperfusion induced necrosis
Perfusion of the hearts with 20-μM Cy3G – an anthocyanin with high reducing capacity – prevented ischemia/reperfusion-induced necrosis; meanwhile, 20-μM Pg3G – an anthocyanin with low reducing activity – had no effect on ischemia/reperfusion-induced necrosis. Coronary effluents during reperfusion were collected for the determination of lactate dehydrogenase activity as described in Methods. *Statistically significant effect of ischemia/reperfusion if compared with control (P<0.05). #Statistically significant effect of added anthocyanin (P<0.05). Data are expressed as means±SE (3–5 separate experiments).

0.695±0.0795 U/L, respectively) suggesting that these anthocyanins at higher concentrations had no protective effect against ischemia/reperfusion-induced necrosis.

Discussion

In recent years, there is growing interest in the pharmacological effects of dietary polyphenolic compounds. Despite, there is still little information about the beneficial effects of anthocyanins on mammalian hearts, since most of the reports were focused on other flavonoid subclasses. The cardioprotective effects of anthocyanins were usually considered to be related to their antioxidant properties (13, 14). In experimental studies investigating the antioxidant properties of anthocyanins, they were usually used at concentrations of 1–100 μM (17, 18), which are similar to the concentrations used in our study. In this study, following our previous work (15), we compared the effects of 2 different anthocyanins – Cy3G and Pg3G – both of which have similar antioxidant properties (18, 19), but differ in their ability to reduce cytochrome c. Based on this, we think that the protective mechanism of Cy3G may be related to its cytochrome c-reducing capacity rather than to antioxidant capacity as both Cy3G and Pg3G have similar antioxidant properties. We demonstrated that Cy3G but not Pg3G exerted protection against ischemia/reperfusion-induced apoptosis and necrosis. It has previously been shown that Cy3G has higher cytochrome c-reducing activity than Pg3G (15). Our findings presented in this study extend our previously published data (15) and confirm that anthocyanins with high cytochrome c-reducing capacity not only suppress caspase activation (without blocking cytochrome c release from mitochondria), but they also can prevent ischemia/reperfusion-induced cell death in the heart. It is well established that heart ischemia/reperfusion induces cytochrome c release from mitochondria to cytosol (3, 4, 20), where cytochrome c can activate caspases via apoptosome formation leading to apoptotic cell death (21). It has been reported that oxidized cytochrome c is more potent in caspase activation than its reduced form (7, 8). The findings of this study are consistent with the proposed mechanism that the reduction of cytosolic cytochrome c (which may be exerted by Cy3G but not Pg3G) prevents or at least delays caspase activation in apoptosome thus preventing apoptotic cell death. Other widely studied cytochrome c-reducing agent, N,N,N',N'-tetramethylphenylene-1,4-diamine (TMPD), which readily crosses cell membranes and rapidly reduces cytochrome c, prevents apoptosis and death of cultured cells (8). As well it was found that TMPD blocks cytochrome c-induced caspase activation, prevents ischemia-induced apoptosis in the perfused rat heart, and inhibits ischemia/reperfusion-induced necrosis (9). The proposed mechanism of this protective effect of TMPD involves the electron reduction of cytochrome c (without decreasing its release), thus inhibiting the activation of caspases. It seems that anthocyanins may possess a similar protective mechanism.

Interestingly, our study demonstrates that Cy3G can also prevent heart ischemia/reperfusion-induced necrosis. This may be explained by assuming that necrosis was secondary to ischemia/reperfusion-induced apoptosis. However, we cannot exclude that Cy3G may exert other protective effects such as the stimulation of mitochondrial respiration after ischemia.

It is also important to note that the protective effect of Cy3G was strongly concentration-depended: only 20-μM concentration prevented cell death in the heart, whereas Cy3G used at higher concentrations (40 μM) had no protective effect. In accordance, Ziberna and colleagues showed that anthocyanins from bilberries at concentrations corresponding to 50–100-μM Cy3G aggravated ischemic/reperfusion injury by increasing lactate dehydrogenase release in the perfused heart (22). This toxic effect is not entirely clear, but anthocyanins can act as pro-oxidants in the systems containing redox active heme and oxygen, leading to the increased formation of reactive oxygen species that damage biologic molecules (23). Under reperfusion conditions, the high concentrations of anthocyanins could further increase oxidative stress, thereby aggravating myocardial injury.

Conclusions

Cy3G protected the rat heart from ischemia/reperfusion-induced apoptosis and necrosis due to the ability of this anthocyanin to reduce cytosolic cytochrome c. Contrary, Pg3G, which possesses low cytochrome c-reducing activity, did not exert any protective effect.

Understanding how cell death is regulated by anthocyanins may have important clinical implications for other pathologies, such as atherosclerosis, which are related to increased apoptosis. Further studies are required to find out whether and how the beneficial effects of anthocyanins depend on their structures, concentrations, duration of treatment, and other factors.

Acknowledgments

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Statement of Conflict of Interest

The authors state no conflict of interest.

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