Free Radical Research, 2003 Vol. 37 (4), pp. 453–460

Cyanidin-3-*O*-β-glucopyranoside Protects Myocardium and Erythrocytes from Oxygen Radical-mediated Damages

ANGELA MARIA AMORINI[a], GIUSEPPE LAZZARINO[a,*], FABIO GALVANO[b], GIOVANNA FAZZINA[a], BARBARA TAVAZZI[c] and GIACOMO GALVANO[d]

[a]*Department of Chemical Sciences, Laboratory of Biochemistry, University of Catania, Viale A. Doria 6, Catania, Italy;* [b]*Department of Agro-Forestry and Environmental Science and Technology, University of Reggio Calabria, P.zza S. Francesco 7, 89061 Reggio Calabria, Italy;* [c]*Institute of Biochemistry and Clinical Biochemistry, Catholic University of Rome "Sacro Cuore", Largo F. Vito 1, 00167 Rome, Italy;* [d]*Department of Agronomical, Agrochemical and Animal Production Sciences, University of Catania, Via Valdisavoja, 5, 95125 Catania, Italy*

Accepted by Professor J.V. Bannister

(Received 5 August 2002; In revised form 21 September 2002)

The cyanidin-3-*O*-β-glucopyranoside (C-3-G) antioxidant capacity towards reactive oxygen species (ROS)-mediated damages was assessed in tissue and cells submitted to increased oxidative stress. In the isolated ischemic and reperfused rat heart, 10 or 30 μM C-3-G protected from both lipid peroxidation (66.7 and 94% inhibition of malondialdehyde (MDA) generation in 10 and 30 μM C-3-G-reperfused hearts, respectively, in comparison with control reperfused hearts) and energy metabolism impairment (higher ATP concentration in 10 and 30 μM C-3-G-reperfused hearts than in control reperfused hearts). These effects were associated to C-3-G permeation within myocardial cells, as indicated by results obtained in the isolated rat heart perfused for 30 min in the recirculating Langendorff mode under normoxia with 10 and 30 μM C-3-G. Protective effects were exerted, in a dose-dependent manner, by C-3-G also in 2 mM hydrogen peroxide-treated human erythrocytes. With respect to MDA formation, an apparent IC_{50} of 5.12 μM was calculated for C-3-G (the polyphenol resveratrol used for comparison showed an apparent IC_{50} of 38.43 μM). The general indications are that C-3-G (largely diffused in dietary plants and fruits, such as pigmented oranges very common in the Mediterranean diet) represents a powerful natural antioxidant with beneficial effects in case of increased oxidative stress, and at pharmacological concentrations it is able to decrease tissue damages occurring in myocardial ischemia and reperfusion.

Keywords: Anthocyanin; Cyanidin-3-*O*-β-glucopyranoside; Nutritional antioxidants; Reactive oxygen species; Oxidative stress; Myocardial ischemia and reperfusion

Abbreviations: C-3-G, Cyanidin-3-*O*-β-glucopyranoside; d.w., Dry weight; HPLC, High-performance liquid chromatography; MDA, Malondialdehyde; RBC, Red blood cells; ROS, Reactive oxygen species

INTRODUCTION

The damaging activity of reactive oxygen species (ROS) towards several biologically important molecules has been clearly evidenced in the last decades. Phenomena such as induction of lipid peroxidation reaction chain,[1] modification of protein structure with change of their activity,[2,3] alteration of nucleic acids[4] have been assessed in various experimental models. Moreover, the demonstration of ROS involvement in different human pathologies, including myocardial ischemia and reperfusion,[5,6] diabetes,[7] viral infections,[8] cerebral trauma,[9,10] has contributed to increase either the interest in finding possible pharmacological remedies, that should be adopted in the clinical practice for avoiding (or at least reducing) the insurgence of the aforementioned ROS-mediated cell and tissue damages, or the attention in determining different nutrients with ROS scavenging activity, that should be assumed with diet for preventing eventual ROS impact by increasing organism antioxidant defenses.

In this light, an always increasing number of studies have evidenced the powerful antioxidant capacity of naturally occurring compounds which

*Corresponding author. Tel.: +39-0957384095. Fax: +39-095337036. E-mail: lazzarig@mbox.unict.it

ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576021000055253

are commonly introduced in human diet, thus underlining the potential benefits of their intake in the prevention of ROS damaging activity. Among the various compounds flavonoids, a large group of polyphenols, including the water-soluble glycosylated forms named anthocyanins, have particularly been studied because of their wide distribution in beverages, fruits and dietary plants. Therefore, it has been demonstrated that polyphenols of red wine increase the antioxidant capacity of serum,[11] thus helping to explain the well known "French paradox" with regard to cardiovascular diseases.[12] Similarly, anthocyanins reduce lipid peroxidation and DNA damage in vitamin E-depleted rats,[13] inhibit the EGF receptor,[14] possess anti-inflammatory properties.[15] Several studies have pointed out the structure–activity relationship of the various anthocyanins,[16,17] evidencing a peculiar antioxidant efficacy for cyanidin-3-*O*-β-glucopyranoside (C-3-G). Very recently, we have shown that the high *in vitro* efficacy of C-3-G to prevent copper-induced human LDL peroxidation is mediated by its effective ROS scavenging capacity rather than to a possible metal chelating activity, the C-3-G ROS scavenging capacity residing in its very negative redox potential ($E_{1/2} = -405$ mV).[18] C-3-G is relatively abundant in the plant kingdom; in fact, strawberry, blackberry, rhubarb, cherry, red cabbage, red onion, cranberry, etc. have a considerable C-3-G content.[19,20] From the nutritional point of view, the importance of characterizing the C-3-G profile of activity is also related to pigmented oranges growing in Sicily (Tarocco, Sanguinello and Moro cultivars) which are certainly peculiar both because of their high C-3-G level[21] and because they represent a typical food largely consumed in the so called Mediterranean diet.

In the present manuscript, we report data on the effect of C-3-G to prevent ROS-mediated tissue and cell damages, as assessed in the isolated Langendorff-perfused rat heart undergoing to ischemia and reperfusion and in isolated human erythrocytes subjected to increased oxidative stress. Possible nutritional relevance and eventual C-3-G pharmacological perspectives are also discussed.

MATERIALS AND METHODS

Chemicals

C-3-G was purchased from Polyphenols AS Laboratories (Hanabryggene Technology Center, Sandnes, Norway). Natural resveratrol, ascorbic acid and 1,1′,3,3′-tetraethoxypropane, which was used to obtain malondialdehyde (MDA) standard as described elsewhere,[22] were obtained from Sigma (St. Louis, Mo, USA). Ultrapure standards for high performance liquid chromatographic (HPLC) analyses were supplied by Boheringer (Mannheim, Germany) and tetrabutylammonium hydroxide, used as the ion-pairing reagent for the HPLC analysis of the various compounds, was purchased as a 55% aqueous solution from Nova Chimica (Cinisello Balsamo, Milan, Italy). All other reagents were of the highest purity available from commercial sources.

Experiments with Isolated Rat Heart

Male Wistar rats of 300–350 g body weight were used in this study. They were fed with a standard laboratory diet and water *ad libitum* in a controlled environment. Animals were anesthetized with an intraperitoneal injection of 50 mg/kg ketamine; after an intravenous administration of 1500 U of heparin into the caudal vena cava, hearts were quickly excised and mounted through the aorta on a stainless steel cannula for a retrograde non-recirculating Langendorff perfusion. Hearts were perfused at a constant hydrostatic pressure of 7.85 kPa (80 cm H_2O) with a Krebs–Ringer bicarbonate solution, pH 7.4, including 2.5 mM $CaCl_2$, 10 mM glucose, 0.6 mM KH_2PO_4 and 1.2 U/l of insulin, saturated with $O_2 + CO_2$ (95/5; v/v). After 20 min of preperfusion, hearts were subjected to 30 min of global normothermic ischemia (obtained by completely abolishing perfusate inflow into the aorta) followed by 30 min of normoxic reperfusion. C-3-G-treated hearts were reperfused with the above described buffer supplemented with 10 or 30 μM C-3-G. At the end of preperfusion or reperfusion, hearts were freeze-clamped by aluminum tongue, pre-cooled in liquid N_2 and immersed in liquid N_2. Atria and non-cardiac tissue were trimmed off, the wet weight was then determined and a piece of myocardial tissue (about 150 mg) was incubated at 150°C for 12 h for the dry weight (d.w.) determination. The remaining heart tissue was deproteinized and processed for HPLC determination of MDA (as index of ROS-mediated lipid peroxidation), ascorbate, nicotinic coenzymes and high-energy phosphates, as previously described.[23,24]

To determine the myocardial cell capability to take up C-3-G, hearts were preperfused for 20 min with the Krebs–Ringer buffer under the non-recirculating Langendorff mode. Afterward, perfusion was continued in the recirculating Langendorff mode for 30 min with 250 ml of the same buffer supplemented with 10 or 30 μM C-3-G. Alternatively, recirculation was effected for 10, 20 or 30 min with 250 ml of 30 μM C-3-G-supplemented Krebs–Ringer medium. After each recirculating time period in presence of C-3-G, hearts were again perfused for 10 min with the Krebs–Ringer buffer under the non-recirculating

Langendorff mode, to ensure the washout of all the extracellular C-3-G. Finally, hearts were freeze-clamped and processed as described above for the HPLC determination of C-3-G concentration within myocardial cells. All the aforementioned perfusions were carried out under normoxic conditions.

Experiments with Isolated Human Erythrocytes

Peripheral venous blood samples were obtained by venipuncture from healthy volunteers and collected into heparinized syringes. Blood was centrifuged at 1850*g* for 10 min at 4°C, plasma and buffy coat were discarded, and packed erythrocytes were washed three times with large volumes of phosphate-buffered saline, pH 7.4, supplemented with 10 mM glucose. After the last washing, red blood cells were resuspended at a 5% hematocrit with the same buffer and incubated at 37°C for 10 min under constant shaking with 1 mM NaN_3, to inhibit catalase activity. Subsequently, erythrocyte suspension was divided into different aliquots to which C-3-G or resveratrol (a different polyphenolic compounds used for comparison) at the following concentrations were added: 0, 1, 2, 5, 10, 20, 50 and 100 μM. Oxidative stress was induced by the addition of 2 mM H_2O_2; this concentration was chosen because it was demonstrated to provoke red blood cell membrane peroxidative damage and energy metabolism alteration without giving rise to significant erythrocyte lysis.[24] Red blood cells incubated with buffer only served as controls. At the end of incubation, erythrocyte suspensions were immersed for 60 s in an ice bath and then centrifuged at 1850*g* for 10 min at 4°C. Deproteinization of packed erythrocytes was carried out by adding ice-cold 1.2 M $HClO_4$ (1/1; w/v) and subsequent sample processing for HPLC determination of MDA and high-energy phosphates was carried out according to Tavazzi *et al.*[24]

HPLC Separation of Metabolites and C-3-G Determination

Determination of different metabolites in both myocardial tissue (100 μl injected) and erythrocyte extracts (200 μl injected) was performed by an ion-pairing HPLC method[23] using a Kromasil 250 × 4.6 mm, 5 μm particle size column (Eka Chemicals AB, Bohus, Sweden), provided of its own guard column, according to a slight modification of the original method.[23,24] Two buffers with the following composition were used: 10 mM tetrabutylammonium hydroxide, 10 mM KH_2PO_4, 0.25% methanol, pH 7.00 (buffer A); 2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol, pH 5.50 (buffer B). A step gradient was obtained as follows: 10 min 100% buffer A; 3 min at up to 90% buffer A; 10 min at up to 70% buffer A; 12 min at up to 55% buffer A; 15 min at up to 45% buffer A; 10 min at up to 25% buffer A; 5 min at up to 0% buffer A. The flow rate of chromatographic runs was 1.2 ml/min and the column temperature was constantly kept at 23°C. The HPLC apparatus consisted of Constametric 2500 pump connected with a SpectraSystem UV6000LP diode array detector (ThermoQuest Italia, Rodano, Milan, Italy) set up between 200 and 300 nm wavelength. Acquisition and analysis of data were performed by a PC provided with the software package (ChromQuest) supplied by HPLC manufacturer. Metabolite concentrations were calculated at 267 nm wavelength (the maximum of MDA absorption) by comparing peak areas of sample runs with those of chromatographic runs of freshly prepared ultrapure standards with known concentrations.

C-3-G determination in the proper myocardial tissue extracts (200 μl injected) was obtained according to Ref. [25], with the diode array detector set up between 200 and 600 nm. Assignment of the C-3-G peak in tissue extracts was performed by comparing retention time and absorption spectrum with those of freshly prepared C-3-G standard with known concentration. Calculation of C-3-G content was carried out at 520 nm (the highest C-3-G maximum of absorption in the visible region).

Statistical Analysis

Differences between control and C-3-G-treated hearts were assessed by the two-tailed Student's *t*-test for unpaired samples, whilst those between control and C-3-G-treated erythrocytes were evaluated by the two-tailed Student's *t*-test for paired samples. A p value of less than 0.05% was considered significant.

RESULTS

In Table I, the effect of C-3-G, at two dose levels, on the ROS-induced peroxidative and metabolic damages of isolated ischemic and reperfused rat hearts is summarized. In control hearts, concomitant increase of MDA (0.069 μmol/g d.w. in reperfused hearts, not detectable in preperfused hearts; $p < 0.001$) and decrease of ascorbate (1.11 μmol/g d.w. in reperfused hearts, 3.03 μmol/g d.w. in preperfused hearts; $p < 0.001$) gave evidence of increased oxidative stress. With respect to these parameters, C-3-G exerted a relevant dose-dependent effect reducing MDA generation (−68 and − 94% in 10 and 30 μM C-3-G-reperfused hearts, respectively, in comparison with control reperfused hearts; $p < 0.001$) and avoiding ascorbate oxidation (+87 and +160% in 10 and 30 μM C-3-G-reperfused hearts, respectively, in comparison with control

TABLE I Effect of cyanidin-3-*O*-β-glucopyranoside (C-3-G) on oxidative damage and modifications of energy metabolism induced by ischemia and reperfusion in the isolated Langendorff-perfused rat heart

	MDA (μmol/g d.w.)	Ascorbate (μmol/g d.w.)	NAD (μmol/g d.w.)	NADP (μmol/g d.w.)	ATP (μmol/g d.w.)	ADP (μmol/g d.w.)	AMP (μmol/g d.w.)
Control preperfused hearts	N.D.[†]	3.03 (0.38)	9.52 (1.41)	0.733 (0.088)	29.35 (2.80)	4.98 (0.91)	1.01 (0.32)
Control reperfused hearts	0.069* (0.011)	1.11* (0.23)	6.04* (1.30)	0.386* (0.033)	14.29* (2.11)	3.40* (0.77)	3.91* (0.53)
10 μM C-3-G-reperfused hearts	0.023*,** (0.010)	2.08*,** (0.19)	6.62*,** (1.12)	0.518*,** (0.031)	19.88*,** (2.56)	3.91*,** (0.46)	2.58*,** (1.13)
30 μM C-3-G-reperfused hearts	0.004*,** (0.001)	2.89*,** (0.36)	8.31*,** (1.40)	0.652*,** (0.055)	23.49*,** (1.81)	4.35*,** (1.31)	1.56*,** (0.83)

Hearts were subjected to 20 min of preperfusion, and after to 30 min of global normothermic ischemia followed by 30 min of normoxic reperfusion by a Krebs–Ringer bicarbonate solution as described in "Materials and Methods" section. C-3-G-treated hearts were reperfused with the above described buffer supplemented with 10 or 30 μM C-3-G. Concentration of various compounds were estimated by direct HPLC measurements. Each value is the mean (S.D.) of five different heart preparations.
*significantly different from control preperfused hearts ($p < 0.05$).
**significantly different from control reperfused hearts ($p < 0.05$).
[†]N.D. = Not Detectable.

reperfused hearts; $p < 0.001$). Concentrations of compounds fundamental for the maintenance of the correct energy state, such as nicotinic coenzymes and high-energy phosphates, were not restored by reperfusion with buffer only. Lower levels of ATP, ADP, NAD and NADP, and higher content of AMP were observed in control reperfused hearts with respect to those recorded at the end of preperfusion (Table I). Again, C-3-G showed a dose-dependent effect, positively influencing all parameters of energy metabolism.

Figures 1 and 2 shows results of experiments effected to establish the extent of the eventual C-3-G uptake by myocardial cells. As shown in Fig. 1, perfusion of isolated rat heart under normoxic conditions in the recirculating Langendorff mode, with a buffer containing 10 or 30 μM C-3-G for 30 min, provoked an intracellular accumulation of 0.021 and 0.056 μmol/g d.w., respectively. The time-course of C-3-G accumulation, obtained by perfusing the hearts with 30 μM C-3-G for 10, 20 or 30 min under normoxic conditions in the recirculating Langendorff mode, suggests the existence of a saturation phenomenon, as indicated by the hyperbolic curve reported in Fig. 2.

The protective effect of increasing C-3-G concentrations on oxidative stress-mediated red blood cell damage, induced by 2 mM H_2O_2 on isolated fresh human erythrocytes, is shown in Fig. 3. A progressive decrease of MDA formation, i.e. of lipid peroxidation caused by ROS, was observed as C-3-G was augmented in red blood cell suspending medium, from a value of 18.45 μmol/l RBC in erythrocyte incubated with 2 mM H_2O_2 only, to a value of 1.02 μmol/l RBC in erythrocyte incubated with 2 mM H_2O_2 and the maximal C-3-G concentration tested, i.e. 100 μM C-3-G produced a 95% reduction of oxidative stress-mediated MDA generation. Resveratrol, at any concentration used, was not as effective as C-3-G in protecting red blood cells from hydrogen peroxide-induced lipid peroxidation; at its maximal concentration resveratrol provoked a decrease by 69% of MDA formation. From data reported in Fig. 3, an apparent IC_{50} of 5.12 and 38.43 μM was calculated for C-3-G and resveratrol, respectively. As a consequence of C-3-G protection towards 2 mM H_2O_2-induced oxidative stress, an amelioration of erythrocyte energy state was also recorded. As shown in Table II, 60 min treatment of erythrocytes with 2 mM H_2O_2 produced a 34.7% ATP decrease, in comparison with the value recorded in control red blood cells; the highest C-3-G concentration used (100 μM) prevented ATP depletion (+44% with respect to erythrocyte incubated with 2 mM H_2O_2 only; $p < 0.001$) and diminished the formation of its catabolites. Also resveratrol had a beneficial effect on energy metabolism of 2 mM hydrogen peroxide-stressed

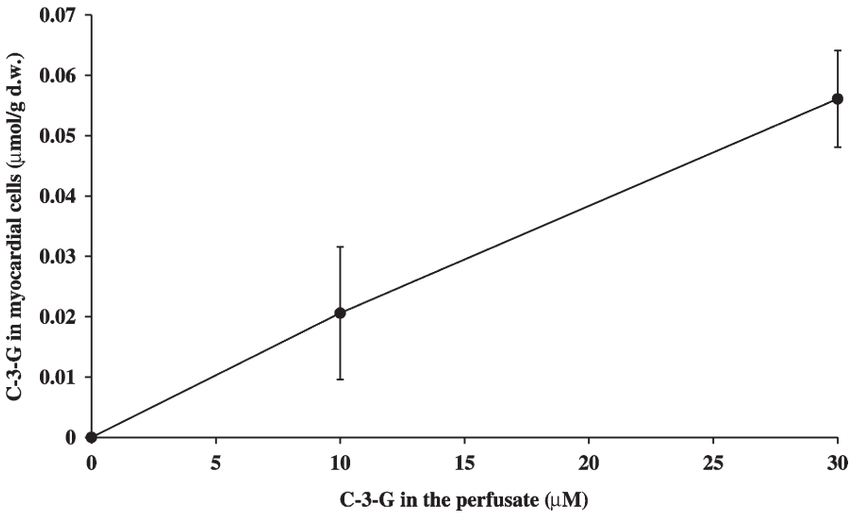

FIGURE 1 Concentration of cyanidin-3-*O*-β-glucopyranoside (C-3-G) in myocardial cells from rat hearts perfused for 30 min in the recirculating Langendorff mode under normoxia with a Krebs–Ringer buffer containing 10 or 30 μM C-3-G. At the end of this perfusion time, to ensure washout of all extracellular C-3-G, hearts were perfused with a C-3-G-free Krebs–Ringer buffer for 10 min in the non-recirculating Langendorff mode and then processed for the HPLC determination of intracellular C-3-G. Each point is the mean of five different heart preparations. Standard deviations are represented by vertical bars.

erythrocyte, even though it was not as efficient as C-3-G (see Table II).

DISCUSSION

Data reported in the present study evidenced the powerful antioxidant capacity of C-3-G to prevent biochemical modifications characteristic of ROS activity in two different cellular and tissue models of increased oxidative stress. In both cases, C-3-G almost completely abolished the onset of lipid peroxidation-based biological membrane damage (decrease of MDA generation), concomitantly preserving cell antioxidant defenses and protecting high-energy metabolism. These findings are particularly remarkable because in both models ROS were produced intracellularly: through mitochondrial malfunctioning[26] and xanthine dehydrogenase into xanthine oxidase conversion,[27] in the isolated rat heart; through the reaction of Fe^{2+} of hemoglobin with hydrogen peroxide, in human erythrocytes

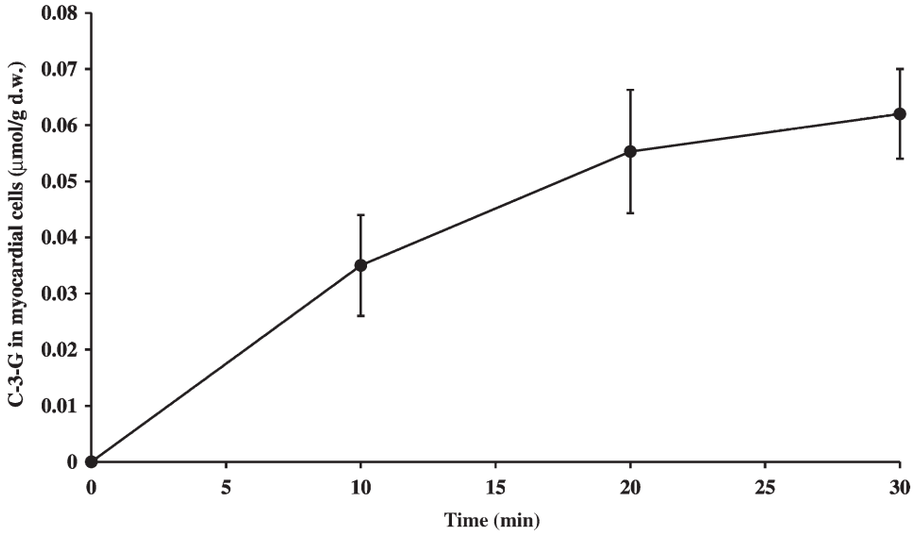

FIGURE 2 Time-course of cyanidin-3-*O*-β-glucopyranoside (C-3-G) accumulation in myocardial cells from rat hearts perfused for 30 min in the recirculating Langendorff mode under normoxia with a Krebs–Ringer buffer containing 30 μM C-3-G. At the end of 10, 20 or 30 min perfusion, to ensure washout of all extracellular C-3-G, hearts were perfused with a C-3-G-free Krebs–Ringer buffer for 10 min in the non-recirculating Langendorff mode and then processed for the HPLC determination of intracellular C-3-G. Each point is the mean of five different heart preparations. Standard deviations are represented by vertical bars.

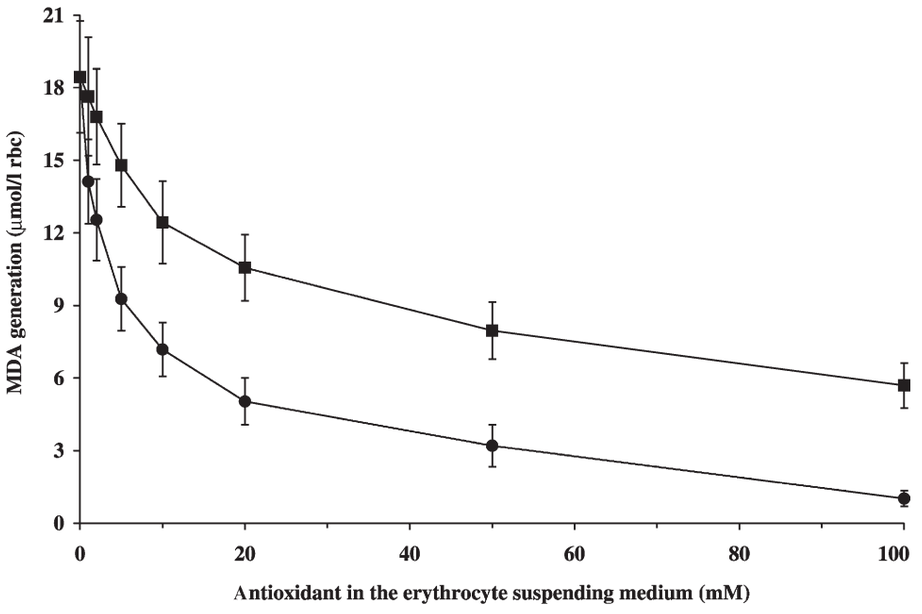

FIGURE 3 Lipid peroxidation of human erythrocytes incubated for 60 min at 37°C under constant shaking with 2 mM hydrogen peroxide and different cyanidin-3-*O*-β-glucopyranoside (C-3-G) (full circles) or resveratrol (full squares) concentrations. Lipid peroxidation was evaluated by a direct HPLC determination of malondialdehyde (MDA). Each point is the mean of six different blood samples. Standard deviations are represented by vertical bars. All values of 2 mM hydrogen peroxide + C-3-G-treated erythrocytes were significantly different from corresponding values of 2 mM hydrogen peroxide + resveratrol-treated erythrocytes ($p < 0.05$).

treated with 2 mM H_2O_2. This implies that in order to exert its antioxidant effects C-3-G should have been able to permeate the plasma membrane, in such an amount to reduce ROS-damaging action. At present, no data have been published showing unequivocally that C-3-G can be found intracellularly in its intact form; at this regard, the only indications on the possibility of this occurrence may be inferred by the detection of C-3-G in plasma (of both rats and human beings) after its oral administration.[28,29] In the isolated rat heart, perfused under normoxic conditions in the recirculating Langendorff mode, we were able to demonstrate that this phenomenon takes place and that the amount of C-3-G detected in the myocardial tissue depended on both the extracellular C-3-G concentration used and the duration of heart perfusion with C-3-G (Figs. 1 and 2). Consequently, the myocardium having received C-3-G during perfusion was more protected than control hearts from ischemia and reperfusion injuries, not only as far as ROS-mediated lipid peroxidation is concerned, but also as far as the cell energy state is pertained. Therefore, it is reasonable to affirm that C-3-G is a powerful antioxidant, the possible pharmacological developments of which in a very relevant clinical field such as that of cardiomyopathies, should be seriously taken into consideration.

From results reported in the present experiments, it is important to observe that C-3-G was effective in counteracting ROS damages mediated by different radical species. In fact, in the isolated rat heart, tissue injuries are supposed to be mainly due to secondarily generated $OH^{\bullet}$ and deriving from $O_2^{\bullet -}$ through the Fenton reaction.[30,31]. Differently, in hydrogen peroxide-treated erythrocytes, oxidative stress has been addressed to the formation of the relatively stable ferryl-hemoglobin radical[32] and, to a lesser extent, to Fenton reaction-produced $OH^{\bullet}$.[33] The direct scavenging action of C-3-G towards

TABLE II Effect of cyanidin-3-*O*-β-glucopyranoside (C-3-G) and resveratrol 100 μM on modifications of energy metabolism induced by 2 mM hydrogen peroxide in isolated human erythrocytes

	ATP (μmol/l RBC)	AMP (μmol/l RBC)	IMP (μmol/l RBC)
Control erythrocytes	1844.51 (280.12)	21.11 (3.45)	9.71 (1.18)
2 mM H_2O_2-treated erythrocytes	1204.31* (182.49)	42.71* (6.28)	197.84* (25.67)
2 mM H_2O_2 + 100 μM C-3-G-treated erythrocytes	1729.55** (212.01)	23.80** (4.31)	24.36*,** (4.46)
2 mM H_2O_2 + 100 μM resveratrol-treated erythrocytes	1501.66*,** (177.78)	31.65*,** (5.27)	89.55*,** (10.36)

Red blood cells incubated with buffer only served as controls. Sample processing for HPLC determination of MDA and high-energy phosphates was carried out as above described in "Materials and Methods" section. Each value is the mean (S.D.) of five different blood samples.
*significantly different from control erythrocytes ($p < 0.05$).
**significantly different from 2 mM-H_2O_2-treated erythrocytes ($p < 0.05$).

superoxide anions (generated by the xanthine–xanthine oxidase system) and hydroxyl radicals (obtained in a Fenton system) has recently been shown *in vitro*, in experiments carried out in our laboratories.[18] Hence, this is the first report demonstrating that C-3-G is capable to reduce ROS-mediated damage to red blood cells, mainly generated by the ferryl-hemoglobin radical, thereby indicating that C-3-G might represent an efficient protection for erythrocytes in case of increased oxidative stress.[3,24,34] The importance of such protective activity is not only due to the diminution of membrane damages caused by ROS-mediated lipid peroxidation, but it is also due to the beneficial effects exerted by C-3-G on red blood cell energy metabolism. It is presumable to hypothesize that higher ATP and lower IMP concentrations found in 2 mM H_2O_2 + 100 μM C-3-G-treated erythrocytes than in 2 mM H_2O_2-stressed erythrocytes might have been caused by the lack of AMP-deaminase activation, which has been shown to be responsible for energy metabolism derangement of oxidatively-stressed red blood cells.[3,24]

C-3-G, as well as several other flavonoids, is largely found in the plant kingdom, in fruits and vegetables of nutritional interest; pigmented oranges, mainly growing in Sicily and being an integral part of the so called Mediterranean diet in Italy, contain at up to 1 mmol/l juice of C-3-G[21,25,35,36] and thus represent one of the main dietary sources of this powerful antioxidant that could easily be found in the Mediterranean area. The assumption of this class of compounds has been shown to increase antioxidant defenses and it has even been supposed that, in populations consuming large quantities of these substances, their regular dietary supplementation might contribute to reduce risks of cardiac attacks. This is the case of the so called "French paradox" for which the daily intake of polyphenol-rich red wines, in spite of an abundant consumption of animal fats, has been suggested to play a role in the significant reduction of cardiovascular disease occurrence in France.[12] It should, however, be recalled that, at present, insufficient information have been produce to unequivocally demonstrate that the regular dietary intake of flavonoid-reach food significantly participates to reduce disease insurgence in human beings. Concerning anthocyanins, additional problems might be related to their not well known bioavailability and eventual organ distribution and accumulation, even though Milbury *et al*.[37] showed that orally-assumed elderberry anthocyanins, mainly containing C-3-G and cyanidin-3-sambubioside, are found in low amounts in their intact glycolsylated forms in plasma of elder women (maximal concentrations reported for C-3-G was about 50 nmol/l plasma). However, it is now well established that the assumption of flavonoid-reach food lead to an increase of the plasma antioxidant capacity,[38] that might represent an effective tool for preventing insurgence of ROS-mediated diseases.

Data reported in the present study indicate that C-3-G antioxidant activity may be exploited at concentrations higher than those obtainable with diet, in acute conditions of severe increase of oxidative stress. This suggests that further studies are necessary not only to better characterize the effects of constant prolonged C-3-G dietary intake, but also to evaluate in depth its possible pharmacological utilization. In this light, it is worth recalling that the antioxidant power of C-3-G, either in the present study or in previous observations,[18] was higher than that of resveratrol, producing better protection of membrane phospholipids and energy metabolism of hydrogen peroxide-stressed erythrocytes as well as of copper-oxidized human LDL.[18].

In conclusion, we have shown that C-3-G is a potent antioxidant in two models of cell and tissue injuries mediated by increased oxidative stress, and we have demonstrated that C-3-G effects are exerted intracellularly thanks to its ability to permeate the myocardial cell plasma membrane. Further studies in different animal models to better characterize the C-3-G pharmacological profile will be performed in the future.

Acknowledgements

This work has been supported by funds of Provincia Regionale di Catania, Italy, research contract on "Antioxidant properties of Sicilian pigmented oranges" (Prof. G. Galvano and G. Lazzarino), by a grant from M.U.R.S.T. (PRIN co-fin 2000) "Studies on antioxidant compounds as protective agents against mycotoxins" (Prof. F. Galvano) and by a grant from Italian National Research Council (C.N.R.) "Progetto Finalizzato Biotecnologie" (Prof. G. Lazzarino).

References

[1] Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) "Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes", *Free Radic. Biol. Med.* **11**, 81–128.

[2] Ravindranath, V. and Reed, D.J. (1990) "Glutathione depletion and formation of glutathione-protein mixed disulfide following exposure of brain mitochondria to oxidative stress", *Biochem. Biophys. Res. Commun.* **169**, 1075–1079.

[3] Tavazzi, B., Amorini, A.M., Fazzina, G., Di Pierro, D., Tuttobene, M., Giardina, B. and Lazzarino, G. (2001) "Oxidative stress induces impairment of human erythrocyte energy metabolism through the oxygen radical-mediated direct activation of AMP-deaminase", *J. Biol. Chem.* **276**, 48083–48092.

[4] Islam, N., Aftabuddin, M., Moriwaki, A. and Hori, Y. (1995) "Detection of DNA damage induced by apoptosis in the rat brain following incomplete ischemia", *Neurosci. Lett.* **188**, 159–162.

[5] Lazzarino, G., Raatikainen, P., Nuutinen, M., Nissinen, J., Tavazzi, B., Di Pierro, D., Giardina, B. and Peuhkurinen, K. (1994) "Myocardial release of malondialdehyde and purine compounds during coronary bypass surgery", *Circulation* **90**, 291–297.

[6] Iqbal, K., Rauoof, M.A., Mir, M.M., Tramboo, N.A., Malik, J.A., Naikoo, B.A., Dar, M.A., Masoodi, S.R. and Khan, A.R. (2002) "Lipid peroxidation during acute coronary syndromes and its intensification at the time of myocardial ischemia reperfusion", *Am. J. Cardiol.* **89**, 334–337.

[7] Uesugi, N., Sakata, N., Horiuchi, S., Nagai, R., Takeya, M., Meng, J., Saito, T. and Takebayashi, S. (2001) "Glycoxidation-modified macrophages and lipid peroxidation products are associated with the progression of human diabetic nephropathy", *Am. J. Kidney Dis.* **38**, 1016–1025.

[8] Treitinger, A., Spada, C., Verdi, J.C., Mirando, A.F., Oliveira, O.V., Silveira, M.V., Moriel, P. and Abballa, D.S. (2000) "Decreased antioxidant defense in individuals infected by the human immunodeficiency virus", *Eur. J. Clin. Investig.* **30**, 454–459.

[9] Cernak, I., Savic, V.J., Kotur, J., Prokic, V., Veljovic, M. and Grbovic, D. (2000) "Characterization of plasma magnesium concentration and oxidative stress following graded traumatic brain injury", *J. Neurotrauma* **17**, 53–68.

[10] Cristofori, L., Tavazzi, B., Gambin, R., Di Pierro, D., Vagnozzi, R., Vivenza, C., Amorini, A.M., Fazzina, G. and Lazzarino, G. (2001) "Early onset of lipid peroxidation following human traumatic brain injury: a fatal limitation for the free-radical scavenger pharmacological therapy?", *J. Investig. Med.* **49**, 450–458.

[11] Whitehead, T.P., Robinson, D., Allaway, S., Syms, J. and Hale, A. (1995) "Effect of red wine on the antioxidant capacity of serum", *Clin. Chem.* **41**, 32–35.

[12] Landrault, N., Poucheret, P., Ravel, P., Gasc, F., Cros, G. and Teissedre, P.-L. (2001) "Antioxidant capacities and phenolics levels of French wines from different varieties and vintages", *J. Agric. Food Chem.* **49**, 3341–3348.

[13] Ramirez-Tortosa, C., Andersen, Ø.M., Gardner, P.T., Morrice, P.C., Wood, S.G., Duthie, S.J., Collins, A.R. and Duthie, G.G. (2001) "Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats", *Free Radic. Biol. Med.* **31**, 1033–1037.

[14] Meiers, S., Kemény, M., Weyand, U., Gastpar, R., von Angerer, E. and Marko, D. (2001) "The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal-growth factor receptor", *J. Agric. Food Chem.* **49**, 958–962.

[15] Wang, H., Nair, M.G., Strasburg, G.M., Chang, Y.C., Booren, A.M., Gray, J.I. and DeWitt, D.L. (1999) "Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries", *J. Nat. Prod.* **62**, 294–296.

[16] Satué-Gracia, M.T., Heinonen, M. and Frankel, E.N. (1997) "Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems", *J. Agric. Food Chem.* **45**, 3362–3367.

[17] Rice-Evans, C., Miller, N.J. and Paganga, G. (1996) "Structure–antioxidant activity relationships of flavonoids and phenolic acids", *Free Radic. Biol. Med.* **20**, 933–956.

[18] Amorini, A.M., Fazzina, G., Lazzarino, G., Tavazzi, B., Di Pierro, D., Santucci, R., Sinibaldi, F., Galvano, F. and Galvano, G. (2001) "Activity and mechanism of the antioxidant properties of cyanidin-3-*O*-β-glucopyranoside", *Free Radic. Res.* **35**, 953–966.

[19] Francis, F.J. (1989) "Food colorants: anthocyanins", *Crit. Rev. Food Sci. Nutr.* **28**, 273–314.

[20] Terahara, N., Yamaguchi, M. and Honda, T. (1994) "Malonylated anthocyanins from bulds of red onion, *Allium cepa L*", *Biosci. Biotechnol. Biochem.* **58**, 1324–1325.

[21] Rapisarda, P., Fallico, B., Izzo, R. and Maccarone, E. (1994) "A simple and reliable method for determining anthocyanins in blood orange juices", *Agrochimica* **38**, 157–164.

[22] Esterbauer, H., Lang, J., Zadravec, S. and Slater, T.F. (1984) "Detection of malonaldehyde by high-performance liquid chromatography", In: Packer, L., ed, *Methods in Enzymology* (Academic Press, New York) **105**, pp 319–328.

[23] Lazzarino, G., Di Pierro, D., Tavazzi, B., Cerroni, L. and Giardina, B. (1991) "Simultaneous separation of malondialdehyde, ascorbic acid and adenine nucleotide derivatives from biological samples by ion-pairing high-performance liquid chromatography", *Anal. Biochem.* **197**, 191–196.

[24] Tavazzi, B., Di Pierro, D., Amorini, A.M., Fazzina, G., Tuttobene, M., Giardina, B. and Lazzarino, G. (2000) "Energy metabolism and lipid peroxidation of human erythrocytes as a function of increased oxidative stress", *Eur. J. Biochem.* **267**, 684–689.

[25] Mondello, L., Cotroneo, A., Errante, G., Dugo, G. and Dugo, P. (2000) "Determination of anthocyanins in blood orange juices by HPLC analysis", *J. Pharmacol. Biomed. Anal.* **23**, 191–195.

[26] Ambrosio, G., Zweier, J.L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P.P., Tritto, I., Cirillo, P., Condorelli, M., Chiariello, M. and Flaherty, J.T. (1993) "Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow", *J. Biol. Chem.* **268**, 18532–18541.

[27] Thompson-Gorman, S.L. and Zweyer, J.L. (1990) "Evaluation of the role of xanthine oxidase in myocardial reperfusion injury", *J. Biol. Chem.* **265**, 6656–6663.

[28] Tsuda, T., Horio, F. and Osawa, T. (1999) "Absorption and metabolism of cyanidin-3-*O*-β-D-glucoside in rats", *FEBS Lett.* **449**, 179–182.

[29] Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K. and Someya, K. (1999) "Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans", *J. Agric. Food Chem.* **47**, 1083–1091.

[30] Dhalla, N.S., Elmoselhi, A.B., Hata, T. and Makino, N. (2000) "Status of myocardial antioxidants in ischemia-reperfusion injury", *Cardiovasc. Res.* **47**, 446–456.

[31] Berenshtein, E., Mayer, B., Goldberg, C., Kitrossky, N. and Chevion, M. (1997) "Patterns of mobilization of copper and iron following myocardial ischemia: possible predictive criteria for tissue injury", *J. Mol. Cell. Cardiol.* **29**, 3025–3034.

[32] Giulivi, C. and Davies, K.J. (1990) "A novel antioxidant role for hemoglobin. The comproportionation of ferrylhemoglobin with oxyhemoglobin", *J. Biol. Chem.* **265**, 19453–19460.

[33] Halliwell, B. and Gutteridge, J.M. (1986) "Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts", *Arch. Biochem. Biophys.* **246**, 501–514.

[34] Comporti, M., Signorini, C., Buonocore, G. and Ciccoli, L. (2002) "Iron release, oxidative stress and erythrocyte ageing(2) (3)", *Free Radic. Biol. Med.* **32**, 568–576.

[35] Rapisarda, P. and Giuffrida, A. (1992) "Anthocyanin levels in Italian blood oranges", *Proc. Int. Soc. Citric.* **3**, 1130–1133.

[36] Rapisarda, P., Carollo, G., Fallico, B., Tomaselli, P. and Maccarone, E. (1998) "Hydroxycinnamic acid as markers of Italian blood orange juices", *J. Agric. Food Chem.* **46**, 464–470.

[37] Milbury, P.E., Cao, G., Prior, R.L. and Blumberg, J. (2002) "Bioavailability of elderberry anthocyanins", *Mech. Ageing Dev.* **123**, 997–1006.

[38] Leighton, F., Cuevas, A., Guasch, V., Perez, D.D., Strobel, P., San Martin, A., Urzua, U., Diez, M.S., Foncea, R., Castillo, O., Mizon, C., Espinoza, M.A., Urquiaga, I., Rozowski, J., Maiz, A. and Germain, A. (1999) "Plasma polyphenols and antioxidants, oxidative DNA damage and endothelial function in a diet and wine intervention study in humans", *Drugs Exp. Clin. Res.* **25**, 133–141.