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Chronic Dietary Intake of Plant-Derived Anthocyanins Protects the Rat Heart against Ischemia-Reperfusion Injury[1–3]

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Abstract

Consumption of flavonoid-rich foods and beverages is thought to reduce the risk of cardiovascular diseases. Whereas the biological activities of flavonoids have been characterized in vitro, there are no clear experimental data demonstrating that chronic dietary intake and intestinal absorption of flavonoids actually protects the heart against ischemia-reperfusion injury. We tested whether long-term consumption of specific flavonoids (anthocyanins) included in normal food could render the heart of rats more resistant to myocardial infarction. Maize kernels that differed specifically in their accumulation of anthocyanins were used to prepare rodent food in which anthocyanins were either present or absent. Male Wistar rats were fed the anthocyanin-rich (ACN-rich) or the anthocyanin-free (ACN-free) diet for a period of 8 wk. Anthocyanins were significantly absorbed and detected in the blood and urine of only rats fed the ACN-rich diet. In Langendorff preparations, the hearts of rats fed the ACN-rich diet were more resistant to regional ischemia and reperfusion insult. Moreover, on an in vivo model of coronary occlusion and reperfusion, infarct size was reduced in rats that ate the ACN-rich diet than in those that consumed the ACN-free diet ($P < 0.01$). Cardioprotection was associated with increased myocardial glutathione levels, suggesting that dietary anthocyanins might modulate cardiac antioxidant defenses. Our findings suggest important potential health benefits of foods rich in anthocyanins and emphasize the need to develop anthocyanin-rich functional foods with protective activities for promoting human health. J. Nutr. 138: 747–752, 2008.

Introduction

Due mainly to the increasing cost of curative medicine, preventive medicine has become crucial for improving health in Western societies. Among nonpharmacological interventions, nutritional recommendations represent a feasible means of developing preventive strategies against chronic degenerative diseases (1–3). Dietary flavonoids have received considerable attention since epidemiological studies suggested that regular consumption of flavonoid-rich foods or beverages is associated with a decreased risk of cardiovascular mortality (4–6). For instance, the Mediterranean diet, which is rich in these bioactive compounds, has been shown to protect against chronic diseases, including coronary heart diseases (2,7,8). Flavonoids include a large group of molecules that are widely distributed in the human diet (9). They are commonly found in fruits and vegetables such as grapes, berries, and onions. Flavonoids have been shown to display a wide range of biological effects although their health benefits have been attributed primarily to their antioxidant properties (10,11). These bioactive molecules have been characterized almost exclusively in vitro, using cell-based assays, in which high concentrations of various conjugates have been used. However, following absorption in the body, flavonoids are metabolized such that plasma and tissues are often not exposed in vivo to high concentrations of the compounds in their original forms (12). Moreover, flavonoids include molecules with a diversity of structures that are often characteristic of a particular plant species. Hence, it is difficult to assess precisely the nature of all the different flavonoids absorbed and metabolized following consumption of plants (vegetables and fruits) present in a human meal. Thus, little is known today about the in vivo health effects following dietary intake of specific flavonoids.

Among the different classes of flavonoids, anthocyanins are a group of water-soluble pigments that may contribute to the

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[3] Supplemental Figure 1 and Supplemental Tables 1–3 are available with the online posting of this paper at jn.nutrition.org.
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health benefits of many fruits, vegetables, and red wine (9). Great effort has been undertaken to understand anthocyanins metabolism (13–15) and several experimental as well as clinical studies suggest that anthocyanins might function as potent in vivo antioxidants (16). In this context, the use of plants that accumulate specific anthocyanins represents a powerful tool for experimental demonstration of the potential health benefits of long-term dietary intake of anthocyanins.

In this study, we aimed to determine whether chronic consumption of plant-derived anthocyanins is cardioprotective. Using isogenic plant material, we produced an anthocyanin-rich (ACN-rich)[9] and an anthocyanin-free (ACN-free) diet for feeding trials in rats. We then evaluated whether absorption of specific anthocyanins could induce resistance against myocardial infarction in both ex vivo and in vivo models of ischemia-reperfusion injury.

Materials and Methods

Maize production. Maize lines used in this study were nongenetically modified and selected from germplasm collections. The maize genotypes were originally in W22 background, homozygous dominant for the *a1*, *a2*, *c1*, *c2*, *bz1*, and *bz2* genes, homozygous recessive for the *pl1* and *b1* genes, and different in their *r1* constitution. The *ACR* genotype carried the *R-r* allele, conferring high anthocyanin accumulation and purple pigmentation to the aleurone of the seed (17,18), whereas the *r-Δ902* genotype (here referred to as *r1*) carried an interstitial deletion involving a region of the long arm of chromosome *10* containing the *r1* locus (kindly provided by J. Kermicle, University of Wisconsin, Madison, WI). Plant and seed tissues homozygous for this deletion are totally devoid of pigment (19). To obtain ears with a high production of kernels, the *ACR* and *r1* genotypes were crossed to a commercial hybrid stock (Dekalb 300) and the F1 progeny seeds were used to produce 2 synthetic populations differing only in their *r1* constitution.

Rats and diets. Special diets were prepared by replacing the maize content (20% of the total) from a standard pellet formula (A04, SAFE) by maize seed powder obtained from either the *r1* (ACN-free) or the *ACR* (ACN-rich) synthetic populations to produce an ACN-free and an ACN-rich diet. Both diets were equal in energy, with macronutrient concentrations of 67% carbohydrate, 23% protein, and 10% lipids (SAFE, France).

Sixty-two male Wistar rats (1 mo old, 75–100 g initial body weight) were purchased from Charles River Laboratories. They were fed a standard diet (A04) for 7 d while acclimating and then randomly assigned to 2 treatment groups for an 8-wk feeding trial, during which they received either the ACN-free or ACN-rich diet, respectively ($n = 31$ per group). Rats from both groups were then randomly assigned to either the ex vivo perfusions, the in vivo study, or the tissue glutathione analysis. Animals were cared for according to the European Communities Council Directive L358–86/609/EEC on the care and use of laboratory animals. All protocols involving living animals were performed under license from the French Ministry of Agriculture (license no. A 38018). Food and tap water were consumed ad libitum. Body weight and food intake were recorded weekly until the end of the protocol.

HPLC and MS analysis of the maize seeds and the diets. Kernels or food pellets (100 mg) were extracted twice with 80% methanol (MeOH). Phenylpropanoids were separated by reverse-phase HPLC (Acquity UPLC with a BEH C18, 1.7 μm, 4.1- $\times$ 50-mm column; Waters) at 25°C. The mobile phase was composed of 98% water, 2% formic solution [5% ammonium formate in formic acid (wt:v)] (solvent A) and 100% acetonitrile (solvent B). The following gradient was applied (flow rate: 0.2 mL/min): initial 0% B; 1.25 min, 0% B; 10 min, 40% B; 11 min, 100% B; 12 min, 100% B; 12.2 min, 0% B; and 13 min, 0% B. Eluted substances were detected with a photodiode array detector (PDA 2996, Waters) between 210 and 600 nm, with a bandwidth of 1.2 nm.

For total anthocyanin determination, 100 mg of food pellet was extracted with 0.1% HCl (vol:vol) MeOH. Anthocyanin concentration was determined spectrophotometrically at 535 nm on 3 replicates, using keracyanin (Extrasynthese, Genay Cedex) for calibration.

For identification of the major anthocyanins, extracts were prepared from 4 g of powdered ACN-rich seeds with 40 mL of 50% MeOH and 20 mL of 100% MeOH. A W600 pump system (Waters) with a preparative HPLC column (X-Bridge MS C18, 5μm, 10 $\times$ 150 mm, Waters) was used for separation. The mobile phase was composed of 87% water, 3% acetonitrile, and 10% acetic acid (solvent A) as well as 40% water, 50% acetonitrile, and 10% acetic acid (solvent B). For elution, the following gradient was used (2 mL/min flow rate): initial 5% B; 80 min, 40% B; 85–90 min, 100% B; and 98 min, 5% B. Separated anthocyanins were collected and subjected to analytical HPLC analysis as described above. The purified anthocyanins were subjected to electron spray ionization tandem MS (ESI-MS/MS) in 50% MeOH with 1% formic acid. The nanoscale effluent from the syringe pump was directed to the NanoLockSpray source of a Q/Tof Premier hybrid orthogonal accelerated time-of-flight mass spectrometer (Waters, MS Technologies Centre). The mass spectrometer operated in positive ion mode with a source temperature of 80°C and a cone gas flow of 30 L/h. A voltage of ~2 kV was applied to the nanoflow sample tip. The MS and MS/MS were acquired in continuum mode with the time-of-flight mass analyser in V-mode of operation and spectra were integrated over 1-s intervals using MassLynx 4.1 software.

HPLC and MS analysis of the animal samples. Urine samples were collected overnight during the last week of animal treatment. After excision of the heart for ex vivo perfusion, blood was collected in the thorax and plasma was obtained after centrifugation. Extraction and analysis of anthocyanins in plasma and urine samples were conducted as previously described (20), using a validated method based on liquid chromatography (LC) MS/MS. Cyanidin-3-glucoside (C3G), cyanidin-3(6-malonyl)glucoside (C3–6MG), cyanidin-3(6-dimalonyl)glucoside (C3–6diMG), and pelargonidin-3(6-malonyl)glucoside (P3–6MG) were quantified in urine samples using calibration curves obtained by adding a commercial standard of C3G to blank rat urine at known concentrations (range 0.5–50 μg/L).

Reduced glutathione/oxidized glutathione analysis. Hearts ($n = 12$ per group) were rapidly excised and the left ventricles were frozen in liquid nitrogen for the glutathione assay. Samples (~100 mg) were homogenized in metaphosphoric acid (5%). Colorimetric determination of reduced (GSH) and oxidized (GSSG) glutathione content was performed using a commercial kit (Bioxytech GSH/GSSG-412, Oxis Research).

Ex vivo perfusion of isolated rat hearts. Rats were anesthetized with pentobarbital (60 mg/kg, intraperitoneally) and heparin (100 IU/100 g body weight) was injected i.v. Hearts ($n = 10$ per group) were excised and perfused according to the Langendorff model as described previously (21). After 15 min of stabilization, regional ischemia was induced by tightening a 5–0 silk suture passed around the left main coronary artery. After 30 min occlusion, hearts were reperfused for 120 min.

In vivo study. Rats ($n = 9$ per group) were anesthetized with ketamine and xylazine mixture (50 and 10 mg/kg, intraperitoneally) and orally intubated. Anesthesia was maintained by ventilating isoflurane (1%) in a mixture of oxygen and air (20%; 80%). The right common carotid artery was cannulated with a PE-30 tubing connected to a pressure transducer to measure peripheral arterial pressure. The catheter was then introduced into the left ventricle for LV recording. The surgical procedure of coronary occlusion was performed as previously described (22). The animals were subjected to 30 min of coronary occlusion followed by 2 h of reperfusion.

Determination of infarct size. On hearts from both the ex vivo and in vivo protocols, the suture was tightened again and Evans Blue (1%) was

[9] Abbreviations used: ACN-free, anthocyanin-free; ACN-rich, anthocyanin-rich; C3G, cyanidin-3-glucoside; C3-6MG, cyanidin-3(6-malonyl)glucoside C3-6diMG, cyanidin-3(6-dimalonyl)glucoside; ESI, electron spray ionization; GSH, reduced glutathione; LC, liquid chromatography; MeOH, methanol; MRM, multiple reaction monitoring; MS/MS, tandem MS; P3-6MG, pelargonidin-3(6-malonyl)glucoside.

injected through the aorta to delineate the nonstained, ischemic zone (risk zone). Infarct size was assessed by triphenyltetrazoliumchloride staining as previously described (22).

Statistical analysis. Data are presented as means ± SEM. We used a nonparametric Kruskal-Wallis 1-way ANOVA followed by a Mann-Whitney U test for data analysis. Differences were considered significant at $P < 0.05$.

Results

Anthocyanin content from the plant material and food pellets. Qualitative HPLC analyses at 535 nm showed that anthocyanins (peaks 1–6) were detected in the purple ACN-rich seeds, whereas they were totally absent from the ACN-free maize seeds (**Supplemental Fig. 1***A*,*B*). Moreover, overlay of typical chromatograms of food pellet extracts showed that the same anthocyanins remained in the ACN-rich diet (Supplemental Fig. 1*C*). HPLC profiles at 280 and 350 nm did not differ in total phenylpropanoids or flavonol composition between the different food pellets (data not shown). Peaks 1, 2 to 4, 5, and 6 (Supplemental Fig. 1*B*,*C*) were respectively identified as C3G, C3–6MG, P3–6MG, C3–6diMG by HPLC and ESI-MS/MS (**Table 1**). Quantitative analysis indicated that the ACN-rich diet contained ~0.24 ± 0.01 mg/g anthocyanin.

Anthocyanin detection in animal samples. Food consumption and body weight gain did not differ between the 2 groups (**Supplemental Table 1**).

Following dietary treatments, anthocyanins were not detected by LC-MS/MS in plasma or urine samples from rats fed the ACN-free diet (data not shown). Unmodified anthocyanin glucosides (C3G, C3–6MG, C3–6diMG, and P3–6MG) were detected in plasma samples from animals fed the ACN-rich diet (data not shown); however, concentrations of these compounds were below the limit of quantification of the analytical method (20). Rats that consumed the ACN-rich diet had quantifiable urinary concentrations of C3G (57.6 ± 21.0 nmol/L), C3–6MG (38.2 ± 15.4 nmol/L), P3–6MG (19.1 ± 11.7 nmol/L), and C3–6diMG (24.6 ± 16.3 nmol/L) (**Fig. 1***A–D*).

Ex vivo study. Hearts of rats fed the ACN-rich or the ACN-free diet were isolated and perfused. Before, during, and after regional ischemia, cardiac function did not differ between the 2 groups (**Supplemental Table 2**). However, the size of myocardial infarction, expressed as percent of the ischemic zone (i.e. risk zone; **Fig. 2***A*) was significantly reduced in hearts of rats fed the ACN-rich diet (Fig. 2*B*).

The effects of the dietary anthocyanins on the cardiac redox status were evaluated by measuring the tissue glutathione content. Preischemic cardiac total glutathione and GSH were greater in rats fed the ACN-rich diet than in those fed the ACN-free diet (Fig. 2*C*).

In vivo study. We analyzed the effects of the 8-wk dietary treatment on the cardiac function in vivo. The groups did not differ in heart rate and arterial and intraventricular pressures (**Supplemental Table 3**). Anesthetized rats were then subjected to in vivo transient coronary occlusion followed by reperfusion. Although the size of the risk zones was the same in the 2 groups (**Fig. 3***A*), infarct size was significantly reduced in rats fed the ACN-rich diet compared with those fed the ACN-free diet (Fig. 3*B*).

Discussion

The present investigation provides, for the first time, to our knowledge, analysis of the specific effects of flavonoids in the diet on cardioprotection through the use of isogenic dietary material. We selected 2 maize lines with the same genetic background, differing only in their capacity to synthesize and accumulate anthocyanins in their seeds (17,18). Incorporation of these seeds into a standard pellet formula allowed the production of an ACN-rich and an ACN-free diet as confirmed by HPLC and mass spectrometry analysis. Following the 8-wk dietary treatment, anthocyanins were detected by LC-MS/MS in blood and urine of animals fed the ACN-rich diet only. These data are consistent with previous reports indicating that anthocyanins can be absorbed intact as glucosides following oral supplementation or stomach infusion with an anthocyanin mixture in human or rat (13–15).

TABLE 1 Identification of the major anthocyanins detected in the ACN-rich maize seeds[1]

Compound	R_t	UV/Vis λmax	LC-ESI MS	ESI MS/MS fragments[2]	Structure
	min	*nm*		*m/z* $[M+H^+]$	
1	4.8	280/516	449	449.39 (Cy+Glc) 287.08 $(Cy)^+$	Cyanidin-glucoside
2	5.1	282/516	535	535.11 (Cy+Glc+Mal)$^+$ 287.08 $(Cy)^+$	Cyanidin-malonylglucoside
3	5.3	282/516	535	535.11 (Cy+Glc+Mal)$^+$ 287.08 $(Cy)^+$	Cyanidin-malonylglucoside
4	5.5	281/519	535	535.11 (Cy+Glc+Mal)$^+$ 287.08 $(Cy)^+$	Cyanidin-malonylglucoside
5	5.8	281/515	519	519.10 (Pg+Glc+Mal)$^+$ 271.06 $(Pg)^+$	Pelargonidin-malonylglucoside
6	6	281/516	621	621.10 (Cy+Glc+2Mal)$^+$ 535.11 (Cy+Glc+Mal)$^+$ 287.08 $(Cy)^+$	Cyanidin-dimalonylglucoside

[1] Peaks 1–6 (as indicated in Supplemental Fig. 1*B*,*C*) were identified based on photo diode array absorbance and mass fragmentation patterns. The purified substances were analyzed by HPLC and ESI-MS/MS.
[2] Fragments: cyanidin (Cy); glucoside (Glc); malonyl (Mal); dimalonyl (2Mal); pelargonidin (Pg).

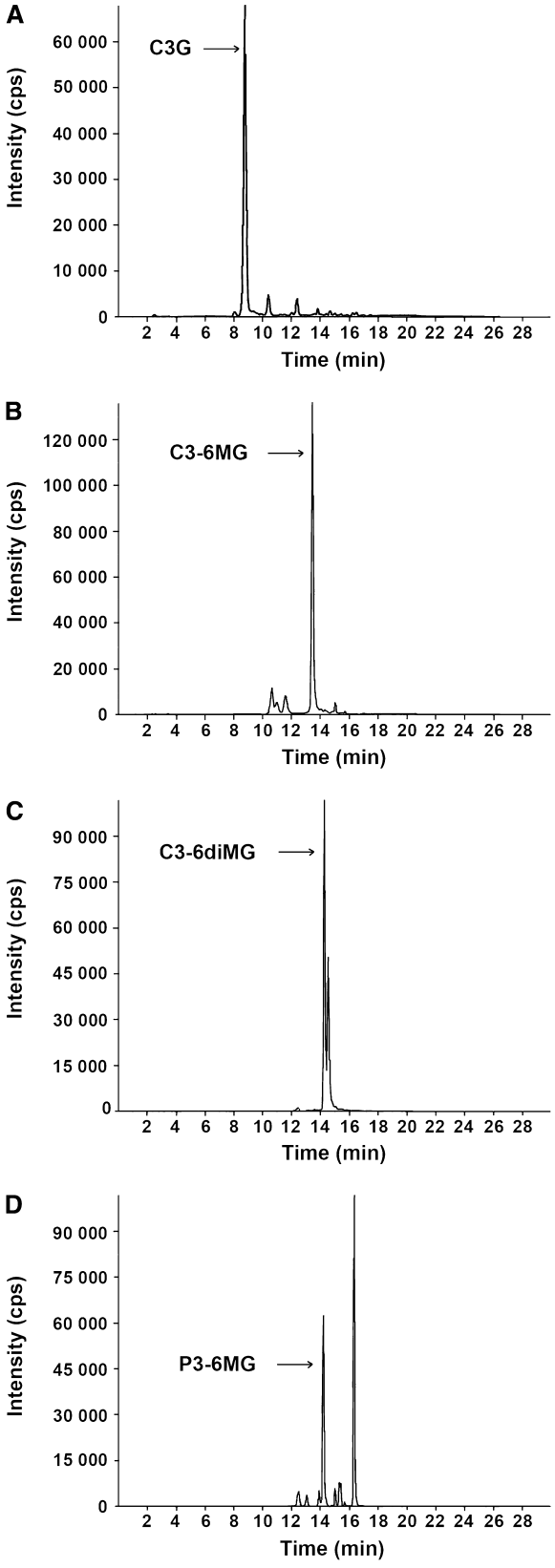

FIGURE 1 Representative multiple reaction monitoring (MRM) chromatograms of urine samples obtained from rats fed the ACN-rich diet. Arrows indicate C3G, MRM transitions 449.1/287.1, retention time 8.8 min (*A*); CMG, MRM transition 535.0/287.0, retention time 13.4 min (*B*); C3-diMG, MRM transition 621.1/287.0, retention time 14.2–14.5 min (*C*); P3–6MG, MRM transition 519.1/271.0, retention time 14.2 min (*D*). CPS, Counts per second.

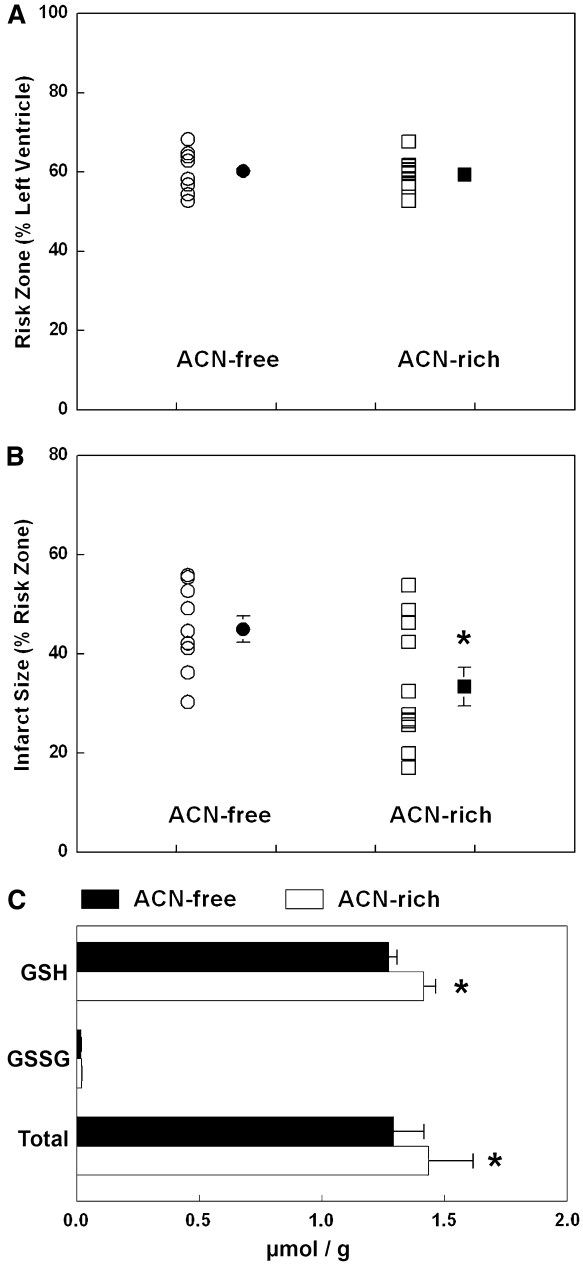

FIGURE 2 Effect of chronic consumption of anthocyanins on the sensitivity to ex vivo ischemia-reperfusion injury as assessed by risk zone (*A*) and infarct size (*B*) and the cardiac glutathione concentrations (*C*) in rats. Open symbols represent individual values and solid symbols are means ± SEM, $n = 10$/group. *Different from ACN-free, $P < 0.05$. GSHt: total glutathione. Bars represent means ± SEM.

We then tested the potential cardioprotective effects of 8-wk dietary intake of flavonoids using an ex vivo model of isolated perfused rat heart. Our results suggest that anthocyanins can induce a state of myocardial resistance as evidenced by a reduced infarct size following regional ischemia and reperfusion. This cardioprotection is independent of any circulating blood cells or of any noncardiac factors such as neural or hormonal regulation, because isolated buffer-perfused preparations were used.

As the beneficial effects of dietary anthocyanins were observed ex vivo in isolated perfused hearts, it is unlikely that the protection might be due to a direct antioxidant effect of the circulating anthocyanins. It has been suggested recently that flavonoids might affect endogenous antioxidant defenses of cells

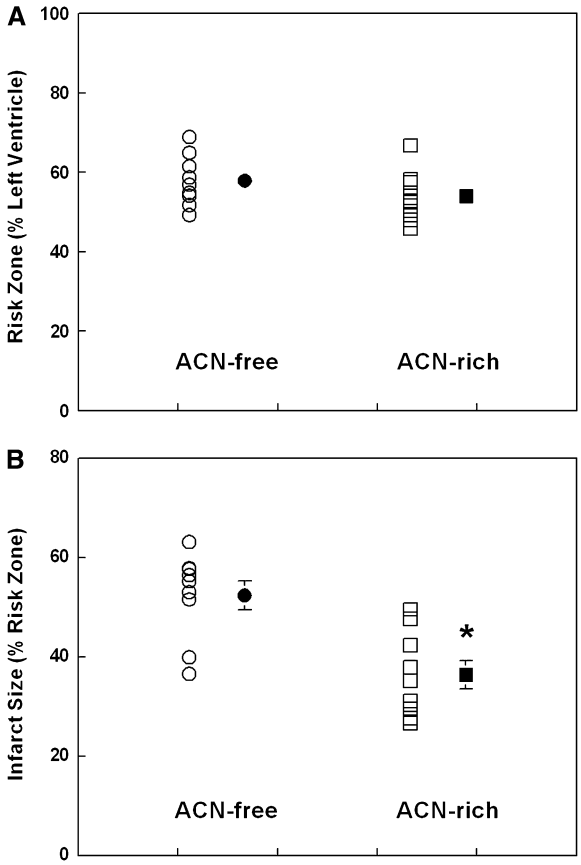

FIGURE 3 Effect of chronic consumption of anthocyanins on the sensitivity to in vivo ischemia-reperfusion injury as assessed by risk zone (*A*) and infarct size (*B*). Open symbols represent individual values and solid symbols are means ± SEM, $n = 9$/group. *Different from ACN-free, $P < 0.01$.

by modulating the glutathione and glutathione-related enzyme systems (23). Glutathione plays an important role in many diseases and regulation of the intracellular levels of glutathione may be one of the mechanisms by which some dietary factors, including anthocyanins, can interfere with disease development. Moreover, flavonoids have been shown to increase the expression of the gene encoding the γ-glutamylcysteine synthetase catalytic subunit, a protein reported to be the rate-limiting step in glutathione synthesis (24,25). The preischemic cardiac total glutathione and GSH in animals that received the ACN-rich diet were greater than in those fed the ACN-free diet. These data therefore suggest that the protection against ischemia-reperfusion injury induced by dietary anthocyanins might be related, at least in part, to an improvement in the antioxidant defenses of the heart.

A critical concern was to determine whether cardioprotection remained operational under more realistic conditions, e.g. when the heart is connected to the brain and nerves and exposed to all the circulatory factors present in the blood. Animal studies that have investigated the cardioprotective effects of various natural or synthetic flavonoids have focused mainly on the acute pharmacological activity of these compounds. Indeed, in vivo studies using mouse (26), rabbit (27), or sheep (28) models of coronary occlusion and reperfusion have demonstrated the acute cardioprotection afforded by i.v. injections of natural or synthetic flavonoids. Similarly, Kim et al. (29) recently showed that oral pretreatment with a single high dose of soybean-derived anthocyanins reduced myocardial damage following in vivo ischemia reperfusion injury in rats. However, to our knowledge, there are no experimental data available on the protective effects of long-term dietary intake of flavonoids or, specifically, anthocyanins, on the heart in vivo. In this study, we showed that in anesthetized rats, following coronary occlusion and reperfusion, infarct size was significantly smaller in rats that received the ACN-rich diet compared with those fed the ACN-free diet. This result therefore suggests that the anthocyanin-induced myocardial resistant state persists in vivo, a critical issue when considering the application of this preventive strategy to human medicine.

One could assume that the cardioprotection observed in vivo may be due to an acute effect of circulating anthocyanins absorbed during the last meal. However, pharmacokinetic studies have shown that in plasma, anthocyanins peak ~1 h after oral consumption and then rapidly decrease (14,15). Anthocyanins were below the limit of quantification in plasma samples from rats fed the ACN-rich diet, suggesting that, at the time of ischemia, in vivo anthocyanin levels were probably too low to exert direct antioxidant effects.

From our data, we calculated that rats fed the ACN-rich diet received ~0.08 $mg \cdot kcal^{-1} \cdot d^{-1}$ anthocyanins. In Western populations, the average intake of anthocyanins is estimated to be 12 mg/d (30), equivalent to a daily intake of 6 $\mu g/kcal^{-1} \cdot d^{-1}$ [6 $\mu g/(4.184\ kJ^{-1} \cdot d)$], assuming a daily energy intake of 2000 kcal (8.4 kJ). In our studies, rats therefore received ~13-fold more anthocyanins than most people following a standard Western-type diet. Many typical Mediterranean foods and beverages such as fruits, vegetables, and wines are known to be rich in anthocyanins (9,30). The anthocyanin content of the traditional Mediterranean diet is much higher than that of the Western diet, which might partly explain why the Mediterranean diet is cardioprotective (2,7,8). Therefore, our data support strongly preventive strategies based on long-term adoption of healthy lifestyles primarily characterized by adoption of a Mediterranean-type diet.

Many flavonoids are available in the human diet and the health-promoting properties of dietary flavonoids suggested by the epidemiological studies have been independently allocated to different subclasses of molecules (4–6). Moreover, the Mediterranean diet offers several sources of various flavonoids that might together contribute to the prevention of cardiovascular diseases. In contrast, in this study, the beneficial effects of dietary anthocyanins involved only a small number of molecules, because the composition of the food pellets was identical except for the maize-derived anthocyanins.

The anthocyanin content in the food pellets was accurately determined and the follow-up of the food consumption allowed a good estimation of the daily anthocyanin intake. Although intact glucosides have been detected in several mammal species following oral consumption (13–15), rats and humans might differ in anthocyanin absorption, because gut microflora has been shown to play an important role in the biotransformation of anthocyanins (31). Therefore, the extrapolation of the beneficial effects of anthocyanin consumption and absorption from rats to humans needs caution.

In summary, our study provides the first experimental evidence, to our knowledge, that long-term dietary intake of plant-derived anthocyanins and actual intestinal absorption of these compounds by animals made the myocardium less susceptible to ischemia-reperfusion injury ex vivo as well as in vivo. The mechanisms of protection are possibly related to improved endogenous antioxidant defenses of the heart. Whatever the general dietary pattern adopted by any population, our results

enhance the interest in recommending anthocyanin-rich foods (and beverages) for the promotion of human health.

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