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Distribution of Betalain Pigments in Red Blood Cells after Consumption of Cactus Pear Fruits and Increased Resistance of the Cells to ex Vivo Induced Oxidative Hemolysis in Humans

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Betalain pigments are bioavailable phytochemicals recently acknowledged as natural radical scavengers. This work, which extends previous research on the postabsorbitive fate of dietary betalains, investigated the distribution of betanin and indicaxanthin in red blood cells (RBCs) isolated from healthy volunteers ($n = 8$), before and during the 1−8 h interval after a cactus pear fruit meal, and the potential antioxidative activity of the pigments in these cells. A peak concentration of indicaxanthin (1.03 ± 0.2 μM) was observed in RBCs isolated at 3 h after fruit feeding, whereas the concentration at 5 h was about half, and even smaller amounts were measured at 8 h. Indicaxanthin was not detected at 1 h. Betanin (30.0 ± 5.2 nM) was found only in RBCs isolated at 3 h from fruit feeding. In comparison with homologous RBCs before fruit ingestion, a significant delay ($P < 0.05$) of the onset of an ex vivo cumene hydroperoxide (cumOOH)-induced hemolysis was evident in the RBCs isolated at 3 h (33.0 ± 4.5 min) and at 5 h (16.0 ± 2.0 min). Neither vitamins C and E nor GSH was modified in the RBCs at any time point. Blood collected from the same volunteers after a 12-h fasting was incubated with the purified betalains in the range of 5−25 μM, to enrich the erythrocytes with either betanin or indicaxanthin, and then the cells were exposed to cumOOH. When compared to the relevant nonenriched cells, the betalain-enriched erythrocytes exhibited an enhanced resistance to the cumOOH-induced hemolysis, which was positively correlated ($r^2 = 0.99$) to the amount of the incorporated compound. On a micromolar basis, betanin and indicaxanthin showed a comparable effectiveness. Taken together, these findings provide evidence that human RBCs incorporate dietary betalains and support the concept that these phytochemicals may offer antioxidative protection to the cells.

KEYWORDS: Betanin; indicaxanthin; cactus pear; antioxidative potential; in vivo; human red blood cells

INTRODUCTION

Diets rich in fruits and vegetables have long been recognized to exert beneficial effects. Apart from essential nutrients and vitamins, plants provide various phytochemicals that are considered to be important contributors to health promotion. Although the mechanisms through which these compounds can act in the body are not completely understood, the radical-scavenging and antioxidant activities of the molecules may play a significant role (*1*−*3*).

The fruits of cactus pear, a plant spread over the Mediterranean and other warm areas of the planet, are characterized by phytochemicals such as betalains (*4*−*6*), unique pigments poorly represented among edible vegetables (*7*−*9*). Beneficial properties of these fruits have recently emerged. We reported that a 2 week supplementation with fruits of the Sicilian cactus pear decreased the level of plasma markers of oxidative stress and of lipid hydroperoxides of circulating low-density lipoprotein (LDL) in healthy humans, an effect which has appeared independent of the consumption of vitamin C with the fruit (*10*).

Betanin and indicaxanthin (**Figure 1**), the betalain pigments occurring in the cactus pear fruit, long known as safe food colorants (*11*−*13*), have recently been investigated as antioxidant compounds. Because of their redox properties (*14*−*18*) these molecules can scavenge effectively various radicals generated in chemical (*18*) as well as biological (*16*, *19*) systems. Both phytochemicals have been shown to inhibit microsomal membrane oxidation (*16*) and the oxidation of human LDL in vitro (*16*, *19*). More importantly, as dietary components, betanin and indicaxanthin are bioavailable (*16*, *20*, *21*) and have recently been evaluated in human plasma and LDL over an 8 h period after the consumption of cactus pear fruits (*21*). To further investigate the postabsorbitive distribution of betalains, in this work we checked the presence of betanin and indicaxanthin in human red blood cells (RBCs)

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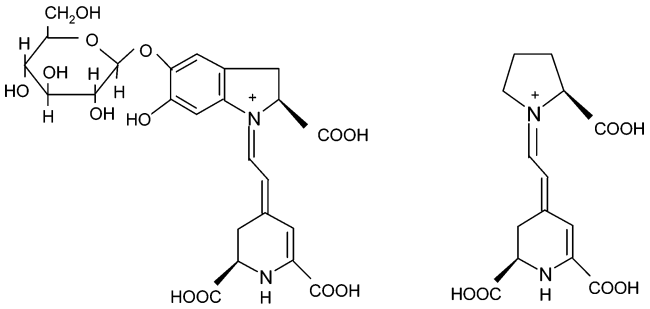

Figure 1. Molecular structures of betanin and indicaxanthin.

after a meal consisting of cactus pear fruit pulp. We then evaluated the resistance to oxidative injury by organic hydroperoxide of the RBCs isolated before and at time intervals following the intake of the fruits. Assays have also been carried out to investigate the antioxidative potential of betalains in a comparable oxidation model of RBCs that had been enriched ex vivo with either purified betanin or indicaxanthin.

EXPERIMENTAL METHODS

Cactus pear fruits from Sicilian cultivars were obtained from a local market at comparable ripening stages and were utilized within 48 h from collection. In the morning of the study, fruits were peeled, and the pulp was minced and divided into eight portions of 500 g. Extraction and analysis of duplicate samples (*18*) showed that each portion provided 20 mg of betanin and 25 mg of indicaxanthin.

Subjects and Experimental Design. Eight healthy nonsmoking volunteers (five females and three males; ages 32.65 ± 10.11 years; body mass index = 21 ± 2.0 kg/m^2; mean ± SD) participated in this study. The study protocol was in accordance with the Helsinki Declaration of 1975, as revised in 1983, and was fully explained to all volunteers, who gave their informed written consent. On the morning of the sampling day, an intravenous catheter was inserted into one forearm of the volunteers after they had fasted overnight. Subjects then consumed one portion of fresh cactus pear fruit pulp. Blood samples (10 mL) were collected in EDTA (1 mg/mL) before (0 h) and at 1, 3, 5, and 8 h after the fruit meal. The subjects were instructed not to eat anything and to drink only water. After each sampling, RBCs were obtained by centrifugation at 2000*g* for 15 min at 4 °C. RBCs were washed three times with phosphate saline buffer (PBS), pH 7.4. Supernatant and buffy coat were carefully removed by aspiration after each wash. Suspensions of RBCs in PBS were carried out to obtain a final hematocrit (HT) suitable for the experiments described below.

Spiking of Blood with Betalains. Indicaxanthin and betanin were purified from the cactus pear fruit pulp as reported (*18*). Blood from each participant in the study was collected in EDTA after an overnight fasting, divided into suitable aliquots, and incubated, at 37 °C, for 15 min, in the absence or in the presence of either betanin or indicaxanthin in PBS (5.0−25 μM). Then the RBCs were isolated, washed, and resuspended in PBS.

HPLC Analysis of Betalains. Either plasma (1 mL) or RBCs (HT 10%, 7.5 mL) were extracted with 3 volumes of chloroform/methanol (2:1, v/v). The methanol phase was dried under nitrogen, resuspended in 1% acetic acid in water, and analyzed on a Varian Microsorb C-18 column (4.6 × 250 mm, Varian, Palo Alto, CA), eluted with a 20 min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with a flow of 1.5 mL/min. Spectrophotometric revelation was at 536 nm for betanin and at 486 nm for indicaxanthin. Under the conditions described, indicaxanthin eluted after 8.15 min and betanin after 11.0 min. An automatic wavelength change after 9.30 min allowed the detection of both compounds in the same sample.

Quantitation was by reference to standard curves constructed with 5−100 ng of purified compound and by relating the amount of the compound under analysis to the peak area.

Oxidative Hemolysis. A suspension of RBCs in PBS (HT 1%; HbO_2 = 170 ± 2 μM per heme group) was incubated at 37 °C in the presence of a 300 μM cumene hydroperoxide (cumOOH) ethanol solution. The volume added never exceeded 0.5% of the total incubation volume. The extent of hemolysis at any given incubation time was determined as follows. A volume (0.2 mL) of the incubation mixture was diluted with 10 volumes of PBS and centrifuged at 1000*g* for 10 min to precipitate the cells. The absorbance of the supernatant was then evaluated at 540 nm. Similarly, a volume of the incubation mixture was treated with 10 volumes of 5 mM sodium phosphate buffer, pH 7.4 (hypotonic PBS), and briefly exposed to an ultrasonic bath to yield complete hemolysis. The supernatant after a centrifugation at 1000*g* for 10 min was evaluated spectrophotometrically at 540 nm. The percentage of hemolysis was calculated from the ratio of the absorbances.

Measurement of Vitamin C, Vitamin E, and Glutathione in RBCs. Vitamin C was measured as follows. RBCs (HT 10%, 300 μL) were treated with 100 μL of 1.0 M perchloric acid. After centrifugation at 1000*g* for 10 min, the supernatant was withdrawn and ascorbic acid determined by HPLC with revelation at 266 nm as reported (*22*). Minor changes included the length of the HPLC column (25 × 0.46 cm) and isocratic elution with 10 mmol/L KH_2PO_4 buffer, containing 10 mmol/L tetrabutylammonium bromide, in 1% methanol in water, pH 7.0, at 1.2 mL min^{-1}. The retention time of ascorbate was 5.3 min.

Vitamin E was measured in RBCs (HT 10%, 5 mL) collected at 1000*g* for 10 min. The cell pellet was resuspended with 1 mL of PBS containing 0.5% pyrogallol and mixed with 2 volumes of absolute ethanol, followed by two successive extractions with 6 and 2 volumes of petroleum ether. The organic extracts were gathered, dried under a nitrogen stream, and resuspended in several microliters of methanol, and vitamin E was separated by HPLC using a Supelco Supelcosil (Bellefonte, PA) LC-18 column (0.46 × 25 cm). The eluent was methanol with a flow rate of 1.0 mL min^{-1}. Fluorometric detection was with excitation at 290 nm and emission at 335 nm. Quantitation was by reference to standard curves constructed with 5−100 ng of purified compounds and by relating the amount of the compound under analysis to the peak area.

Intracellular GSH was determined by titration with DTNB. Briefly, RBCs (HT 1%, 3 mL) were collected by centrifugation at 1000*g* for 10 min, and 0.5 mL of H_2O was added to the RBC pellet to lyse the cells. Proteins were precipitated by the addition of 0.5 mL of a metaphosphoric acid solution (1.67 g of metaphosphoric acid, 0.20 g of EDTA, and 30 g of NaCl in 100 mL of H_2O). After centrifugation at 3000*g* for 10 min, 400 μL of the clear supernatant was combined with 500 μL of 300 mM Na_2HPO4, pH 8.0, and the absorbance at 412 nm was read against a blank consisting of 400 μL of supernatant and 500 μL of H_2O. Then, 100 μL of DTNB solution (20 mg of DTNB in 100 mL of 1% sodium citrate) was added to both the blank and the sample, and the absorbance of the sample was read against the blank at 412 nm, after 5 min at 37 °C in a thermostatic cuvette to allow color development. Under these conditions the molar extinction coefficient of GSH is 13600 (*23*).

Statistical Analysis. All determinations were carried out in duplicate. Calculations and drawings were performed by Instat-3 statistical software (GraphPad Software Inc., San Diego, CA), using a repeated-measures ANOVA test, with Bonferroni's correction for multiple comparisons. A value of $P < 0.05$ indicated significance.

RESULTS

The presence of indicaxanthin and betanin was checked in RBCs during the 1−8 h interval from the consumption of cactus pear fruits. A peak concentration of indicaxanthin (1.03 ± 0.2 μmol/L of packed RBCs) was measured in the cells isolated at 3 h after fruit feeding, whereas the concentration at 5 h was about half. Small amounts were measured at 8 h, and the pigment was not found, or it was below the detection level of our system (13 nmol/L of packed RBCs), at 1 h. Very low amounts of betanin were detected only in RBCs isolated at 3 h after fruit feeding (**Table 1**).

Table 1. Betalain, Vitamin C, Vitamin E, and GSH Contents in RBCs and Resistance to the Cumene Hydroperoxide-Induced Oxidative Hemolysis of the Cells Isolated before and at Time Intervals after Ingestion of Cactus Pear Fruit Pulp[a]

time	indicaxanthin (μM)	betanin (μM)	resistance to hemolysis (min)	vitamin C (μM)	vitamin E (μM)	GSH (mM)
before ingestion	nd	nd	62 ± 7.5^{a}	44.0 ± 5.2	0.81 ± 0.09	2.1 ± 0.20
after ingestion						
1 h	nd	nd	61 ± 7.2^{a}	42.0 ± 5.0	0.82 ± 0.10	2.2 ± 0.31
3 h	1.03 ± 0.2^{a}	0.03 ± 0.005	95 ± 10^{b}	43.5 ± 4.4	0.83 ± 0.08	2.0 ± 0.19
5 h	0.55 ± 0.06^{b}	nd	78 ± 9.0^{c}	44.0 ± 4.9	0.81 ± 0.09	2.3 ± 0.21
8 h	0.11 ± 0.02^{c}	nd	66 ± 8.1^{a}	44.1 ± 4.9	0.80 ± 0.09	2.2 ± 0.22

[a] Values are the mean ± SD of separate determinations performed in duplicate on RBC samples from different subjects ($n = 8$). Means in a column with different letters are significantly different, $P < 0.05$ (repeated-measures ANOVA followed by a Bonferroni corrected t test). nd, not detectable.

Table 2. Betalain Content and Resistance to Cumene Hydroperoxide-Induced Hemolysis of Betalain-Enriched RBCs Following ex Vivo Spiking of Blood with the Purified Compounds[a]

betalain added (nmol/mL of blood)	RBC-incorporated betalains (nmol/mL of packed cells)	resistance to hemolysis (min)
no addition	nd	60 ± 5.0^{a}
indicaxanthin		
5	0.51 ± 0.08^{a}	77 ± 6.8^{b}
10	0.78 ± 0.12^{b}	85 ± 9.3^{c}
25	0.98 ± 0.14^{c}	93 ± 9.5^{d}
betanin		
5	0.45 ± 0.05^{a}	76 ± 7.0^{b}
10	0.80 ± 0.12^{b}	88 ± 9.5^{c}
25	1.10 ± 0.11^{c}	95 ± 8.9^{d}

[a] Values are the mean ± SD of separate determinations performed in duplicate on RBC samples from different subjects ($n = 8$). Means in a column with different letters are significantly different, $P < 0.05$ (repeated-measures ANOVA followed by a Bonferroni corrected t test). nd, not detectable.

RBCs isolated at time intervals after feeding with cactus pear fruits were submitted to oxidative injury by cumOOH, and their resistance to oxidation was evaluated as the length of time required to start hemolysis. In comparison with the homologous RBCs isolated before the fruit intake, the cells isolated at 1 h did not show any variation (**Table 1**). A delay of 33 ± 4.5 min in the onset of hemolysis was evident in the RBCs isolated at 3 h, whereas the increase of resistance of the RBCs at 5 h was smaller than that at 3 h. Finally, the resistance to hemolysis of the cells isolated 8 h after the fruit meal was slightly, although not significantly, higher with respect to that of the homologous cells before fruit consumption (**Table 1**).

The resistance of cells to oxidation is related to the level of their antioxidants. The absorption of vitamin C, or possibly other substances from the fruit, may have affected the endogenous RBC antioxidants. We then measured the amounts of vitamin C, vitamin E, and GSH in the RBCs isolated before and at time intervals after the fruit meal. With respect to the amount before fruit ingestion, no significant modification was observed at any time point (**Table 1**).

To help delineate whether the betalains incorporated in the RBCs after fruit consumption may be concerned with the increased resistance to the oxidative hemolysis, other experiments were performed. Ex vivo spiking of blood with either purified betanin or indicaxanthin, in the range of $5.0-25$ μM, resulted in the incorporation of increasing amounts of both compounds in the RBCs (**Table 2**). The betalain-enriched erythrocytes were more resistant to the cumOOH-induced oxidative hemolysis than the relevant nonenriched erythrocytes, whereas a significant relationship ($r^2 = 0.99$) existed between the increase of resistance and the amount of the incorporated betanin or indicaxanthin (**Table 2**). On the basis of the incorporated amount, betanin and indicaxanthin exhibited a comparable effectiveness in delaying the oxidative hemolysis (32.8 ± 5.9 min μM^{-1}).

DISCUSSION

A remarkable number of phytochemicals are currently investigated as bioactive components of fruits and vegetables. We have recently demonstrated that betalains such as betanin and indicaxanthin are bioavailable in humans and bind to circulating LDLs after a meal consisting of cactus pear fruit pulp (*21*). This work extends our previous findings on the postabsorbitive distribution of betanin and indicaxanthin and provides evidence that both compounds incorporate in the RBCs time-dependently, with the concentration of indicaxanthin much higher than that of betanin. Because the fruits ingested provided comparable amounts of the two pigments, these results appear to be in accordance with the knowledge that the bioavailability of indicaxanthin, as well as its plasma half-life in humans after ingestion of cactus pear fruits, is higher than that of betanin (*21*). Therefore, the amounts of the two compounds in the RBCs can be considered the mere reflection of their plasma concentrations. On the other hand, a selective storage of the compounds in cells is ruled out because the plasma disposal of indicaxanthin and betanin in humans has been observed to occur with first-order kinetics (*21*). It must be pointed out, however, that because we do not know if the washing procedures during the preparation of the RBCs may cause a release of the compounds, the amount of betalains evaluated in the RBCs may be an underestimation of the compounds incorporated.

The location of these compounds in the cells may be a matter of speculation. Although betanin and indicaxanthin are hydrophilic, these betalains have been shown to bind to LDL either in vitro (*19*) or in vivo (*21*) and to microsomal membranes (*16*). Not yet published results from our laboratory provide evidence that betanin binds to bilayers of dipalmitoylphosphatidylcholine liposomes, with a binding constant of 3×10^3 M^{-1} at 37 °C. The positive charge of the pyrrolidinic nitrogen may be important to favor interactions with polar sites of LDL or membranes. In principle, it cannot be ruled out that the compounds possibly traverse the membrane and locate in the RBC cytosol.

In comparison with the homologous erythrocytes isolated before the fruit meal, the RBCs isolated at 3 and 5 h after the fruit ingestion were more resistant to the oxidative hemolysis by cumOOH, an effect that did not appear to involve variations of the amount of the major RBC either soluble or membrane antioxidants. Indeed, neither reduced glutathione, vitamin C, nor vitamin E was modified by the fruit consumption at any of the considered time points. It appears of interest that the

resistance of the RBCs to the oxidative injury varied with the amount of betalains incorporated after the fruit meal, the higher the amount, the higher the resistance, which leads us to consider an involvement of these fruit components. Betanin and indicaxanthin are effective scavengers of a number of radicals in chemical settings, from the cation radical of ABTS (*14*, *18*) to the radical of DPPH (*17*), and of lipoperoxyl radicals in biological matrices in vitro, from microsomes to LDL (*16*, *19*); however, the radical-scavenging activity and the antioxidant effects of these compounds are still largely unexplored. The oxidative destruction of RBCs by organic hydroperoxides results from a series of events involving the production of a number of oxidants, including hydrogen peroxide, cumene-derived oxyradicals, and hemoglobin-derived oxoferryl radicals (*24*−*26*). In the present study evidence has been provided that erythrocytes enriched ex vivo with either purified betanin or indicaxanthin are more resistant to the cumOOH-stimulated hemolysis than nonenriched erythrocytes, which shows that these phytochemicals may behave as protective agents even in such a cell oxidation model. Moreover, indicaxanthin and betanin incorporated ex vivo in the RBCs were shown to act in a concentration-dependent manner within a low micromolar range and exhibited a quite comparable effectiveness in increasing the resistance of the cells to the cumOOH-induced oxidation. When corrected for the amount incorporated, the increase of the time required to start the hemolysis is calculated as 32.8 ± 5.9 min μM^{-1} for either betanin or indicaxanthin. It deserves to be mentioned that a 33 min delay in the onset of the cumOOH-induced hemolysis was observed in the RBCs that incorporated a total betalain amount of 1.06 μmol/L of packed cells after ingestion of the cactus pear fruits.

In contrast with the present findings, previous in vitro characterization of the radical-scavenging activity of betanin and indicaxanthin (*18*) had shown that betanin was more effective than indicaxanthin at reacting with the ABTS cation radical, possibly due to the different chemistries of the two compounds. Nevertheless, molecules with potential antioxidant effects can be strongly affected by the biological environment, by interaction with various cell components, thus enhancing or reducing their activity. This indeed has appeared to be the case of betanin and indicaxanthin in the RBCs and in LDL as well (*19*).

Fruits and herbs are expected to possess antioxidant properties because they are sources of phenolic compounds (*27*). It has recently been reported that small amounts of polyphenols such as rutin and isorhamnetin derivatives occur in the juice of the whole fruit, including the peel, of the Sicilian cultivars of cactus pear (*28*). A modest protective effect of rutin against the cumOOH-induced lipid oxidation of erythrocyte membrane has been shown in vitro (*29*). In other studies (*30*, *31*) conflicting results have been reported on the effects of quercetin on the erythrocyte membrane. In any instance, because the bioavailability of these compounds from cactus pear fruits remains unknown, it is difficult at this time to exclude or eventually evaluate their importance in the protection of erythrocytes observed under our experimental conditions.

Natural compounds that may prevent and/or mitigate the effects of the oxidative stress on the human body are presently the object of active research. As a consequence of high oxygen tension and large amounts of iron, a transition metal promoting the formation of oxygen free radicals (*32*), erythrocytes are highly susceptible to oxidation even in healthy individuals. Thus, these cells may be a peculiar target of dietary antioxidant components. Our findings provide unequivocal evidence that betanin and indicaxanthin incorporate in RBCs after the consumption of cactus pear fruit pulp and may support the hypothesis that these compounds are involved in the increased resistance of the cells to induced oxidative injury. At this stage, however, the activity of other components absorbed from the cactus pear fruit cannot be ruled out. Further in vivo studies with the purified compounds are necessary to fully evaluate the bioactive potential of betanin and indicaxanthin in red blood cells.

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