Absorption, excretion, and distribution of dietary antioxidant betalains in LDLs: potential health effects of betalains in humans[1–3]

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ABSTRACT

Background: Betalains were recently identified as natural antioxidants. However, little is known about their bioavailability from dietary sources.

Objective: The objective was to evaluate the bioavailability of betalains from dietary sources.

Design: The plasma kinetics and urinary excretion of betalains were studied in healthy volunteers ($n = 8$) after a single ingestion of 500 g cactus pear fruit pulp, which provided 28 and 16 mg indicaxanthin and betanin, respectively. The incorporation of betalains in LDL and the resistance of the particles to ex vivo–induced oxidation was also researched.

Results: Betanin and indicaxanthin reached their maximum plasma concentrations 3 h after the fruit meal and declined according to first-order kinetics. The half-life of betanin (0.94 ± 0.07 h) was shorter than that of indicaxanthin (2.36 ± 0.17 h). Both compounds had disappeared from plasma by 12 h after intake. The urinary excretion of indicaxanthin and betanin over 12 h represented $76 \pm 3.0\%$ and $3.7 \pm 0.2\%$, respectively, of the ingested compounds. LDL isolated 3 and 5 h after the fruit meal incorporated betalains at concentrations of 100.5 ± 11 and 50 ± 7.2 pmol/mg LDL protein, respectively. In addition, the particles appeared more resistant to ex vivo–induced oxidative injury than did the samples isolated before fruit ingestion ($P < 0.05$)—the higher the amount of betalains incorporated, the higher the resistance. The concentrations of vitamin E and β-carotene in LDL did not change significantly after fruit ingestion.

Conclusion: Our results show that cactus pear fruit is a source of bioavailable betalains and suggest that indicaxanthin and betanin may be involved in the observed protection of LDL against ex vivo–induced oxidative modifications. *Am J Clin Nutr* 2004;80:941–5.

KEY WORDS Betanin, cactus pear, dietary betalains, human health, indicaxanthin, LDL

INTRODUCTION

Various bioavailable compounds from plant food, acting independently of known nutrients and micronutrients, have recently attracted much attention as important factors in protecting human health (1, 2). The remarkable antioxidant activity of these phytochemicals, among which polyphenol compounds predominate (3), has become an important issue for explaining the beneficial effects of foods containing these compounds.

Betalains, known for a long time as safe colorants for food or other industrial purposes (4, 5), are phytochemicals that were recently classified as antioxidants (6–10). Contained in some families of the Caryophyllales order of plants, including the edible red beet and cactus pear, betalains are betalamic acid derivatives and they encompass 2 classes of compounds (11): betacyanins and betaxanthins. Betalamic acid may condense either with cyclic 3,4-dihydroxyphenylalanine (cyclo-DOPA), which may or may not be glycosylated, to produce betacyanins, or with various amino acids or amine derivatives to produce betaxanthins (**Figure 1**). Betalains are cationized compounds with a positive nitrogen in a polyene system. Their cyclic amine, which is similar to that of the antioxidant ethoxyquine (12, 13), has reasonably been considered to be the reactive group conferring to this class of molecules reducing properties. The yellow indicaxanthin, the adduct of betalamic acid with proline (14, 15), and the purple-red betanin, 5-*O*-glucose betanidine (16–18), are the pigments of the cactus pear fruit (Figure 1). The redox potential and radical scavenging activity, recently measured for these compounds (10), suggest that they may behave as effective reducing species, whereas a clear antioxidant activity has been shown in biological environments such as membranes and LDLs (8, 19).

Bioavailability is a major issue when considering the potential health effects of dietary antioxidants. Betacyanins have appeared to be absorbed and have been detected in the urine of subjects who consumed red beet juice (8) or beetroot (20). However, in-depth studies of the biokinetics of dietary betalains and of the potential significance in humans are lacking. In this study, we investigated the plasma kinetics and disposal of betanin and indicaxanthin in humans after ingestion of the fresh fruit of cactus pear. We also researched the postabsorptive distribution of these compounds in circulating LDL and evaluated the susceptibility of the particles isolated at time intervals ranging from the time of ingestion of fruit to the time of ex vivo–induced oxidation.

SUBJECTS AND METHODS

Subjects

Eight nonsmoking volunteers (5 women and 3 men) with a mean ($\pm$SD) age of 32.65 ± 10.11 y and a body mass index (in
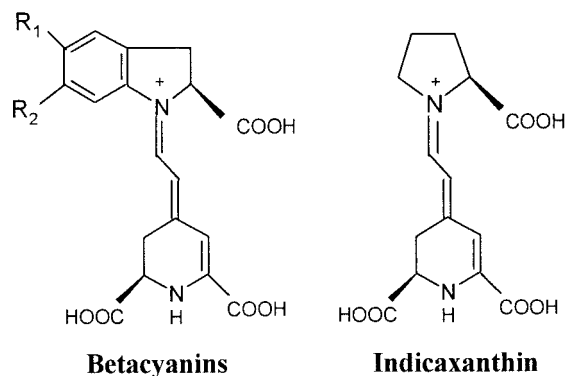
FIGURE 1. Molecular structures of betacyanins and indicaxanthin.

Betanidin: R_1 and $R_2 = OH$
Betanin (5-O-glucose betanindin): $R_1 = $ glucose; $R_2 = OH$

kg/m^2) of 21 ± 2.0 were recruited to participate in this study. All subjects were considered healthy on the basis of a medical questionnaire and none were taking any medications or vitamin supplements. 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.

Fruit

Cactus pear fruits from Sicilian cultivars were obtained from a local market at comparable ripening stages and were used within 48 h of collection. In the morning of the study, the fruit was peeled and the pulp minced and collected. The pulp was then divided in 8 portions of 500 g each. The contents of indicaxanthin and betanin were measured according to the methods reported elsewhere (10). Analysis of duplicate samples showed that each portion provided 16 mg betanin and 28 mg indicaxanthin.

Experimental design

Subjects followed a betalain-free diet for 7 d. On the morning of the sampling day, an intravenous catheter was inserted into one forearm of the subjects 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 the fruit meal (0 h) and at the time intervals depicted in **Figure 2**. The subjects were instructed not to eat anything and to drink only water after the fruit consumption until lunch. Lunch was provided 6 h after the consumption of fruit and was designed to be highly enriched in carbohydrates (200 g boiled rice).

After each sampling, plasma—separated from red blood cells by centrifugation at 2000 × g for 15 min at 4 °C—was processed to evaluate betanin and indicaxanthin and to prepare LDL. The LDL was stored at −80 °C and processed within 24 h. Urine was collected from the subjects over 12 h after consumption of the fruit meal. The samples were stored at −80 °C before analysis for betalains, which was performed within 24 h.

Preparation of LDL

LDL (density: 1.019–1.063 g/mL) was isolated from EDTA plasma by ultracentrifugation at 110 000 × g for 4 h at 4 °C in a Beckman L8-70 ultracentrifuge that was fitted with a 50 Ti rotor
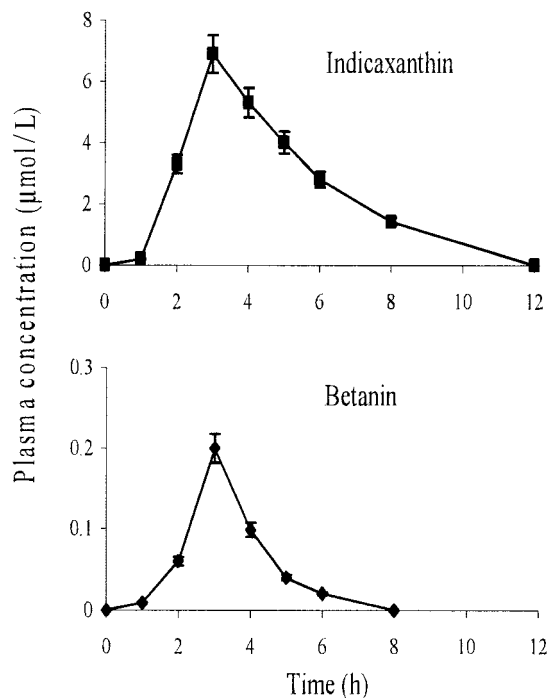

FIGURE 2. Plasma betalain concentrations in the subjects ($n = 8$) after ingestion of 500 g fresh cactus pear pulp that provided 28 mg indicaxanthin and 16 mg betanin. Values are the mean ($\pm$SD) of separate determinations performed in duplicate in samples from different subjects.

and used potassium bromide for density adjustments according to Kleinveld et al (21). The LDL fraction was shown to be free of other lipoproteins by electrophoresis on agarose gel. EDTA, salts, and plasma components were removed from LDL by gel filtration on a Sephadex G-25 medium (Pharmacia Biotech, Milan, Italy). Proteins were determined by using the Bio-Rad colorimetric method (22).

Oxidation of LDL

LDL (0.2 mg protein/mL) was incubated in oxygen-saturated EDTA-free phosphate-buffered saline (PBS), pH 7.4, supplemented with 10 μmol CuCl$_2$/L as a prooxidant in a 1-mL quartz cuvette. LDL oxidation was followed by continuously monitoring the formation at 37 °C of conjugated diene (CD) lipid hydroperoxides at 234 nm (23). The lag phase was determined as the intercept with the extrapolations of the parts of the curve representing the lag and propagation phases.

HPLC measurement of betalains

Betanin and indicaxanthin were purified from fruit of *Opuntia ficus indica* as reported (10). Enzyme hydrolysis with 3 nkat β-glycosidase (Sigma Chemical Co, St Louis) was carried out to obtain betanidin from 1.0 mmol betanin/L, in 50 mmol phosphate buffer/L, pH 3.5. The substrate was hydrolyzed within 60 min. Urine and plasma (1 mL) and LDL (4 mg protein) were extracted with 3 volumes of chloroform:methanol (2:1, by vol). 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) and 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 rate

of 1.5 mL/min. Spectrophotometric revelation was at 536 nm for betanin and betanidin and at 486 nm for indicaxanthin. Under the conditions described, indicaxanthin eluted after 8.15 min, betanin after 11.0 min, and betanidin after 15 min. An automatic wavelength change after 9.30 min allowed the detection of all compounds in the same sample. Quantitation of betalains was by reference to standard curves constructed with 5–100 ng purified compounds and by relating the amount of the compound under analysis to the peak area.

HPLC measurement of vitamin E and β-carotene in LDL

LDL (0.1 mg protein) was diluted to 1.0 mL with PBS, pH 7.4, and then 2 volumes of absolute ethanol and 8 volumes of petroleum ether were added. The organic extracts containing vitamin E were dried under nitrogen, resuspended with several microliters of methanol, and analyzed on a Supelco LC-18 column (0.46 × 25 cm; Supelcosil Bellefonte, PA) with methanol as eluent at a flow rate of 1.0 mL/min. Detection was at 290 nm. β-carotene was extracted from 500 μg LDL protein in a final volume of 1.0 mL PBS by mixing with 1 vol methanol and 3 vol hexane-diethyl ether (1:1, by vol). The extracts were then dried under nitrogen, resuspended with several microliters of a mixture of acetonitrile:methanol:tetrahydrofuran (58.5:35:6.5, by vol) and analyzed with the same solvent with an LC-18 Supelco column as above at a flow rate of 2.5 mL/min. Revelation was at 450 nm.

Vitamin E and β-carotene were quantified by reference to standard curves constructed with 5–100 ng purified compounds and by relating the amount of the compound under analysis to the peak area.

Statistical analysis

All determinations were carried out in duplicate. Calculations and graphs were obtained by INSTAT-3 statistical software (GraphPad Software Inc, San Diego) with the use of repeated-measures analysis of variance, with Bonferroni's correction for multiple comparisons. In all cases, significance was accepted if the null hypothesis was rejected at the $P < 0.05$ level.

RESULTS

The plasma kinetics and urinary excretion of betalains were investigated after ingestion of 500 g cactus pear fruit pulp, which provided 28 and 16 mg indicaxanthin and betanin, respectively. Plasma extracts were analyzed by using HPLC to identify indicaxanthin, betanin, and betanidin, the betanin aglycone. Betanin and indicaxanthin were detectable after 60 min, and plasma peak concentrations were reached 3 h after ingestion (Figure 2). Both compounds had disappeared from plasma at 12 h. Betanidin was absent or under the detection limit of our system (1.0 pmol/mL plasma). Log transformation of the plasma concentrations during the period 3–12 h after fruit ingestion indicated that the disposal of both betalains followed first-order kinetics and had a calculated half-life of 2.36 ± 0.17 and 0.94 ± 0.07 h for indicaxanthin and betanin, respectively. No significant interindividual variation of the pharmacokinetics of the 2 compounds was observed in our study of 8 subjects. The calculated plasma pharmacokinetic parameters are reported in **Table 1**.

Urine samples were collected from the subjects over 12 h, and the amounts of betanin and indicaxanthin excreted are reported in **Table 2**. Betalains were excreted to the extent of 76 ± 3.0% and

TABLE 1
Main plasma pharmacokinetics of betalains in humans after ingestion of 500 g fresh pulp of cactus pear that provided 28 mg indicaxanthin and 16 mg betanin[1]

	Betanin	Indicaxanthin
C_{max} (nmol/mL)	0.20 ± 0.02	6.90 ± 0.54
t_{max} (h)	3.10 ± 0.25	3.00 ± 0.27
AUC (nmol · h/mL)	0.46 ± 0.03	29.24 ± 2.60
$t_{1/2}$ (h)	0.94 ± 0.07	2.36 ± 0.17
k (h^{-1})	0.73 ± 0.09	0.29 ± 0.02

[1] All values are $\bar{x}$ ± SD; $n = 8$. C_{max}, maximum plasma concentration; t_{max}, time to reach the maximum plasma concentration; AUC, area under the plasma concentration versus time curve; k, terminal elimination rate constant [slope x (−2.303); the slope was derived from linear regression of the log plasma concentration of the period 3–12 h after the moles versus time profile]; $t_{1/2}$, elimination half-life (ln2/k).

3.7 ± 0.2% of the ingested compound for indicaxanthin and betanin, respectively.

Recent in vitro studies have shown that betalains can bind to LDL (19). The postabsorptive distribution of betalains was researched in LDL during the 3–8-h interval after fruit feeding. Extracts of LDL isolated at 3 h showed that indicaxanthin was incorporated to the extent of 98 ± 12 pmol/mg LDL protein (49 ± 8 mmol/mol LDL, assuming a 1:1 molar ratio of apolipoprotein B 100 to LDL and a molecular weight of 500 000 for apolipoprotein B 100; 24). In parallel with its plasma concentration, bound indicaxanthin was lower at 5 h than at 3 h and was undetectable at 8 h (**Table 3**). Betanin was only detected in LDL isolated 3 h after the fruit meal (Table 3). When exposed to copper-induced oxidation, the LDL isolated 3 h after fruit ingestion showed a marked elongation of the time preceding the onset of oxidation (lag phase) with respect to the homologous LDL isolated before fruit feeding. The elongation of the lag phase was shorter in the LDL isolated at 5 h than at 3 h, and no significant increment of the time required to start oxidation was observed in LDL isolated at 8 h (Table 3).

We then measured the vitamin E and β-carotene contents in LDL isolated before and at time intervals after the fruit meal. With respect to the amount at time 0, no significant modification was observed at any time point (Table 3).

DISCUSSION

Phytochemicals from food are continuously inspected for potential bioactivity. The betalain pigments have recently emerged as a novel class of antioxidants (8–10, 19). Because their presence is limited to a few edible vegetables, such as red beet and cactus pear (24, 25), these substances have been poorly studied as

TABLE 2
Betalain excretion in subjects within 12 h after ingestion of 500 g fresh pulp of cactus pear that provided 28 mg indicaxanthin and 16 mg betanin[1]

	Urinary excretion
	μmol/12 h (% of ingested compound)
Indicaxanthin	68.4 ± 2.5 (76 ± 3)
Betanin	1.06 ± 0.08 (3.7 ± 0.2)

[1] All values are the $\bar{x}$ ± SD of separate determinations performed in duplicate in samples from different subjects. $n = 8$. Urine was collected and betalains were extracted and measured as reported in Subjects and Methods.

TABLE 3
Betalain, vitamin E, and β-carotene contents in LDL and the resistance of LDL to copper-induced oxidation (lag phase) before and at time intervals after the ingestion of 500 g fresh pulp of cactus pear that provided 28 mg indicaxanthin and 16 mg betanin[1]

Time	Indicaxanthin	Betanin	Lag phase	Vitamin E	β-Carotene
	pmol/mg LDL protein	*pmol/mg LDL protein*	*min*	*nmol/mg LDL protein*	*nmol/mg LDL protein*
Before ingestion	ND	ND	40 ± 4.5^a	13.5 ± 1.4	0.65 ± 0.31
After ingestion					
3 h	98 ± 12	2.5 ± 1.2	58 ± 6.5^c	12.98 ± 1.5	0.64 ± 0.25
5 h	50 ± 7.2	ND	49 ± 5.5^b	13.1 ± 1.3	0.65 ± 0.43
8 h	ND	ND	41 ± 5.0^a	13.3 ± 1.4	0.66 ± 0.53

[1] All values are the $\bar{x} \pm$ SD of separate determinations performed in duplicate in LDL samples from different subjects. $n = 8$. LDL was isolated from the blood of subjects before and at the indicated time points after fruit ingestion. ND, not detectable. Means in a column with different superscript letters are significantly different, $P < 0.05$ (repeated-measures ANOVA followed by a Bonferroni corrected t test).

dietary phytochemicals. This study investigated the plasma kinetics and urinary excretion of betanin and indicaxanthin in humans and provided evidence that these compounds bind to circulating LDL.

Various phytochemicals can be structurally modified during metabolism by conjugation with glucuronic acid, sulfate, or both. Because it has been shown that the absorbed betalains are not metabolized by the hepatic cells (26) and are excreted as such via the urine (20), deconjugated plasma or urine was not considered in our study. After fresh cactus pear fruit pulp was ingested, indicaxanthin and betanin reached their plasma peak concentration 3 h after the fruit meal, which suggested that they were absorbed by the same intestinal portion. Betanidin, the betanin aglycone, was not detected in plasma, which indicated that, as recently shown with some glycosylated flavonoids (27), hydrolysis of the glycosyl moiety is not a prerequisite for absorption. Both betalains declined according to first-order kinetics and were undetectable after 12 h. The half-life of betanin was shorter than that of indicaxanthin, which suggested a lower apparent distribution volume, a higher clearance, or both. The glycosyl moiety of betanin may somewhat prevent this compound from accumulating at the level of lipid compartments. On the other hand, the molecular structure of indicaxanthin may permit a higher tubular permeability and resorption.

The absorption and bioavailability are major points to characterize dietary components. The ratio between the calculated area under the curve at 0-12 h (AUC_{0-12}) for indicaxanthin and betanin was much higher than the ratio between the amounts of the 2 compounds in the fruit, which suggests better absorption of indicaxanthin than of betanin. Because degradation products from betalains were not researched, the urinary recovery of the 2 compounds over 12 h might represent an underestimation of their absorption. At any instance, the molar ratio between the excreted betalains is quite similar to the ratio between the AUC_{0-12} of the 2 compounds, which is evidence that indicaxanthin and betanin undergo similar metabolic pathways. On the whole, our data show that the bioavailability of indicaxanthin is 20 times that of betanin, at least when the cactus pear fruit is the source of these phytochemicals. As far as we know, there is no available information about the bioavailability of indicaxanthin from other sources. On the other hand, it has been shown that the urinary recovery of betanin in humans ranges from 0.5% to 1% after ingestion of red beet juice (8) or beetroot extract (20). The extent of absorption of phytochemicals varies greatly because of many factors, including the dietary source, instability of the molecule in the digestive environment, bacterial degradation in the gut, and

mechanisms of absorption. Betanin has appeared easily degraded in the gastrointestinal tract (26, 28). In addition, as a glycosylated phytochemical, betanin could need the intestinal glucose transporter (8, 27, 29) and is thereby in competition with the fruit sugar. On the other hand, diffusion processes of indicaxanthin through the intestinal membrane may be favored.

Knowledge of the body distribution may aid the investigation of the eventual bioactivity of absorbed dietary components. We observed that small amounts of betanin and indicaxanthin bind to LDL, in parallel with their plasma concentrations. Although it is not possible to assess whether the centrifugation procedure to prepare LDL would release betalains from the particles, when considering a 1.2 μmol/L plasma LDL concentration (30), the LDL-bound betalains would account for 0.62% of their plasma content—a percentage distribution that is comparable with that observed after ex vivo spiking of human plasma with 25 and 50 μmol/L of either betanin or indicaxanthin (19).

In comparison with the particles isolated before, LDL isolated 3 and 5 h after the fruit meal were more resistant to ex vivo–induced oxidation. Variations in the amount of the major LDL endogenous antioxidants did not appear to be involved in this event. Indeed, neither vitamin E nor β-carotene was modified as a consequence of fruit ingestion, at least not during the experimental time. On the other hand, the resistance of LDL to oxidation varied with the amount of the incorporated betalains—the higher the amount, the higher the resistance. Betanin and indicaxanthin have been shown in vitro to be very effective lipoperoxyl radical scavengers in microsomal membranes (8) and in human LDLs ex vivo (19). Suggesting that these phytochemicals may be concerned with the observed protection of LDL is an attractive hypothesis. Much of the antioxidant activity of fruit and vegetables is generally considered to be related to the flavonoid content. Small amounts of rutin and isorhamnetin derivatives were recently reported in the whole fruit juice, including the peel, of the Sicilian cultivars of cactus pear (31). However the metabolic fate of these compounds in vivo, or their eventual accumulation in LDL after ingestion of cactus pear fruit, is unknown.

An important goal of recent nutritional studies has been to recognize the properties of components of vegetables and fruit that may positively affect human health. The current findings may encourage further in vivo studies with the pure molecules to investigate potential bioactivities of betalains. ☙

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