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Increased Resistance to Oxidation of Betalain-enriched Human Low Density Lipoproteins

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Betalains are natural pigments recently considered as compounds with potential antioxidative properties. In this work, *ex vivo* plasma spiking of pure either betanin or indicaxanthin, followed by isolation of low density lipoprotein (LDL), and measurement of its resistance to copper-induced oxidation, has been used to research if these betalains can bind to LDL and prevent oxidation of LDL lipids. When pooled human plasma from 10 healthy volunteers was incubated in the presence of 25–100 μM either betanin or indicaxanthin, incorporation of both compounds in LDL was observed, with a maximum binding of 0.52 ± 0.08, and 0.51 ± 0.06 nmoles of indicaxanthin and betanin, respectively, per mg LDL protein. Indicaxanthin-enriched and betanin-enriched LDL were more resistant than homologous native LDL to copper-induced oxidation, as assessed by the elongation of the induction period. The incorporated indicaxanthin, however, appeared twice as effective as betanin in increasing the length of the lag phase, while both compounds did not affect the propagation rate. Both betalains were consumed during the inhibition period of lipid oxidation, and delayed consumption of LDL-beta carotene. Indicaxanthin, but not betanin, prevented vitamin E consumption at the beginning of LDL oxidation, and prolonged the time of its utilization. The resistance of LDL to oxidation when vitamin E and indicaxanthin acted separately in a sequence, was lower than that measured when they were allowed to act in combination, indicating some synergistic interaction between the two molecules. No prooxidant effect over a large concentration range of either betanin or indicaxanthin was observed, when either betalain was added to the LDL system undergoing a copper-induced oxidation.

These results show than indicaxanthin and betanin may bind to LDL, and are highly effective in preventing copper-induced lipid oxidation. Interaction with vitamin E appears to add a remarkable potential to indicaxanthin in the protection of LDL. Although molecular mechanisms remain uncompletely understood, various aspects of the action of betanin and indicaxanthin in preventing LDL lipid oxidation are discussed.

Keywords: Betalains; Betanin; Human LDL; Indicaxanthin; Natural antioxidants; Prickly pear

INTRODUCTION

Interest in antioxidant molecules occurring in fruits and vegetables raised enormously in the latest years. Focus has been on phenolic derivatives such as catechins and other flavonoids, whose properties and activities have actively been researched. More recently, other naturally occurring compounds such as betalains have been investigated as natural antioxidants.[1,2] These water-soluble cationic pigments, that have long been used as a natural food-coloring stuff,[3–5] occur in fruits and flowers of plants of the order of Caryophyllales,[6] and include two groups of compounds having in common the structure of betalamic acid (Fig. 1). This may condense with either cyclic DOPA to produce the violet–red betacyanins, or aminoacids to give the less polar yellow betaxanthins.[6–8] Protective effects of betanin have been shown in a number of *in vitro* models of lipid oxidation.[1] In addition, reducing properties of betanin and indicaxanthin have recently been evaluated as the capacity of scavenging the ABTS cation radical,[2,9] and redox potential measured by cyclic voltammetry.[2] Data collected so

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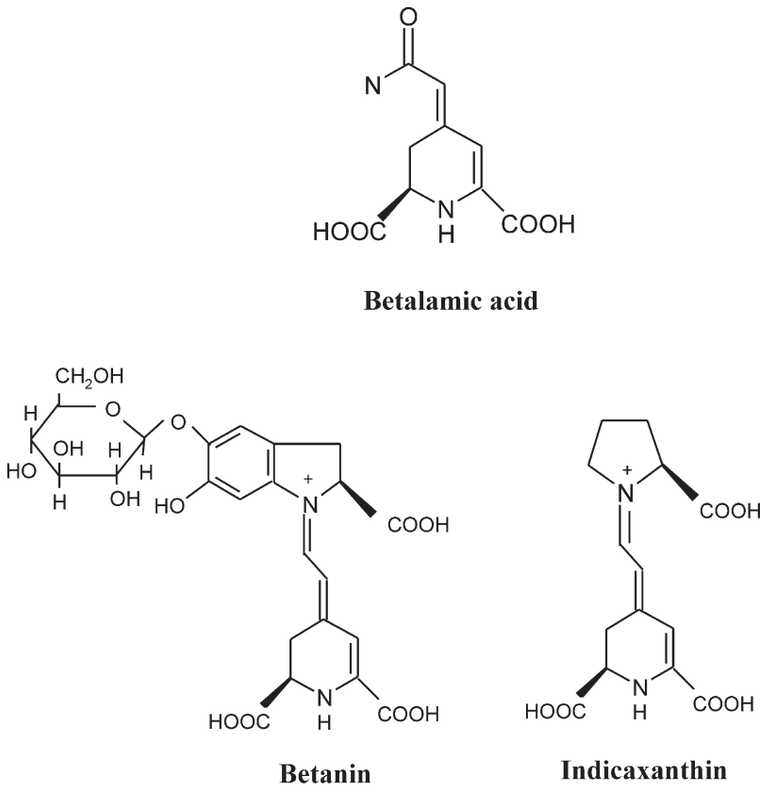

FIGURE 1 Structures of betalamic acid, betanin and indicaxanthin.

far suggest that these compounds could play a role as antioxidants in biologically relevant systems.

The cationic nature of betalains would favour the interaction of these water-soluble molecules with polar moieties of membranes and low density lipoproteins (LDL). With this in mind, we performed an *ex vivo* spiking of human plasma with either betanin or indicaxanthin to investigate if these compounds can enrich the LDL fraction, and eventually protect the lipid compartment lipids against the oxidation induced by copper ions. We show here that both compounds bind to LDL and act as effective antioxidants, and speculate about mechanisms underlying their activity in this system.

MATERIALS AND METHODS

α-Tocopherol, β-carotene, ethylenediaminetetraacetic acid (EDTA), Sephadex G-25 were from Sigma Chemical Company (St. Louis, MO). All other materials and solvents were of the highest purity or high-performance liquid chromatography (HPLC) grade.

Isolation of Betalains

Opuntia ficus indica fruits were obtained from a local market within one day from the collection. Betanin and indicaxanthin were purified from the methanolic extract of the pulp by liquid chromatography on Sephadex G-25.[2] Fractions containing the separate pigments were submitted to cryodessication, and betalains were resuspended at a suitable concentration in phosphate-buffered saline (PBS), pH 7.4. Purity was confirmed by HPLC, using the system reported below. Quantitation of the two pigments was by spectrophotometry (betanin $A_{536} = 65,000$;[10] indicaxanthin $A_{486} = 42,600$).[7]

Experimental Protocol

Blood from healthy volunteers (6 females and 4 males) was withdrawn by venipuncture after overnight fasting, and collected in EDTA ($1\,mg\,ml^{-1}$). Plasma, separated by centrifugation at $2000\,g$ for 15 min at 4°C was pooled, and aliquots were incubated 15 min in the dark at room temperature, with either indicaxanthin or betanin in PBS, or with PBS alone. The procedure to isolate LDL started immediately after. LDL was either used immediately or stock solutions were stored in light-shielded tubes at −80°C and used within 48 h.

Preparation and Oxidation of LDL

LDL (d 1.019–$1.063\,g\,ml^{-1}$) was isolated from EDTA plasma by stepwise ultracentrifugation at

4°C in a Beckman L8-70 M ultracentrifuge, fitted with a 50 Ti rotor using potassium bromide for density adjustments, according to Kleinveld *et al.*[11] LDL fraction was shown to be free of other lipoproteins by electrophoresis on agarose gel. EDTA and salts were removed from LDL by gel filtration on Sephadex G-25. Proteins were determined by the Bio Rad colorimetric method.[12] LDL (0.2 mg protein ml^{-1}) was incubated in oxygen-saturated EDTA-free PBS, pH 7.4, supplemented with 40 μM $CuCl_2$ 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, using a Beckman DU 640 spectrophotometer. The lag phase was determined as the intercept with the extrapolations of the parts of the curve representing the lag and propagation phases. The propagation rate was calculated as the amount of CD hydroperoxides formed per minute, and per mg LDL protein, during the propagation phase. In other experiments, the CD lipid hydroperoxides in the lipid fraction of LDL were extracted from 1 ml aliquots of reaction mixture after 2 h incubation by 3.0 ml CH_3Cl_3/MeOH (2:1,v:v). The organic extract was dried under a nitrogen stream, re-suspended in cyclohexane and quantified spectrophotometrically at 234 nm, with a molar absorption coefficient of 27,000.[13]

Consumption of Antioxidants

LDL was incubated in the presence of copper ions as described above in a water bath at 37°C, in the dark, under air. Portions were taken at intervals from 0 to a maximum 90 min and simultaneously extracted to reveal α-tocopherol, beta-carotene and betalains by HPLC analysis. α-Tocopherol was extracted by mixing aliquots of the incubation mixture (50 μg protein in 1.0 ml PBS, pH 7.4) with two volumes of absolute ethanol and eight volumes of petroleum ether. The organic extracts were then dried under nitrogen, re-suspended with several microliters of methanol and analysed on Supelco Supelcosil™ (Bellefonte, PA) LC-18 column (0.46 × 25 cm), with methanol at 1.0 ml min^{-1}. Revelation was at 290 nm. Beta-carotene was extracted from 500 μg LDL protein by mixing with one volume of methanol and three volumes of hexane:diethyl ether (1:1, v:v). The extracts dried under nitrogen and re-suspended with several microliters of a mixture of acetonitrile/methanol/tetrahydrofurane (58.5:35:6.5, v:v:v), were analysed with the same solvent by a LC-18 Supelco column as above, at a flow rate of 2.5 ml min^{-1}. Revelation was at 450 nm. Betalains were extracted from 1 mg LDL protein with three volume of chloroform/methanol (2:1, v/v). The methanol phase was dried under nitrogen, re-suspended in 1% acetic acid in water, and analysed on a Varian Microsorb C-18 column (4.6 × 250 mm, Varian, Palo Alto, CA, USA), 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^{-1}. Spectrophotometric revelation was at 536 and 486 nm for betanin and indicaxanthin, respectively. Quantitation of all compounds was by reference to standard curves constructed with 5–100 ng of each compound, and by relating the amount of the compound under analysis to the peak area. All procedures were performed under dim red light to avoid artifactual photooxidation of lipids and to preserve light sensitive vitamins.

RESULTS

Binding of betalains to human LDL was investigated by spiking of plasma with purified either betanin or indicaxanthin to an end concentration of 25–100 μM, followed by LDL isolation and HPLC measurement of the pigments extracted therefrom. A maximum binding of 0.52 ± 0.08 nmol indicaxanthin, and 0.51 ± 0.06 nmol betanin per milligram of LDL protein was observed, by spiking with 50 μM betalains (Fig. 2). These betalain-enriched preparations were used for further experiments. When submitted to copper-induced oxidation, betalain-enriched LDL showed a prolonged lag phase with respect to the native control-LDL. The incorporated indicaxanthin appeared twice as effective as betanin in delaying the onset of the Cu^{2+}-promoted lipid oxidation (Fig. 3), while both betalains did not affect the propagation rate (4.6 nmoles CD lipid hydroperoxides min^{-1} mg LDL $prot^{-1}$), nor

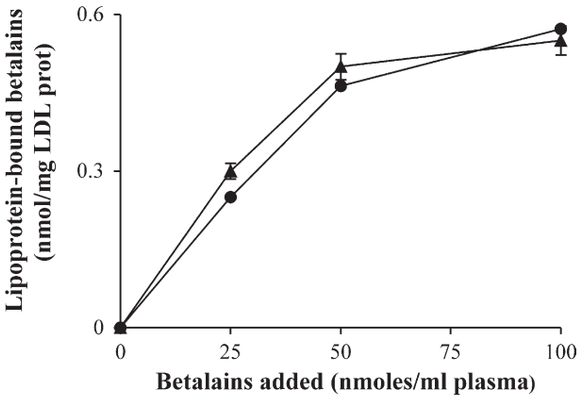

FIGURE 2 Binding of either indicaxanthin (●), or betanin (▲), to human LDL following spiking of plasma with the betalains. Incubation conditions, isolation of LDL, extraction of betalains from the particles, and quantization by HPLC, were as reported in "Methods Section". Values are the mean ± SD of three separate experiments carried out in duplicate.

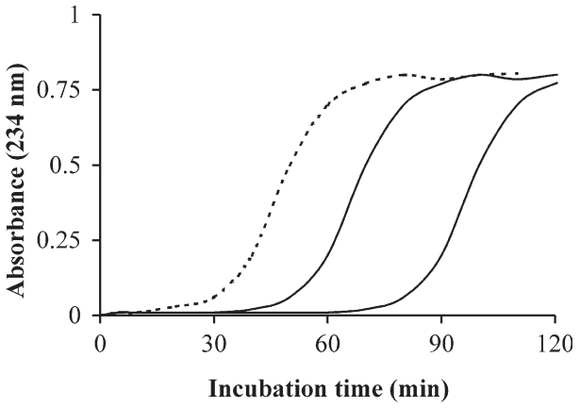

FIGURE 3 Time course of the copper-induced oxidation of control-LDL (broken line), betanin-(B-LDL) (thin line) and indicaxanthin-(I-LDL) (thick line) and enriched LDL. The LDL preparations were oxidised as reported in "Methods Section". The variation of the 234 nm absorption was recorded in a Beckman DU-640 spectrophotometer, at 37°C, in 10-s intervals. The figure is representative of three separate experiments carried out with different preparations, for each LDL-preparation.

the maximum amount of hydroperoxides formed (148 nmoles mg LDL prot^{-1}).

Vitamin E and carotenoids are the major LDL antioxidants.[14] Vitamin E and β-carotene in control-LDL were 13 ± 1.2 nmol and 0.65 ± 0.07 nmol mg LDL prot^{-1} ($n = 6$), respectively, and their amount was not modified by the bound betalains. Antioxidant compounds are usually consumed during the oxidation process to which they offer protection. The temporal disappearance of vitamin E and β-carotene was investigated in the betalain-enriched LDL, in comparison with control-LDL. A total loss of vitamin E was evident within 10 min, while consumption of β-carotene occurred within 20 min, in control-LDL (Fig. 4a). The time-course of vitamin E consumption was not modified in betanin-enriched LDL (Fig. 4b). On the contrary, vitamin E was spared completely for 10 min, then slowly declined within the following 40 min, in indicaxanthin-enriched LDL (Fig. 4c). Both the LDL-bound betanin and indicaxanthin caused a significant delay of β-carotene consumption (Fig. 4b,c). Consumption of betalains was also monitored. Decrease of betanin started after vitamin E exhaustion, and it decreased to zero at the end of the 50 min lag phase, in betanin-enriched LDL (Fig. 4b). In contrast, indicaxanthin underwent a progressive slow disappearance since the beginning of oxidation in indicaxanthin-enriched LDL, and a total loss was observed after vitamin E depletion, by the 80 min lag phase (Fig. 4c).

To segregate the effect of indicaxanthin, and to investigate about interactions between indicaxanthin and vitamin E, native-LDL were incubated either in the absence, or in the presence of 0.10 μM indicaxanthin added in the medium either before, or 10 min after starting a copper-stimulated oxidation, i.e. after vitamin E exhaustion. With respect to the length of lag phase of native-LDL, the coincubation with indicaxanthin when vitamin E was already depleted caused an additional 15 min inhibition period, during which indicaxanthin was totally consumed (Fig. 5a). In contrast, when indicaxanthin was added prior starting the LDL oxidation, the observed increment of lag phase was 45 min (Fig. 5b), providing evidence that indicaxanthin had established cooperative interactions with LDL components. Under such conditions, vitamin E was preserved for 10 min, and its total consumption occurred in 50 min, while indicaxanthin slowly disappeared within the entire length of the lag phase (Fig. 5b).

Antioxidants may become prooxidant under certain conditions. Concentration-dependent effects of indicaxanthin and betanin were investigated by measuring the formation of CD hydroperoxides formed within 120 min, after incubation of LDL in the presence of variable betalain amounts. No prooxidant effect was found, at the lower, as well as at the higher betalain concentrations (Table I). Indicaxanthin showed a higher antioxidant activity than betanin at concentrations between 0.05 and 1.0 μM. However, above 1.0 μM both compounds completely inhibited the formation of conjugated diene lipid hydroperoxides for the time of observation.

DISCUSSION

Cationic pigments such as betalains are distributed in a number of vegetal species,[6] but beets and prickly pear fruits are the only food containing them.[15,16] We have recently shown that, in addition to vitamin C, these compounds were potentially important contributors to the activity of aqueous extracts from prickly pear fruits against LDL oxidation.[2] By *ex-vivo* plasma spiking of pure either betanin or indicaxanthin, we now show that these compounds can bind to human LDL, and provide a very effective antioxidant protection.

Binding of betalains such as betanin and betanidin, its *o*-diphenol, to microsomal membranes has been shown,[1] however binding of betalains with plasma lipoproteins had not been reported yet. Under our conditions, binding of either betanin or indicaxanthin to LDL has appeared saturable, with a maximum binding of 0.5 nmol mg LDL prot^{-1} (0.25 mol/LDL mol). In principle, incorporation of molecules in lipid moieties would proceed according to lipophilicity, the more the lipophilicity the easier the incorporation. The positive charge of betalains could favour interactions with polar head groups of

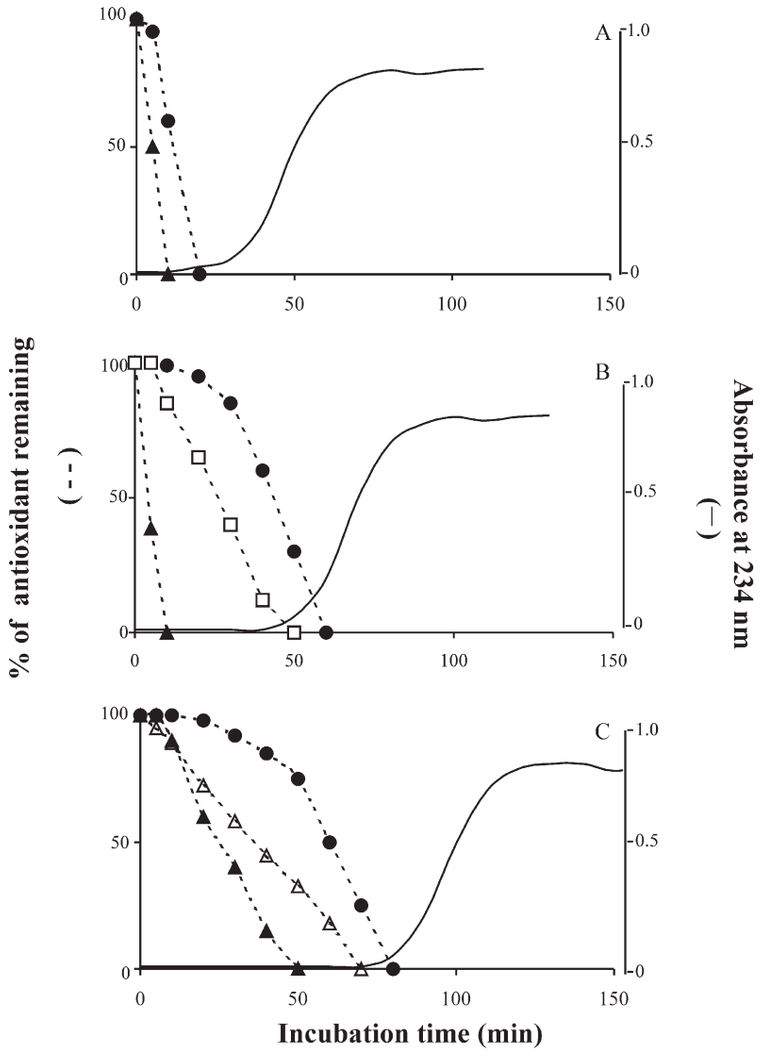

FIGURE 4 Time course of the consumption of antioxidants during the copper-induced oxidation of control-LDL (A), betanin-enriched LDL (B), and indicaxanthin-enriched LDL (C). LDL oxidation was as reported in "Methods Section". In parallel assays, the antioxidants were extracted at the indicated time points, and evaluated by HPLC. The figure is representative of three separate experiments carried out with different preparations, for each LDL preparation. ▲, α-tocopherol; ●, β-carotene; □, betanin; △, indicaxanthin.

lipids and/or possibly polar sites of apo B-100. Indeed, although solubility would favour the incorporation of the more lipophilic indicaxanthin over betanin, both molecules appear to bind to the same extent.

LDL-bound either betanin or indicaxanthin produce significant effects on LDL oxidation. Both betalains cause a marked elongation of the period preceding the onset of lipoperoxide accumulation, with indicaxanthin much more effective than betanin, while do not have any effect on the rate of the conjugated diene lipid hydroperoxide formation during the propagation phase. Although betanin has been shown capable of chelating Cu^{2+} ions,[2] these findings would rule out that both betalains interfere with binding of copper to apo-B 100, and provide evidence that neither indicaxanthin nor betanin acts as a preventative antioxidant[14] in the present model.

The lag phase, and hence the oxidation resistance of LDL, is mainly determined by its antioxidant content, with α-tocopherol as the most significant. During the lag phase the LDL becomes progressively depleted of its antioxidants, with α-tocopherol as the first and β-carotene as the last one.[14] Either hydrophilic,[17] or lipophilic[18] compounds enhance the resistance of LDL to oxidation by protecting LDL vitamin E and β-carotene and/or regenerating α-tocopherol from its tocopheroxyl radical. The incorporated betanin and indicaxanthin are both able to delay consumption of β-carotene, but affect in a different way the temporal disappearance of the LDL-vitamin E. Betanin does not modify the time-course of vitamin E depletion, begins to disappear

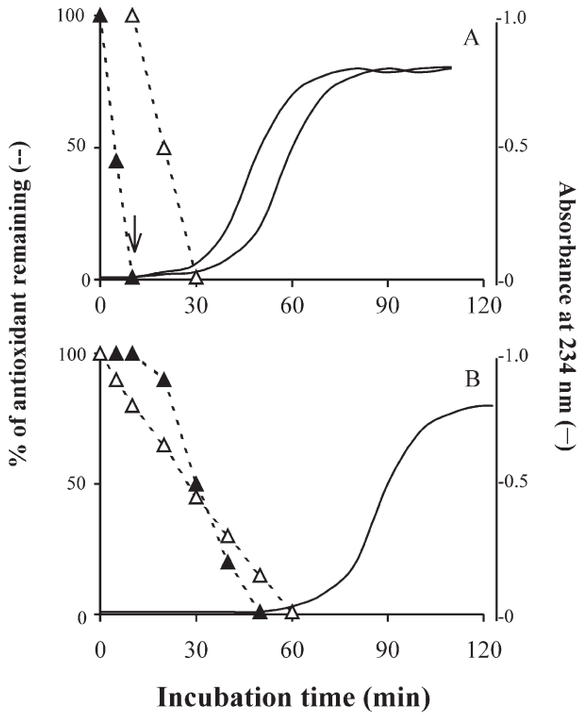

FIGURE 5 Time-course of the copper-induced oxidation and antioxidant consumption in human LDL incubated in the absence (thin line), or in the presence (thick line) of indicaxanthin, added either 10 min after (arrow, A), or immediately before (B) starting oxidation. Oxidation of LDL, and consumption of vitamin E (▲), and of indicaxanthin (△), were monitored as reported in "Methods Section". The figure is representative of three separate experiments carried out with the same LDL preparation.

TABLE I Concentration-dependent effect of betanin and indicaxanthin on the CD hydroperoxide formation, after a copper-induced oxidation of human LDL

Betalain concentration (μM)	CD hydroperoxides (nmol mg^{-1} LDL protein)	
	Betanin	Indicaxanthin
0	143 ± 11	
0.05	142 ± 8.5	145 ± 11
0.5	100 ± 13	72 ± 9.0
1.0	48 ± 5.3	1.1 ± 0.9
5	1.3 ± 1.0	2.0 ± 0.3
20	1.5 ± 0.1	1.3 ± 0.3
50	1.2 ± 0.2	1.6 ± 0.2

LDL (200 μg protein ml^{-1}) were incubated with 40 μM $CuCl_2$ at 37°C, in the absence or in the presence of either betanin or indicaxanthin at the indicated concentrations. After 2 h incubation, 1 ml aliquots were taken and CD hydroperoxides extracted and spectrophotometrically quantified as reported in "Methods Section". Values are the mean ± SD of three experiments carried out with the same LDL preparation.

only after vitamin E exhaustion, and causes an additional lag period during which it decreases to zero. While showing that the protective effects of betanin are independent of endogenous LDL α-tocopherol, these results would confirm, as observed with other models,[1] that betanin may act as a lipoperoxyl radical-scavenger, with an apparent scavenging capacity between vitamin E and β-carotene in our system. By contrast, indicaxanthin starts to decline since the beginning of LDL oxidation, and its consumption extends beyond that of vitamin E. Moreover, it modifies the time-course of vitamin E consumption. An early complete sparing of vitamin E is followed by a marked reduction of its temporal disappearance, which may suggest recycling of α-tocopherol and/or competition by the incorporated indicaxanthin for scavenging of lipoperoxyl radicals. If either mechanism or both are involved is not possible to assess at this stage. We observed that indicaxanthin, coincubated with vitamin E-deprived LDL, was able to act as a real lipoperoxyl radical-scavenger. On the other hand, though interactions with other LDL components cannot be ruled out, the synergistically increased resistance of LDL to oxidation, and sparing of vitamin E, observed when indicaxanthin was coincubated with vitamin E-containing native LDL, would suggest regeneration of α-tocopherol. Theoretically, the reported reduction potential of indicaxanthin (611 mV)[2] would allow reduction of lipoperoxyl radicals, but would not support reduction of the tocopheroxyl radical ($E^{o\prime}$(α − tocopherol, H^+/α − tocopheroxyl radical) = 480 mV).[19] As discussed below, local interactions at the lipid microenvironment could significantly increase the reductive force of this molecule.

Dynamic interactions with vitamin E may amplify the antioxidant effectiveness of indicaxanthin, which would make indicaxanthin much more protective than betanin in the present system. It should also be noted that the antioxidant capacity may ultimately depend not only on the structural features and redox capacity, but also on the orientation of the molecules at the lipid phase of LDL. In this context, the monoglycosylated catechol ring of betanin could play a critical role, "pulling" the betanin backbone far from LDL surface in the water environment.

No concentration-dependent prooxidant effect was found when LDL underwent a copper-dependent oxidation in the presence of either betanin or indicaxanthin, added at concentrations ranging from 0.05 to 50 μM. Thus, differently from most phenolic derivatives which become prooxidant at low concentrations in systems using Cu^{2+} as catalyst,[20,21] neither betalain did undergo redox reactions with Cu^{2+} ions, nor adverse effects were caused by radicals eventually generated from both compounds.

A number of questions are to be answered to understand chemical and molecular mechanisms underlying the observed effects, which may be matter of speculation. While scavenging of lipoperoxyl radicals by both betalains is thermodynamically feasible according to their redox potentials,[2] it is difficult to envisage how the small concentrations

incorporated in the LDL particles are consumed so slowly while inducing the observed extension of lag time. Some recycling of betalains should in turn be hypothesised.

Biochemical pathways leading to betalain synthesis in vegetal cells have been well established,[22–24] however the redox chemistry of betalains is less known. Either reaction with lipoperoxyl radicals or recycling of tocopheroxyl radicals would require donation of a hydrogen atom. Indicaxanthin and betanin contain a cyclic amine group, the structure of which resembles that of the ethoxyquine, a very strong antioxidant,[25,26] and a nitrogen positively charged in a polyene system. The reactivity of these molecules may be limited by the positive nitrogen. The interaction with negative head groups of lipids can increase the capacity of the amine group to donate a hydrogen atom. Though the monoglycosylated phenolic ring could confer betanin reducing properties, other studies seem to rule out that the phenolic group of betanin may be involved in the scavenging of lipoperoxyl radicals,[1] suggesting an important role of the cyclic amine for the antioxidant activity. The cyclic amine group of both betanin and indicaxanthin could only be the one able to work as a hydrogen atom donor to repair lipoperoxyl- or eventually tocopheroxyl-radicals, with formation of a delocalised betalain radical. Regeneration from their radicals would permit a much longer protection of the LDL, and explain the observed slow temporal disappearance of the two betalains. Similarly to other observations with isoflavone-derived phenoxyl radicals in lipid systems,[27] recycling of betalains at the expenses of unsaturated fatty acids of the particle may be involved, a potentially prooxidant event. We had no evidence of a prooxidant behaviour within a large range of betalain concentrations, which would indicate that, under the conditions applied, the rate at which the betalain radical initiates oxidation events is slower as compared to the rate at which betalains scavenge a lipid peroxyl radical. In other terms, the "prooxidant" regeneration should be slower than the antioxidant activity. Therefore, in spite of an effective recycling, a slow irreversible oxidation of betalains by lipoperoxyl radicals appears to prevail, leading to a progressive consumption of betalains during LDL oxidation. Additional studies with simple chemical models are required to shed light on these reactions.

In conclusions, although the underlying molecular mechanisms remain incompletely understood, our study shows that betanin and indicaxanthin can incorporate in LDL, and are highly effective in preventing the oxidation of LDL lipids. While both compounds appear capable of scavenging lipoperoxyl radicals, indicaxanthin shows synergistic interactions with LDL components, which adds a remarkable potential to delay LDL oxidation. Reported results,[1] and still unpublished data from our laboratory provide evidence that these compounds are readily absorbed after consumption of red beet juice,[1] or fresh prickly pear fruits, and a micromolar peak is reached in human plasma in about 3 h (data not shown). Although we do not still have a clear picture about metabolic transformations of betalains, and the impact of structural modifications on their antioxidative properties, our findings would suggest betanin and indicaxanthin as potential contributing factors to the antioxidant activity of the diet.

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