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Cytoprotective effects of the antioxidant phytochemical indicaxanthin in β-thalassemia red blood cells

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Abstract

Antioxidant phytochemicals are investigated as novel treatments for supportive therapy in β-thalassemia. The dietary indicaxanthin was assessed for its protective effects on human β-thalassemic RBCs submitted *in vitro* to oxidative haemolysis by cumene hydroperoxide. Indicaxanthin at 1.0–10 μM enhanced the resistance to haemolysis dose-dependently. In addition, it prevented lipid and haemoglobin (Hb) oxidation, and retarded vitamin E and GSH depletion. After *ex vivo* spiking of blood from thalassemia patients with indicaxanthin, the phytochemical was recovered in the soluble cell compartment of the RBCs. A spectrophotometric study showed that indicaxanthin can reduce perferryl-Hb generated in solution from met-Hb and hydrogen peroxide (H_2O_2), more effectively than either Trolox or vitamin C.

Collectively our results demonstrate that indicaxanthin can be incorporated into the redox machinery of β-thalassemic RBC and defend the cell from oxidation, possibly interfering with perferryl-Hb, a reactive intermediate in the hydroperoxide-dependent Hb degradation. Opportunities of therapeutic interest for β-thalassemia may be considered.

Keywords: *Antioxidants, betalains, haemoglobin, indicaxanthin, phytochemicals, red blood cells*

Introduction

Beta-thalassemia is a genetic haemolytic disorder characterised by an increased generation of reactive oxygen species, first caused by haemoglobin (Hb) auto-oxidation and precipitation [1,2]. This is associated to depletion of the red blood cell (RBC) antioxidant defence, which results in damage to cell components, impairment of morphology and function of cell membrane and accelerated RBC destruction [2–6]. In addition, the transfusion therapy, which is the standard treatment for these patients, generates a state of iron overload that further affects the oxidative status [7]. Although early studies had shown that supplementation with antioxidant vitamins did not cause substantial benefit in the requirement of transfusions nor in the Hb levels [8], recent evidence has been provided that it may be of help to β-thalassemia patients [9,10]. It has been observed that administration of vitamin E improves the antioxidant/oxidant balance in plasma, and counteracts the oxidative processes in erythrocytes, thus contributing to their longevity. In addition, natural antioxidants such as rutin [11], curcumin, and the tea polyphenols [12], have recently been shown to have salutary effects on thalassemic erythrocytes [13], suggesting that phytochemicals, possibly used in combination with vitamin E and/or iron chelators, may offer new co-therapeutic opportunities.

Betalains, nitrogen eterocycles the redox properties of which have been reported in a number of *in vitro* studies [14–18], recently emerged as another class of phytochemicals to be considered as potential natural antioxidants. Among them, the betaxanthin indicaxanthin, the yellow pigment of the cactus pear fruit,

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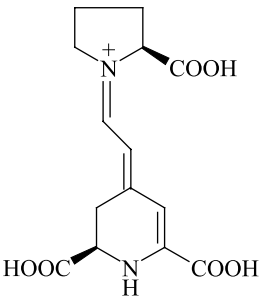

Indicaxanthin

Figure 1. Molecular structure of indicaxanthin.

is an immonium conjugate of proline with betalamic acid (Figure 1). A number of investigations have shown the radical-scavenging activity of this compound in either aqueous or hydrophobic environments [17,19]. Studies with humans showed that dietary indicaxanthin is highly bioavailable, and attains a peak plasma concentration of 2.2 μg/ml (7 μM) after ingestion of a fruit meal containing 28 mg [20]. In addition, in parallel with its plasma concentrations, it can distribute in body compartments such as low density lipoproteins and RBCs [20,21].

This study investigated the activity of indicaxanthin in RBCs from β-thalassemia patients challenged *in vitro* with cumene hydroperoxide (cumOOH), an organic compound promoting a sequel of oxidative events mimicking the pathophysiological pathway leading these cells to haemolysis. A number of measurements, including the resistance to haemolysis, evaluation of oxidation products from lipids and Hb, and relationships with the major cell antioxidants such as GSH and vitamin E, were performed to assess antioxidative effects of the phytochemical. Additional experiments in solution aimed at studying the reaction of indicaxanthin with perferryl-Hb, a hypervalent-iron highly reactive species, were carried out to have insights into mechanisms involved in the cytoprotective effects.

Methods

Chemicals

Ascorbic acid, bovine met-Hb, butylated hydroxytoluene (BHT), cumOOH, 5,5′-dithiobis(2-nitro benzoic acid) (DTNB), ethylendiaminetetraacetic acid (EDTA), hemin, hydrogen peroxide (H_2O_2), tetraethoxypropane (TEP), thiobarbituric acid (TBA), α-tocopherol and Trolox, were from Sigma (St Louis, MO). All other chemicals were of research highest purity grade. Ion-free water and buffers used throughout this study were filtered through Chelex-100 (Sigma), and suitable plastic labware was used to avoid the effect of adventitious metals.

Isolation of indicaxanthin

Indicaxanthin was isolated from cactus pear (*Opuntia ficus-indica*) fruits (yellow cultivar). The phytochemical was separated from the methanolic extract of the pulp by liquid chromatography on Sephadex G-25 [17]. Fractions containing the pigment were submitted to cryoessiccation. The essiccated material was re-suspended in 1% acetic acid in water [22] and submitted to semi-preparative HPLC using a Varian Pursuit C18 column (250 × 10 mm i.d.; 5 μm; 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 3 ml/min [17]. Spectrophotometric revelation was at 482 nm. The elution volumes relevant to indicaxanthin were collected. Samples after cryoessiccation were re-suspended in PBS at a suitable concentration.

Red blood cells

Blood samples from β-thalassemia intermedia patients ($n = 15$), previously characterized for β-globin gene mutation, were obtained from the patients by venipuncture, with informed consent. With the exception of 5 patients, who had never been transfused, they had received blood transfusions at least 2 years before this study. The mean value of Hb was 12.5 ± 1.8 mM (per heme group). EDTA (1 mg/ml blood) was used as an anticoagulant. RBCs were sedimented at 1000*g* for 10 min and washed three times with phosphate saline buffer (PBS), pH 7.4. Similarly, RBCs were obtained from blood samples of healthy volunteers ($n = 9$). The cells were suspended in PBS, to obtain a suitable hematocrit (HT).

Oxidative hemolysis

RBC oxidation was carried out by incubating RBC suspensions (HT 1%) with 300 μM cumOOH at 37°C, in the absence or in the presence of indicaxanthin. CumOOH was added to the erythrocyte suspensions as ethanol solution. The volume added never exceeded 0.5% of the total incubation volume. The extent of the time-dependent haemolysis was determined as follows. A volume (0.2 ml) of the incubation mixture at any given incubation time 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 same incubation mixture was treated with 10 volumes of 5 mM sodium phosphate buffer, pH 7.4 (hypotonic buffer) and

briefly exposed to an ultrasonic bath to yield complete haemolysis. After a centrifugation at 1000*g* for 10 min the absorbance of the supernatant was evaluated at 540 nm. The percentage of haemolysis was calculated from the ratio of the absorbances.

Consumption of indicaxanthin

Indicaxanthin was extracted from 1 ml oxidation mixture 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 (250 × 4.6 mm i.d., 5 μm), eluted with the same gradient reported above, with a flow of 1.5 ml/min. Spectrophotometric revelation was at 482 nm. Quantitation of indicaxanthin was by reference to standard curves constructed with 5–100 ng of the purified compound, and by relating the amount of the compound under analysis to the peak area.

Measurement of vitamin E and GSH

Vitamin E was measured in RBCs (HT 1%, 5 ml) collected at 1000*g* for 10 min. The cell pellet was re-suspended with 1 ml of PBS, and mixed with two volumes of absolute ethanol, followed by two successive extractions with six and two volumes of petroleum ether. The organic extracts were gathered, dried under a nitrogen stream, resuspended in several microliters of methanol, and vitamin E was separated by HPLC using a Supelco Supelcosil^{TM} (Bellefonte, PA) LC-18 column (0.46 × 25 cm). The eluent was methanol with a flow rate of 1.0 ml min^{-1} [20]. 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 α-tocopherol, 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, then 0.5 ml of H_2O were added to the RBC pellet to lyse the cells. Proteins were precipitated by addition of 0.5 ml of a metaphosphoric acid solution (1.67 g metaphosphoric acid, 0.20 g EDTA and 30 g NaCl in 100 ml of H_2O). After centrifugation at 3000*g* for 10 min, 400 μl of the clear supernatant were combined with 500 μl of 300 mM Na_2HPO_4, 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 DTNB in 100 ml of 1% sodium citrate) were added to both blank and 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 13,600 [23].

Measurement of lipid oxidation

The conjugated dienes (CD) lipid hydroperoxides were extracted from 2.0 ml of RBC suspension (HT 2%), with 6.0 ml chloroform/methanol (2:1, v:v). The organic extract was dried under nitrogen, re-suspended in cyclohexane and quantitated spectrophotometrically at 234 nm, using a molar absorption coefficient of 27,000 [24].

MDA was measured in RBCs (HT 1%, 0.5 ml) mixed with 1.5 ml H_2O, 5 μl of 40 mM BHT and 5 μl of 1 M NaOH. After cell homogenization, 250 μl of 0.6% TBA in 35% $HClO_4$ were added and the suspension heated for 60 min. Then, samples were cooled, centrifuged at 1000*g* for 10 min, and the MDA–TBA adduct in the supernatant was separated by isocratic HPLC, using a Supelco Supelcosil LC-18 column (0.46 × 25 cm) (Bellafonte, PA). Eluent was 40% methanol in 50 mM potassium phosphate buffer, pH 6.8, at 1.5 ml min^{-1}. The MDA–TBA adduct was revealed spectrophotometrically at 532 nm and quantified by reference to a calibration curve of TEP submitted to the TBA colorimetric procedure.

Measurement of haemoglobin and hemin

RBCs (HT 1%, 1 ml) were collected by centrifugation, and the cell pellet, re-suspended in 2.0 ml of hypotonic buffer, was briefly exposed to an ultrasonic bath to yield complete haemolysis. After centrifugation, aliquots of supernatant were scanned at 500–700 nm, and concentrations of the oxidation products of Hb in mixtures of HbO_2, met-Hb and hemichrome were calculated according to Winterbourn [25], and expressed per heme group. The amount of hemin associated with RBC membranes was measured in the membrane pellet re-suspended in 1% SDS in PBS, by spectrophotometry at 408 nm. Concentrations were evaluated from a calibration curve of 2–10 μM hemin in the same SDS buffer.

Ex vivo *spiking of blood from β-thalassemic patients with indicaxanthin and binding to red blood cells*

Blood from each β-thalassemia patient was incubated at 37°C, for 15 min, in the presence of PBS solutions of indicaxanthin, to obtain final concentrations of 5.0–50 μM. RBCs were isolated by centrifugation, washed and re-suspended in PBS to obtain a 10% hematocrit. Aliquots (7.5 ml) were extracted with 3 volumes of chloroform/methanol (2:1, v:v), the methanol phase was dried and re-suspended in 1% acetic acid in water, then indicaxanthin was measured by HPLC as above reported.

In other experiments, RBCs isolated after spiking of plasma with 25 μM indicaxanthin were re-suspended in hypotonic buffer to obtain a 10% hematocrit and briefly exposed to an ultrasonic bath to achieve complete haemolysis. Membrane were precipitated by centrifugation (100,000*g* for 1 h, at 4°C), and re-suspended with hypotonic PBS. Either membrane or supernatant preparations were extracted and analysed for indicaxanthin.

Reaction of indicaxanthin with hypervalent-iron Hb species

Bovine met-Hb (100 μM) in PBS was oxidized to perferryl-Hb (·Hb[Fe^{IV}=O]) by 100 μM H_2O_2, at 37°C, either in the absence or in the presence of 25 μM either indicaxanthin or other antioxidants. The reaction was carried out in a Beckman DU 640 UV/vis spectrophotometer, equipped with a temperature controller. Spectrophotometric scans were monitored at 500–700 nm [26] and the concentrations of the oxoferryl species was calculated from the absorbance at 556 nm, the point at which met- and ferryl-forms differ most ($\Delta = 3.6\,mM\,cm^{-1}$) [11]. The absorption spectrum of indicaxanthin, from 360 to 500 nm with a maximum at 482 nm [27], did not interfere with the spectrophotometric measurements of the Hb species.

Results

The oxidative state of the β-thalassemic RBCs was first assessed in terms of cell antioxidants, and level of oxidation products from lipids and haemoglobin in the soluble cell fraction and in membrane. The results are reported in Table I in comparison with healthy RBCs. Vitamin E and GSH were remarkably reduced in the pathological RBCs, whereas, substantial amounts of CD lipid hydroperoxides and MDA, and of oxidised products from Hb, including met-Hb, hemichrome, and hemin in the membrane, were found. Because of the wide spectrum of disease severity, inter-individual differences of the redox conditions of the RBCs are expected. As a consequence, the coefficient of variation of the data from thalassemia cells is quite high with respect to healthy RBCs.

Cumene hydroperoxide is a lipophilic oxidant requiring iron to promote radical production and start a cascade of events including oxidation of membrane components and degradation of haemoglobin. Because of the limited antioxidant defence and higher amounts of hemin in membrane, thalassemic RBCs were more susceptible than healthy RBCs to the cumOOH-induced oxidative haemolysis, and a complete cytolysis was evident when only 20% of healthy RBCs were broken. In addition, in accordance with the wide variability of the cell redox conditions, the kinetic curve of haemolysis showed a slow progression and did not exhibit a definite time of resistance (Figure 2(a)). When added to the incubation mixture at concentrations ranging from 1 to 10 μM, indicaxanthin enhanced the resistance of RBCs to haemolysis in a concentration-dependent manner (Figure 2(b)). The phytochemical appeared to be totally consumed during the RBC oxidation (Figure 2(b)).

GSH and vitamin E were rapidly exhausted after oxidation of thalassemic RBCs with cumOOH. Co-incubation of the cells with 1–10 μM indicaxanthin resulted in a decreased rate of consumption of both antioxidants, the higher the concentration the slower the consumption (Figure 3(a) and (b)).

A rapid accumulation of MDA, hemichrome and hemin in the membrane (Figure 4) was evident in the cumOOH-treated thalassemic RBCs. Indicaxanthin delayed MDA formation, and inhibited the formation of Hb degradation products, with the effect being related to the concentration added (Figure 4).

Binding of indicaxanthin to thalassemic erythrocytes and cell location were investigated. *Ex vivo* spiking of blood from thalassemia patients with indicaxanthin, in the range of 5.0–50.0 μM, resulted in the incorporation of increasing amounts of the compound, with a maximum amount of 1.03 ± 0.1 nmoles/ml packed RBCs, at 25 μM (Figure 5). When researched in either membrane or soluble cell preparations, indicaxanthin was found entirely associated with the aqueous cell compartment.

Table I. Major antioxidants and oxidised products from lipids and Hb in RBCs from β thalassemia patients and healthy volunteers.

	β-thalassemic RBCs, $n = 15$	c.v. (%)	Healthy RBCs, $n = 9$	c.v. (%)
Vitamin E (μM)	0.80 ± 0.19⋆	23.7	1.9 ± 0.15	7.8
GSH (mM)	0.85 ± 0.25⋆	29.4	2.21 ± 0.2	9.0
CD-lipid hydroperoxides (μM)	210 ± 41⋆	19.5	91 ± 8	8.8
MDA (μM)	12 ± 2.7⋆	22.5	1.5 ± 0.18	12.0
Met-Hb (mM)†	1.6 ± 0.35⋆	21.8	0.8 ± 00.9	11.2
Hemicrome (μM)†	22 ± 4	18.1	n.d.	
Hemin (mM)	0.21 ± 0.05	23.8	n.d.	

Values are the mean ± S.D. of *n* separate determinations. c.v., coefficient of variation (S.D. × 100%); n.d., not detectable.
⋆ Significantly different from healthy RBCs, $P < 0.0001$ (Student's-*t* test); † Concentration was expressed per heme group.

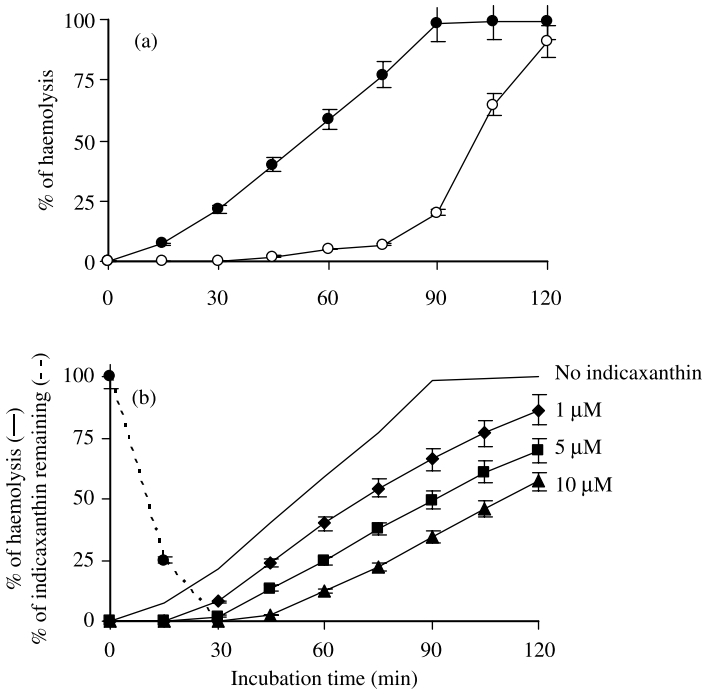

Figure 2. (a) CumOOH-induced haemolysis of healthy (open symbols) and thalassemic RBCs (full symbols); (b) effect of 1–10 μM indicaxanthin on the cumOOH-induced haemolysis of thalassemic RBCs and time course of the consumption of 10 μM indicaxanthin (dotted line).

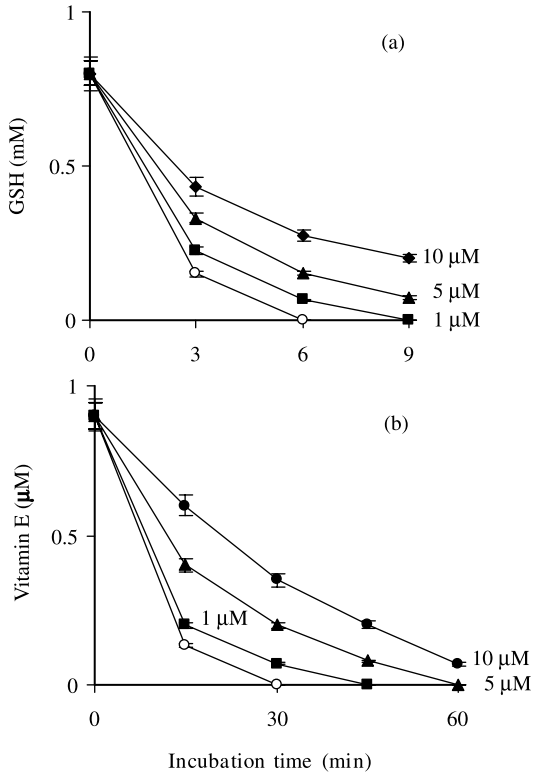

Figure 3. CumOOH-induced GSH (a) and vitamin E (b) consumption in thalassemic RBCs incubated in the absence (open symbols) or in the presence (full symbols) of 1–10 μM indicaxanthin.

Reaction of indicaxanthin with hypervalent-iron Hb species

The hydroperoxide-mediated degradation of heme-proteins, including Hb, proceeds through the transient formation of highly reactive radical species. The primary oxidation of the heme iron of oxy-Hb to met-Hb is followed by the formation of perferryl-Hb [28–30]. This species, two oxidation equivalents above the met-Hb, includes a hypervalent-iron oxoferryl heme group [Fe^{IV}=O] detected spectrophotometrically [28,29,31], and a radical species localized on the gobin [29,32]. Decay of the radical to the oxoferryl form [26], followed by slow auto-reduction to met-Hb, involves intramolecular electron transfer and modification of the globin moiety [28,29]. Experiments in solution were designed to check whether indicaxanthin would reduce perferryl-Hb.

Purified met-Hb was treated with H_2O_2 to generate perferryl-Hb, either in the absence or in the presence of indicaxanthin, and the reaction monitored by the spectral changes between 500 and 700 nm. When 100 μM met-Hb and 100 μM H_2O_2 were incubated at 37°C, the characteristic peak of met-Hb at 630 nm (Figure 6, dotted line) was almost completely lost, while two new peaks at 545 and 580 nm, characteristic of the oxoferryl moiety, appeared (Figure 6, line 1). The amount of oxoferryl after a 1-min incubation was calculated as 50 ± 4 μM ($n = 10$) from the changes in the absorption at 556 nm. Spontaneous reduction

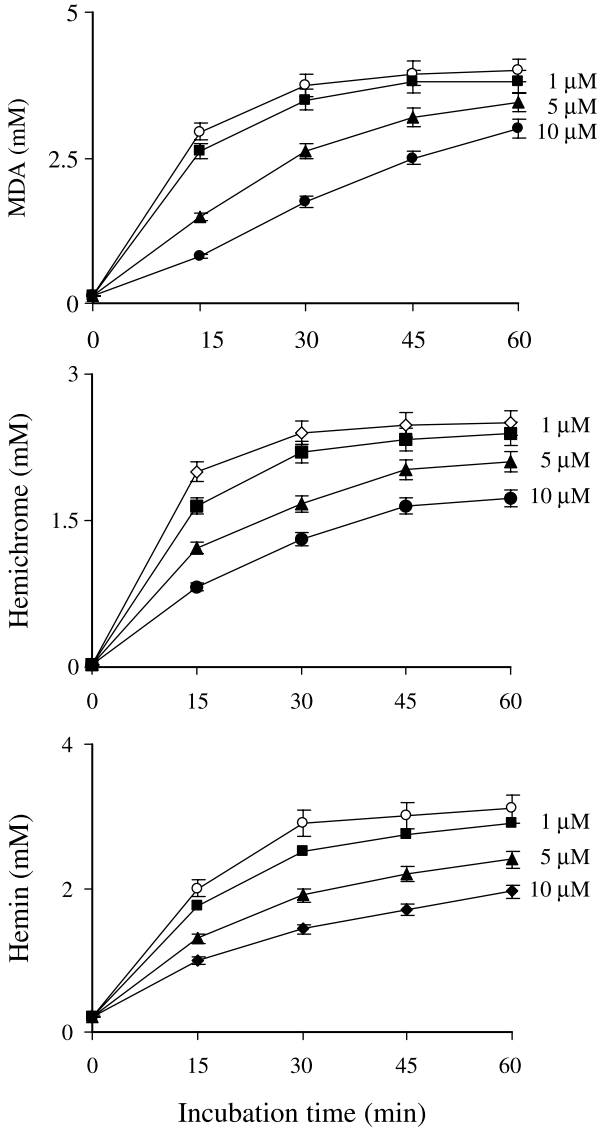

Figure 4. CumOOH-induced formation of MDA (a), hemichrome (b) and hemin (c) in thalassemic RBCs incubated in the absence (open symbols) or in the presence (full symbols) of 1–10 μM indicaxanthin.

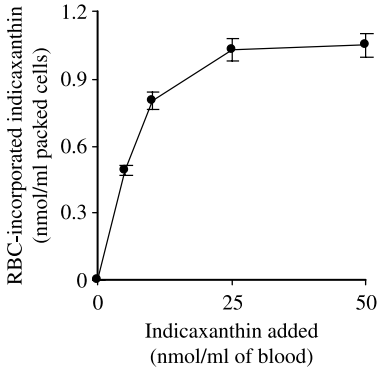

Figure 5. Incorporation of indicaxanthin in thalassemic RBCs following *ex vivo* spiking of blood with the purified compound.

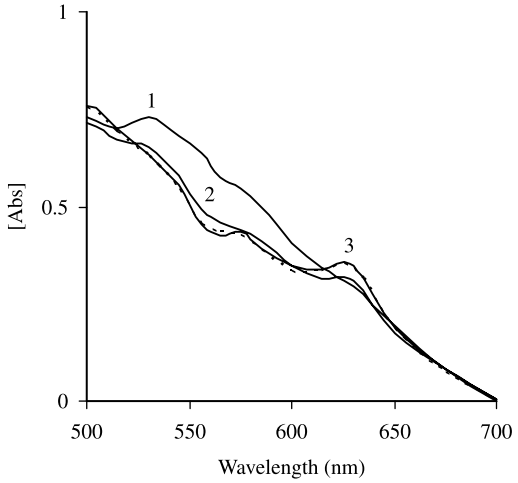

Figure 6. Spectral changes of perferryl-Hb upon reaction with indicaxanthin. Met-Hb (100 μM; dotted line) was converted to perferryl-Hb by a 1 min incubation with H_2O_2 (100 μM; line 1). Indicaxanthin (25 μM) was added to the incubation mixture before H_2O_2 and scans were taken at 1 (line 2) and 3 (line 3) min of incubation.

of the oxoferryl moiety started soon after 1 min, however, because of the globin denaturation, the spectrophotometric trace of met-Hb after 20 min did not overlap the original met-Hb spectrum (not shown). When 25 μM indicaxanthin was added to the incubation mixture before the oxidant (Figure 6, line 2), the amount of oxoferryl moiety after 1 min was 10 ± 1 μM ($n = 8$). Furthermore, the spectrum recorded after 3 min entirely fitted the original met-Hb spectrum (Figure 6, line 3). A reduction rate of the perferryl species of 660 ± 50 nM s^{-1} was calculated from the spectrophotometric data collected within the first minute. As a comparison, antioxidants known to reduce perferryl moieties were assayed [33–35]. Trolox, the water-soluble analog of vitamin E and vitamin C, at 25 μM, reduced the perferryl-Hb at a rate of 66 ± 5.7 and 83 ± 7.9 nM s^{-1}, respectively.

Discussion

The sequel of events consequent to the precipitation of Hb α-chains remarkably affects the redox status of β-thalassemia RBCs. Indeed, depletion of the main cell antioxidants, and accumulation of products from oxidised lipids and Hb, have been observed in RBCs from thalassemia patients in the present as well as in previous studies [7,36]. This paper shows that indicaxanthin, a dietary phytochemical whose reducing and antioxidant properties [17,19], as well as bioavailability in humans [20] have recently been reported, has cytoprotective activity in β-thalassemia RBCs submitted to an oxidative injury *in vitro*.

A cascade of oxidative reactions, that mimick and accelerate the pathophysiologic events leading thalassemic RBCs to haemolysis, is triggered by treatment

with cumOOH in the soluble as well as in the membrane cell compartment [37]. In the presence of redox-active iron and membrane-adhering Hb, cumOOH generates H_2O_2, met-Hb and a number of cumOOH-derived oxyradicals starting oxidation of membrane lipids [38]. Further oxidation of met-Hb by hydroperoxides, results in formation of hemichrome followed by precipitation of hemin, which is crucial to the membrane damage ensuing in haemolysis [39–42]. Indeed, the rate of formation of these products determines the rate of haemolysis [1].

Our work shows that indicaxanthin is capable of enhancing dose-dependently the resistance of thalassemic RBCs to the cumOOH-induced haemolysis, by preventing oxidation of both membrane and soluble cell components. Indicaxanthin has been shown to be incorporated in human healthy erythrocytes *in vivo* [20] and *ex vivo* [21]. As a result of oxidative modifications and cross-linking of lipid and protein components, thalassemia RBCs have an altered membrane morphology, including phospholipid asymmetry [43,44], and dysfunction of transport systems [45]. This study provides evidence that spiking of blood from thalassemia patients with indicaxanthin is followed by its incorporation in the RBCs, indicating that the pathological alterations do not affect a trans-bilayer movement of this phytochemical. In addition, the incorporated amount is quite comparable with that measured in RBCs from healthy volunteers after spiking of blood under conditions similar to those of the present work [21].

Indicaxanthin has been recovered in the aqueous compartment of thalassemia cells. Though it is water-soluble, indicaxanthin has appeared capable of binding to lipid moieties such as LDL [19,20], while other results from our laboratory indicate a preferential distribution of indicaxanthin at the bilayer/water interface in a dipalmitoyl phosphatidylcholine liposomal system (manuscript in preparation). The evidence that indicaxanthin is cell-permeating is consistent with an amphiphilic character of the molecule, which may be crucial to locate the compound in the cell, possibly at the water–lipid interface. This could eventually allow interactions with aqueous, as well as with lipid-derived radicals floating to the lipid–water interface [46].

Scavenging of lipoperoxyl radicals by indicaxanthin, has been reported [19]. Such an activity could account for the observed decrease of MDA production as well as of vitamin E consumption, but cannot entirely explain the anti-haemolytic effects [47,48].

The net decrease of the cumOOH-induced hemin and hemichrome production provided evidence that indicaxanthin effectively prevented Hb degradation, an autoxidation process involving the intermediate formation of the highly reactive perferryl-Hb, a species that comprises a radical localised on the globin, possibly an aromatic amino acid radical [29,32,49], and an oxoferryl heme group [28,29,31]. Our spectrophotometric measurements in solution showed that indicaxanthin is highly effective in reducing perferryl-Hb generated from met-Hb and H_2O_2, inducing a quite rapid reduction of the hypervalent-iron, and producing met-Hb. Whether reduction by indicaxanthin may occur at the oxoferryl moiety or the unpaired electron electrophile center at the globin moiety, or both, is indistinguishable by the spectrophotometric evidence. It is noteworthy that the spectrum of met-Hb induced by indicaxanthin entirely fitted the spectrum of native met-Hb. While indicating reaction with the oxoferryl moiety, this also suggests that a reduction of the globin radical occurs, otherwise the oxidation of globin residues by perferryl-Hb would make the met- to ferryl-Hb transformation irreversible [50].

On a molar basis, the reducing activity of indicaxanthin towards perferryl-Hb was one order of magnitude higher than that exhibited by effective reductants of perferryl-Hb such as ascorbate [35] or Trolox [33,34]. In accordance with these results, recent work showed that the hypervalent-iron porphyrin moieties of either compound I or II, formed during the catalytic cycle of the human heme-enzyme myeloperoxidase [51], can be reduced by this phytochemical [52], with calculated rate constants of the order of 10^6 and $10^5\,M^{-1}\,s^{-1}$, respectively, whereas, vitamin C exhibits rate constants of the order of 10^5 and $10^3\,M^{-1}\,s^{-1}$ for the reduction of the same intermediates [53]. Then indicaxanthin seems to be highly effective in reducing protein-bound hypervalent-iron heme species. This suggests that the observed cytoprotective effects in thalassemia red blood cells could be accounted for by the inhibition of the autoxidative chain reaction of Hb. Prevention of this self-amplifying oxidation process is consistent with the minute amounts of indicaxanthin required to delay cell lysis. In addition, protection of haemoglobin would in turn spare GSH, a major RBC component to regulate cell redox environment and counteract Hb oxidation [54,55]. Finally, the deleterious reactions triggered by precipitation of Hb, including heme-iron-induced lipid peroxidation [56–58], would also be delayed.

The redox properties and radical-scavenging activity of indicaxanthin have been proved by a number of assays [14–19], however, the molecular mechanism is still unknown. It is accepted that the conjugated π-orbitals of indicaxanthin are responsible for the reducing properties of the molecule [14,15,17]. Though the withdrawal of an electron may be hindered by the positive charge present in the nitrogen atom, a neutral pH, by stabilizing a deprotonated species, may favour oxidation of the molecule, leading to a delocalised betalain radical. Regeneration from its radical, would remarkably amplify the molecule effectiveness, and has been

discussed to explain the protective activity of indicaxanthin in a low-density lipoprotein oxidation model [19]. In the present study, the time-course of consumption of indicaxanthin seems to indicate other fate of indicaxanthin after the oxidative challenges. The conditions applied and/or the nature of the radicals generated apparently make the oxidation of indicaxanthin a rapid and irreversible process. Isolation and identification of the product(s) in simple oxidation models will be of valuable help to shed light in the redox chemistry of indicaxanthin.

In conclusion, indicaxanthin may be incorporated in the redox machinery of beta-thalassemic RBCs, remarkably improving their antioxidant potential. It seems noticeable that protective effects are evident at concentrations consistent with the plasma concentrations achieved in humans by ingestion of dietary indicaxanthin [20]. In view of the absence of toxicity of indicaxanthin [59] and of its potential antioxidative effects in humans [20,21], present data emphasize the potential interest of this molecule in the supportive therapy of thalassemia, while stimulating further studies involving patients.

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