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Betanin inhibits the myeloperoxidase/nitrite-induced oxidation of human low-density lipoproteins

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Abstract

Production of nitrogen dioxide by the activity of myeloperoxidase (MPO) in the presence of nitrite is now considered a key step in the pathophysiology of low-density lipoprotein (LDL) oxidation. This study shows that betanin, a phytochemical of the betalain class, inhibits the production of lipid hydroperoxides in human LDL submitted to a MPO/nitrite-induced oxidation. Kinetic measurements including time-course of particle oxidation and betanin consumption, either in the presence or in the absence of nitrite, suggest that the antioxidant effect is possibly the result of various actions. Betanin scavenges the initiator radical nitrogen dioxide and can also act as a lipoperoxyl radical-scavenger. In addition, unidentified oxidation product(s) of betanin by MPO/nitrite inhibit(s) the MPO/nitrite-induced LDL oxidation as effectively as the parent compound. In the light of betanin bioavailability and post-absorbtion distribution in humans, present findings may suggest favourable *in vivo* activity of this phytochemical.

Keywords: *Betanin, myeloperoxidase, nitrite, low-density lipoproteins, atherosclerosis*

Introduction

Oxidation of low-density lipoproteins (LDL), considered a pivotal process in the progression and eventual development of atherosclerosis [1–3], has been studied for years. It has recently been suggested that myeloperoxidase (MPO) has a prominent role in promoting oxidative reactions in LDL *in vivo* [4,5]. MPO, a heme-enzyme which occurs in neutrophils and monocytes, utilizes hydrogen peroxide and a variety of co-substrates to generate reactive oxidants as intermediates [2,4,6]. The enzyme follows the classical peroxidase cycle [7], in accordance with equations (1)–(3):

$$\mathrm{MPO} + \mathrm{H_2O_2} \leftrightarrow \mathrm{MPO\text{-}I} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{MPO\text{-}I} + \mathrm{AH_2} \leftrightarrow \mathrm{MPO\text{-}II} + \mathrm{AH^{\cdot}} \quad (2)$$

$$\mathrm{MPO\text{-}II} + \mathrm{AH_2} \leftrightarrow \mathrm{MPO} + \mathrm{AH^{\cdot}} + \mathrm{H_2O} \quad (3)$$

A single two-electrons oxidation of the native enzyme to compound I (MPO-I) by H_2O_2 is followed by two successive one-electron reductions by reducing substrates (AH_2) to native enzyme, via compound II (MPO-II). MPO-I, which is two oxidative equivalents above the ground state, is an extremely reactive species but cannot easily oxidize large molecular complexes, including LDL, because its active site is buried in a hydrophobic cleft. Indeed, it relies on low molecular weight compounds to convey oxidizing equivalents from the heme group to its target [8–10]. The hypochlorous acid formed via a single two-electrons oxidation of chloride ions by MPO-I [11], has been considered implicated in the pro-oxidant activity of MPO on the LDL protein (LDL prot) [12–14]. On the other hand, it has recently been shown that the MPO activity also depends on the metabolism of nitric oxide (NO) being nitrite, the final oxidation product of NO metabolism, a substrate for the enzyme [15–18]. Nitrogen dioxide radical ($NO_2^{\cdot}$), the one-electron oxidation product of nitrite by MPO-I, has

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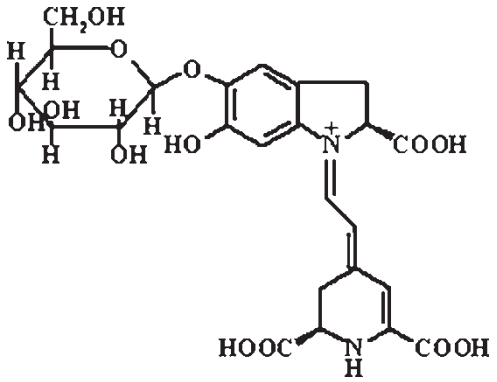

Figure 1. Chemical structure of betanin.

been proposed as the reactive species to start massive oxidation of the LDL lipids [19–20].

Bioactive components of fruits and vegetables are considered of great importance in the prevention of various degenerative pathologies, including cardiovascular diseases [21]. Betalains are phytochemicals characteristic of flowers and fruits of ten families of *Cariophyllalae* plants, including red beet and cactus pear, and of few superior fungi of the genus *Amanita* of the *Basidiomycetes* [22]. These pigments are betalamic acid derivatives and depending on the components bonded to the main structure, betacyanins or betaxanthins arise, the former when the group is 3,4-dihydroxyphenylalanine which may or may not be glycosylated and the latter if the conjugation partners are amino acids or the derived amines. Various *in vitro* studies showed that the betacyanin betanin (5-*O*-glucose betanidine) (Figure 1), a molecule with three redox potentials at 404, 616, and 998 mV [23], reduces numerous radical species in both chemical and biological environments. This molecule can partition in lipid compartments [24,25] and increases the resistance of human LDL to oxidation induced by copper [25] as well as by H_2O_2-activated metmyoglobin [24]. This study assessed the antioxidant activity of betanin the MPO/nitrite-dependent oxidation of human LDL.

Materials and methods

Betanin was extracted from cactus pear fruits (red cultivar) as reported by Butera et al. [23] and then purified according to Stintzing et al. [26].

Unless stated otherwise, all materials and solvents were from Sigma (Milan, Italy) and of the highest purity or high-performance liquid chromatography (HPLC) grade.

LDL preparation

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 L8-70 ultracentrifuge (Beckman, CA, USA) fitted with a 50 Ti rotor and using potassium bromide for density adjustments according to Kleinveld et al. [27]. 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 column (Pharmacia Biotech, Milan, Italy). Proteins were determined by using the Bio-Rad colorimetric method [28].

Lipoprotein oxidation

Reactions were carried out at 37°C in 0.1 M potassium phosphate buffer, pH 7.4, supplemented with 0.1 mM diethylenetriaminepentacetic acid. The reaction mixture contained 50 μg LDL prot, 50 μM nitrite, 53 nM MPO ($\varepsilon_{430} = 170\,mM^{-1}\,cm^{-1}$, Planta Natural Products, Vienna, Austria), 310 ng/ml (1.29 nkat/ml) glucose oxidase (GOD) and 0.56 mM D-glucose (final concentrations). In some experiments nitrite and/or LDL were omitted. Betanin was added to the reaction mixture dissolved in PBS. The oxidation of LDL was followed spectrophotometrically by measuring the formation of conjugated diene (CD) lipid hydroperoxides at 234 nm. The absorbance of the sample was recorded every 2 min for a total of 100 min on a Beckman DU 640 spectrophotometer, equipped with a temperature control device.

Betanin analysis

Betanin was extracted from the incubation mixtures by mixing one volume of the incubation medium with three volumes of chloroform/methanol (2:1, v/v). The methanol phase was then dried under nitrogen and re-suspended in 1% acetic acid in water and analysed on a Varian Microsorb C-18 column (250 × 4.6 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 and revelation at 536 nm [22]. Quantitation was by reference to a standard curve constructed with 5–100 ng of betanin. A computer-assisted analysis (TableCurve 2D, Jandel, CA, USA) of the linear portion of the kinetic traces of the molecule decay was used to determine the initial rate of betanin consumption (v_i).

Spectral changes of betanin during oxidation were evaluated by analysing the pigment methanol extracts in the range 250–750 nm, before and at time-intervals from the reagent mixing.

LDL-vitamin E consumption

α-Tocopherol was extracted from LDL by mixing aliquots of the incubation mixture (50 μg LDL prot) with two volumes of absolute ethanol and eight volumes of petroleum ether. The organic extracts

containing vitamin E were then dried under nitrogen, re-suspended with several microliters of methanol and analyzed on a Supelco Supelcosil (Bellefonte, PA) LC-18 column (0.46 × 25 cm), with methanol as eluent at a flow rate of 1.0 ml/min. Detection was at 290 nm. Quantitation was by reference to a standard curve constructed with 5–100 ng of α-tocopherol.

Statistical analysis

Conventional methods were used for calculation of means and standard errors.

Results

When nitric dioxide, produced by the catalytic activity of MPO on nitrite, attacks LDL lipids, formation of CDs is first counteracted by the endogenous LDL antioxidants, which is spectrophotometrically monitored as a lag phase, followed by a net rise of the absorbance at 234 nm. The time-course of the MPO/nitrite-induced LDL oxidation is shown in Figure 2. A lag phase of 25 min is followed by a linear increase of the sample absorbance as a result of the chain propagation process. Betanin in a 0.5–5.0 μM range inhibited the MPO/nitrite-induced LDL oxidation, decreasing the propagation rate in a dose-dependent manner, without affecting the lag period. At the highest concentration tested (10 μM), betanin prevented the CD formation over a period of 100 min (Figure 2).

Spectral changes of the pigment were assessed during LDL oxidation in the presence of 5 μM betanin. Scanning of the absorbance spectrum at time-intervals showed a net decrease of the 536 nm peak (Figure 3), while did not provide evidence of absorbance peaks revealing formation of betanin product(s). The spectrophotometric traces showed that depletion of the molecule occurred within 10 min.

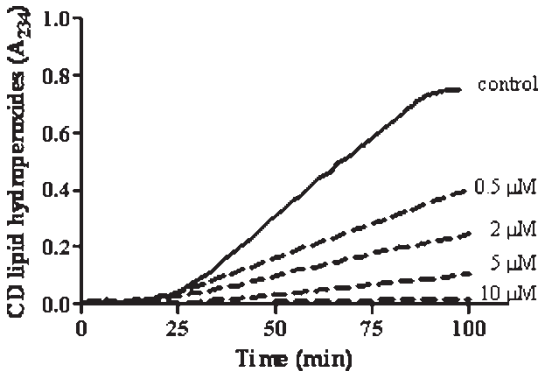

Figure 2. Time-course of LDL lipid oxidation by MPO/GOD/glucose/nitrite either in the absence (control) or in the presence of various betanin concentrations (0.5–10 μM). That reported is representative of six experiments with different LDL preparations. Oxidation of LDL was as reported in Methods.

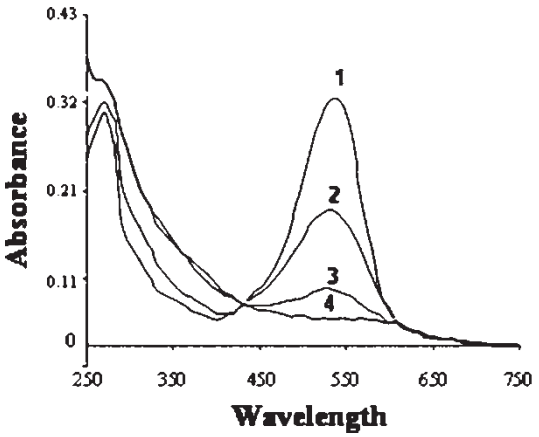

Figure 3. Spectral evidence of the consumption of betanin (5 μM) during LDL oxidation by MPO/nitrite. The spectral traces before (1) and after 2 min (2); 5 min (3); and 10 min (4) represent one of a large series of experiments. Oxidation of LDL was as reported in Methods.

Consumption of the endogenous vitamin E, the main LDL antioxidant, was monitored during LDL oxidation, either in the absence or in the presence of 5 μM betanin. Vitamin E was consumed in 20 min and betanin did not modify its temporal disappearance (not shown).

The MPO/nitrite-dependent LDL oxidation model entails the formation and interaction of various reactive species in succession. For a fuller understanding of the protective action of betanin in counteracting the LDL oxidation, a number of experiments were performed. Betanin has recently been shown as a substrate for the highly reactive intermediates of the MPO cycle [29]. To distinguish consumption of betanin by peroxidase activity from oxidation by nitrogen dioxide, the consumption of the molecule in a MPO-dependent nitrogen dioxide generating system was monitored in the absence of LDL and compared to its consumption in the presence of MPO and hydrogen peroxide alone. The time-course of betanin disappearance (Figure 4) provides evidence that the consumption rate is much higher in the presence of nitrite ($v_i = 1.01 \times 10^{-8}\,M\,s^{-1}$) than in its absence ($v_i = 0.75 \times 10^{-8}\,M\,s^{-1}$), thus suggesting reaction with nitrogen dioxide. On the other hand, betanin was not consumed by hydrogen peroxide alone (not shown). It should be noted that consumption of betanin during the MPO/nitrite-dependent LDL oxidation was even faster ($v_i = 1.8 \times 10^{-8}\,M\,s^{-1}$, Figure 4).

Oxidation of LDL lipids by MPO can occur even in the absence of nitrite [30,31], though at much a lower rate [20]. Then assays were performed to check about the activity of betanin against the MPO-induced LDL oxidation in the absence of nitrite. The time-course profile of LDL oxidation in this system is shown in Figure 5. Betanin (0.5–10 μM) inhibited the CD lipid hydroperoxide formation in a dose-dependent

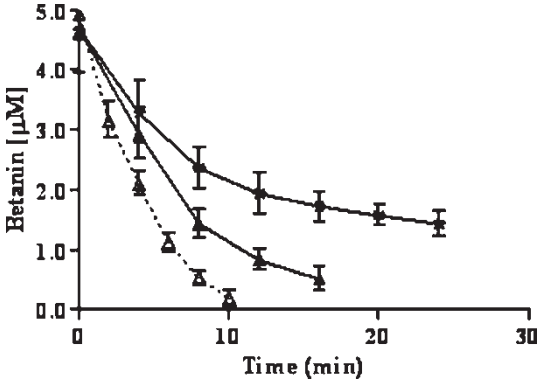

Figure 4. Time-course of the consumption of betanin (5 μM) during the catalytic cycle of MPO, either in the absence (✳) or in the presence (▲) of nitrite and during the MPO/nitrite-dependent LDL oxidation (△, dotted line). Values are means ± SE of three separate experiments. The experimental conditions are reported in Methods.

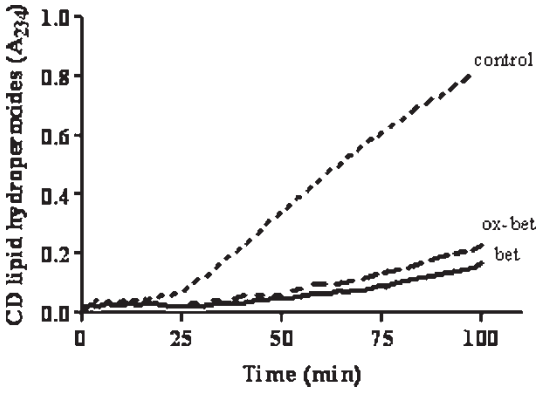

Figure 6. Time-course of the MPO/nitrite-dependent LDL lipid oxidation in the absence (control) or in the presence of either 5 μM betanin (BET), or the betanin oxidation product(s) (ox-bet). LDL and ox-betanin were as reported in Methods. That reported is representative of six experiments with different LDL preparations.

manner, with a lag phase the length of which linearly increased at the increase of the betanin concentration. On the other hand, betanin did not have any effect on the propagation rate and was consumed within the lag phase (not shown).

Activity of betanin oxidation product(s) on the MPO/nitrite-stimulated LDL oxidation was assessed by incubating 5 μM betanin with a MPO-dependent NO_2-generating system for 15 min, to obtain oxidation of the molecule and then adding the LDL. The oxidation kinetics compared with those in the presence of the native compound, provided evidence that the oxidised betanin was as effective as betanin in counteracting LDL oxidation (Figure 6).

Discussion

Current knowledge on the nutrition's role in disease prevention, highlights the importance of phytochemicals in health promotion. Characterization of the activity of these substances in various either chemical or bio-mimetic models may suggest eventual actions of these molecules in the body. This study shows that betanin, a betalain with recently acknowledged radical-scavenging properties [23–25,29,32,33], protects human LDLs from oxidative damage in a MPO/nitrite-dependent oxidation model, which is considered relevant to the pathophysiological conditions supporting the atherogenic process [34–36].

Because of its physicochemical properties, betanin intercepts radicals in the water phase [23], or can interact with lipid moieties, and affords antioxidant protection [24,25,32]. We have previously shown that *ex vivo* spiking of human plasma with varied betanin concentrations resulted in a concentration-dependent binding to LDLs, while the incorporated compound prevented a copper-induced oxidation of the isolated particles, acting as a lipoperoxyl radical-scavenging and chain-breaking antioxidant [25]. The inhibition kinetics and the time-course of betanin consumption during the MPO/nitrite-induced LDL oxidation suggested more complex antioxidative mechanisms.

Nitrogen dioxide, the oxidation product of nitrite with MPO-I, has been proposed as the main reactive intermediate able to promote LDL lipid oxidation [19,20]. This amphyphilic molecule can attack lipid moieties and start oxidation by abstracting hydrogen atoms from bis-allylic methylene groups of polyenoic fatty acids [37]. Since betanin can partition between LDL and water, it is supposed to be able to protect lipids by reacting with either lipoperoxyl radicals [25] and/or possibly with hydrophilic species, including NO_2 radicals. Our data show that betanin is consumed by nitrogen dioxide in a MPO-dependent NO_2^- generating system, supporting the idea that it can indeed inhibit the MPO/nitrite-stimulated LDL oxidation by intercepting NO_2 radicals, though additional radical scavenging activity in the lipid phase cannot be ruled out. The faster rate of consumption of the molecule in the MPO/nitrite–LDL system, as

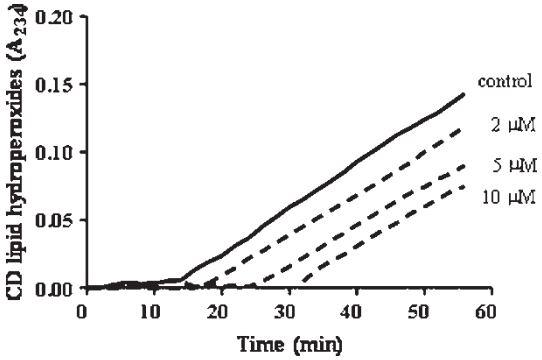

Figure 5. Time-course of LDL lipid oxidation by MPO/GOD/glucose, either in the absence (control) or in the presence of various betanin (BET) concentrations (2.0–10 μM). That reported is representative of six experiments with different LDL preparations. Oxidation of LDL was as reported in Methods.

compared to its disappearance in the presence of MPO/nitrite alone, suggests that betanin reacts with other radical species in the presence of LDL.

Betanin has been shown to act as a reducing substrate for the intermediates of the peroxidative MPO cycle, namely compound I and II, with a rate constant of the order of 10^6 and $10^5\,M^{-1}\,s^{-1}$, respectively [29], which could have affected the activity of the betalain in the MPO/nitrite-dependent-LDL oxidation. In particular, reduction of MPO compound I/II could result in formation of diffusible oxidants that may actually enhance the efficacy of the enzyme to oxidise the particles [38]. On the contrary, when assayed in the absence of nitrite, betanin was not pro-oxidant, providing evidence that, though capable of activating the catalytic cycle of MPO [29], it does not export the oxidizing power of the enzyme from the active protein site to the medium. Rather, betanin clearly counteracted the activity of the peroxidase to oxidize LDL. Thus, these data ruled out that the effects of betanin in the MPO/nitrite-induced LDL oxidation, could result from overlapping of antioxidant actions and the eventual pro-oxidative activity from the MPO-mediated conversion of the betalain to a reactive species.

In contrast with the kinetics observed in the presence of nitrite, the inhibition kinetics of LDL oxidation by MPO in the absence of nitrite revealed a classical chain-breaking antioxidant behaviour of betanin, with dose-dependent prolongation of the lag phase and consumption of the molecule within the lag phase. In accordance with previous findings [25], amounts of betanin partitioned in the LDL particle can account for this evidence. Apparently, such a lipoperoxyl radical-scavenging activity is masked in the presence of nitrite by the effects from scavenging nitrogen dioxide.

Finally, the early consumption of betanin, as compared with the extent of the protective effects during the MPO/nitrite-induced LDL oxidation, revealed peculiar aspects of the molecular activity of the phytochemical. Interactions and recycling are common features in the action of antioxidant molecules and cooperative interactions with the endogenous vitamin E, the main antioxidant in biological membranes and LDL [39,40], can amplify the effects of various compounds [41,42]. As already observed in other metal ion-dependent LDL oxidation model [25], the time-course of vitamin E consumption, either in the presence or in the absence of betanin, ruled out that interactions with the LDL–vitamin E were involved. On the other hand, oxidation product(s) from the reaction of betanin with MPO have appeared to prevent LDL oxidation as effectively as the parent compound, which allows to extend the antioxidative protection beyond the time in which betanin is consumed. Oxidation products of betanin by MPO are not known. Products from the reaction

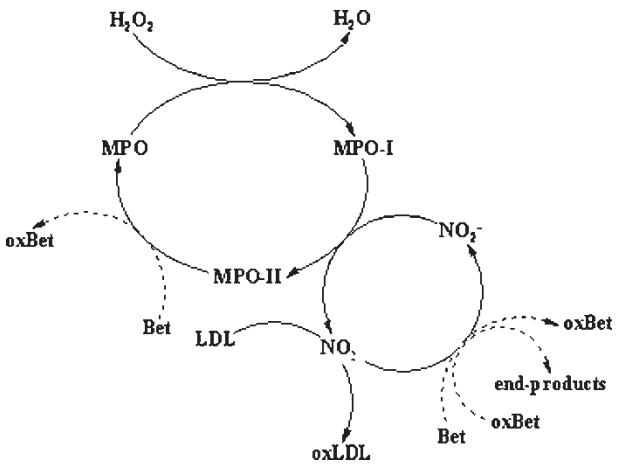

Figure 7. Catalytic cycle of MPO and putative sites of action of betanin (Bet). Oxidised betanin(OxBet).

between betanin and horseradish peroxidase have been described as stable phenoxyl radicals and betalamic acid [43], however, we failed to have any spectral evidence of such compounds in our system. It is tempting to speculate that a rapid reaction of betanin and/or MPO-oxidised betanin with $NO_2^{\cdot}$ led to the accumulation of end-products that do not exhibit a characteristic absorbance spectrum.

On the whole, our work shows that betanin may protect human LDLs from a MPO/nitrite-induced oxidation and suggests that it may affect the oxidative process at various levels. Based on the present findings and previous observations [25,29], a proposal as to how betanin may act in this system is shown in Figure 7. It is hypothesized that, after partitioning between water phase and LDL lipid, betanin could act as an electron donor towards what are currently believed to be the focal oxidants generated in this system, i.e. the MPO catalytic intermediates and their main downstream oxidation product nitrogen dioxide. The rate constant for the reaction between MPO-I and nitrite [15], of the same magnitude order than that of MPO-I and betanin [29] and the actual concentrations of the reductants in our assays, prevent betanin from competing with nitrite for the enzyme compound I, which results in production of nitrogen dioxide radical. By contrast, being the rate constant for the reduction of nitrite by MPO-II of the order of $10^2\,M^{-1}\,s^{-1}$ [15], betanin can effectively reduce the MPO-II intermediate, being oxidised and promoting the enzyme cycle. This potentially pro-oxidant behaviour appears to be counteracted by the antioxidant activity of betanin and/or possibly a betanin oxidised product, through scavenging of $NO_2^{\cdot}$. Finally, it is not possible to rule out that betanin partitioned in the LDL may be involved in additional lipoperoxyl radical-scavenging activity within the particle.

By blunting undesirable MPO-mediated actions of nitrite, betanin could modulate NO metabolism in

a favourable direction, thus counteracting LDL oxidation. In this regard, it is remarkable that recent nutritional studies have shown that betanin is bioavailable in humans [24,44], accumulates time-dependently in circulating LDL and attains a 0.2 μM plasma concentration after a single ingestion of a fruit meal providing 16 mg of the molecule.

Acknowledgements

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