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Article

Phytate Decreases Oxidative Damage Caused by Labile Forms of Iron in Solution, Blood Plasma and in HeLa Cells

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Fitato (PHYT, mio-inositol 1,2,3,4,5,6-hexakisfosfato) é um produto natural com forte efeito sobre a biodisponibilidade de minerais, especialmente o ferro. Neste trabalho, investigamos os efeitos antioxidantes do PHYT em modelos de transtornos de sobrecarga de ferro (ferro lábil plasmático e reservatório de ferro lábil). PHYT apresentou um efeito antioxidante considerável, com a vantagem de ser permeável às células e ser um constituinte normal da dieta humana. Nossos resultados sugerem que o PHYT pode auxiliar as defesas do organismo contra estresse induzido por sobrecarga de ferro.

Phytate (PHYT, myo-inositol 1,2,3,4,5,6-hexakisphosphate) is a natural product with strong effect on the bioavailability of minerals, especially iron. In this work, we investigated the antioxidant effects of PHYT on models of iron overload disorders (labile plasma iron and labile iron pool) both in solution and in HeLa cells. PHYT has a considerable antioxidant effect, with the benefit of being cell permeant and a normal constituent of human diet. Our results suggest that PHYT may assist organism defenses against iron-overload stress.

Keywords: iron bioavailability, phytate, overload, antioxidant effect

Introduction

Phytate (PHYT, myo-inositol 1,2,3,4,5,6-hexakisphosphate, Figure 1) is the main reservoir of phosphorus in seeds. It is a secondary messenger in intracellular signal transduction systems and, in animals, acts as a neuromodulator. Although initially thought to be of vegetal origin only, it has been demonstrated that mammalian cells such as HeLa cells or rat tissues also produce their own PHYT, ca. 10-20 μmol L^{-1}. In humans, white blood cells display measurable amounts of this molecule, but serum and platelet-free plasma are devoid of it.[1] Intracellular phytate probably resides in the cytosol.[2] PHYT has a major impact on the bioavailability of minerals such as zinc, calcium and iron. The six phosphate groups (Pearson hard base) constitute an excellent coordination environment for the Fe^{3+} ion (Pearson hard acid), which makes the absorption of dietary iron from PHYT-enriched food less efficient.[3,4]

Iron is the most abundant transition metal in superior organisms, being a key factor in O_2 transport and storage, cell respiration and deoxyribonucleotide formation. However, iron has a very low bioavailability, as it occurs naturally as stable and poorly soluble oxides and hydroxides. For these reasons, organisms developed strategies to accumulate iron in high affinity compartments that, in one hand, protect the metal against competition from infectious agents, and in the other hand block the iron-catalyzed generation of reactive oxygen species (ROS) such as hydroxyl radical through Fenton reaction.[5,6]

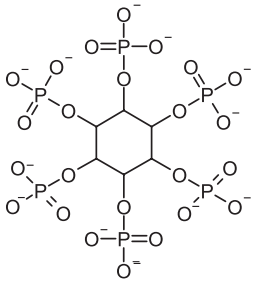

Figure 1. Structure of phytate (PHYT).

Iron overload disorders (IOD) occur when, by any reason, the organism accumulates more iron than it can safely store in proper biochemical compartments. IOD may be of genetic origin (e.g., hereditary hemochromatosis) or acquired (e.g., a result of multiple blood transfusions).

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This surplus iron catalyzes the formation of ROS in the plasma (labile plasma iron; LPI) or in cells (labile pool of iron; LIP),[7,8] leading to a cascade of oxidative damage that may result in tissue necrosis. Chelation therapy by means of desferrioxamine, deferiprone or deferasirox is the best approach for the treatment of IOD. However, all these chelators display side effects such as gastrointestinal, renal and liver damage that often decrease patient quality of life.[9]

Therefore, the pursuit of new chelators for the treatment of IOD with reduced side effects is justified. In this work, we evaluated the effect of PHYT both on iron redistribution to endogenous high-affinity reservoirs, and as an antioxidant in models of LPI and LIP (in HeLa cells). Comparison with the high affinity chelator diethylenetriaminepentaacetic acid (DTPA) was conducted as well.

Experimental

Reagents

Phytic acid dodecasodium salt (PHYT), ferrous ammonium sulfate (FAS), calcein, apo-transferrin, ascorbic acid, hepes, Chelex®, nitrilotriacetic acid (NTA), DTPA and 2',7'-dichlorofluorescin diacetate (DCFDA) were obtained from Sigma-Aldrich or Fluka. Dihydrorhodamine 1,2,3 dihydrochloride (DHR) was from Biotium.

Competitive iron binding monitored by fluorescence

Fluorescent transferrin (FlTf) was prepared by a method described elsewhere.[10] Stock solutions of iron-loaded calcein and FlTf were prepared by the reaction of solid FAS with 10 mmol L^{-1} aqueous solution of either calcein or FlTf in order to achieve 1:1 Fe:calcein or 2:1 Fe:transferrin molar ratios (transferrin binds two equivalents of iron per molecule). In flat, transparent 96-well microplates 10 μL aliquots of aqueous solutions of the chelator (DTPA or PHYT) and 190 μL of 2 μmol L^{-1} iron-calcein or iron-FlTf in Chelex-treated HBS buffer (NaCl 150 mmol L^{-1}, hepes 20 mmol L^{-1}, Chelex® 1 g/100 mL, pH 7.4) were mixed and allowed to react for 2 h at 37 °C in the dark. The final concentrations of the chelators were in the range of 0 to 400 μmol L^{-1}. Fluorescence measurements were performed in a FluoStar Optima (BMG) microplate reader with $\lambda_{exc}/\lambda_{em}$ = 485/520 nm. The experiment was conducted in duplicate.[8]

Antioxidant activity

This assay is based on the effect of a chelator on the rate of oxidation of the probe DHR mediated by the Fe(NTA)/ascorbic acid system.[7] Fe(NTA) complex was prepared by the addition of FAS to a stock solution of aqueous NTA in order to attain a 1:3 Fe:NTA molar ratio. This reaction was allowed to proceed for 1 h at 37 °C. Aliquots of 10 μL of 10 μmol L^{-1} Fe(NTA) were transferred to 96-wells microplate readers and treated with 10 μL of chelators (DTPA or PHYT in the range of 0 to 100 μmol L^{-1}) and 180 μL of DHR (50 μmol L^{-1}) and ascorbic acid (40 μmol L^{-1}) in HBS/Chelex® buffer. Kinetic curves were registered immediately in a FluoStar Optima (BMG) microplate reader with $\lambda_{exc}/\lambda_{em}$ = 485/520 nm during 40 minutes. The slopes of the kinetic curves (in fluorescence units F min^{-1}) calculated from 15 to 40 minutes were then plotted against chelator concentration. The experiment was conducted in duplicate.

Serum samples from iron-overloaded patients were also assessed. Patients undergoing hematopoietic stem cell transplantation gave informed consent, and the samples were kindly provided by Dr Flavio Naoum (Bone Marrow Transplant Service, Hospital Beneficência Portuguesa, São José do Rio Preto, Brazil). Aliquots of 10 μL of these samples were studied in the same setting described above, with serum samples used in the place of the Fe(NTA) standard and chelator concentration adjusted to 100 μmol L^{-1}.

Cell studies

Phytate loading to cells: HeLa cells (1×10^5 cells mL^{-1}) were grown in MEM-Earl medium containing 10% fetal calf serum, glutamine and antibiotics, in homemade Petri dishes with glass coverslips. Cells were cultivated in incubator at 37 °C, 5% CO_2 and 3% humidity until confluence was attained. On the day of the experiment, the dishes were washed once with 1.0 mL HBS buffer and then treated with 1.0 mL of 0.3 μmol L^{-1} acetometoxy-calcein (CAL-AM), a cell-permeant form of calcein, for 10 minutes.[11] Dishes were again washed and treated with 1.0 mL of FAS/ascorbic acid aqueous solution (10/100 μmol L^{-1}, respectively) for 10 minutes to induce iron overload. The dishes were washed and mounted on a Zeiss confocal fluorescence microscope (magnification 400×). When indicated, 1.0 mL of PHYT in HBS buffer (20 μmol L^{-1}) was added immediately before fluorescence was registered.[12]

Antioxidant effect of phytate on iron-overloaded cells: HeLa (5×10^4 cells mL^{-1}) cells were cultivated and grown as described above, in 24-wells flat bottom transparent microplates. After confluence was attained, cells were incubated at 37 °C in the dark with FAS/ascorbic acid with or without H_2O_2 (35 μmol L^{-1})[13] for 10 min. Whenever required, 20 μmol L^{-1} PHYT was added after washing the wells. After the final wash, cells were incubated with 5 μmol L^{-1} DCFDA in HBS for 40 min. The microplate

was bottom-read in a FluoStar Optima (BMG) microplate reader with $\lambda_{exc}/\lambda_{em}$ = 485/520 nm with gains manually adjusted to 1000 or 2000, in a 15 × 15 matrix for each well. Statistical analysis was conducted by Two Sample Independent t-Test (Microcalc Origin®) and significant differences were considered at the 0.05 level.

Results and Discussion

Competitive iron binding monitored by fluorescence

Calcein and transferrin are both models of high affinity binding sites for iron (pK_f = 24 and 22, respectively).[14,15] Calcein is a fluorescent chelator which is stoichiometrically quenched upon coordinating iron. Transferrin, the serum protein involved in iron transport in vertebrates, was derivatized to the fluorescent form FlTf so that iron binding could also be monitored by fluorescence quenching. To assess the relative ability of PHYT to remove iron from these ligands, we pre-loaded calcein and FlTf with iron (rendering both non-fluorescent) and added increasing amounts of PHYT or DTPA as competitive chelators. The recovery of 50% of fluorescence of the probes (Figure 2) is attained at [DTPA] = 4 μmol L^{-1} and [PHYT] = 24 μmol L^{-1} (for calcein) and [DTPA] = 19 μmol L^{-1} and [PHYT] = 162 μmol L^{-1} (for transferrin). These data show that the Fe(PHYT) complex is less stable than Fe(DTPA) and only at very high (and maybe unrealistic) concentrations PHYT would be able to successfully compete for iron with endogenous chelators. Therefore, plasmatic iron in suitable biochemical compartments is not accessible to PHYT as a competing chelator.

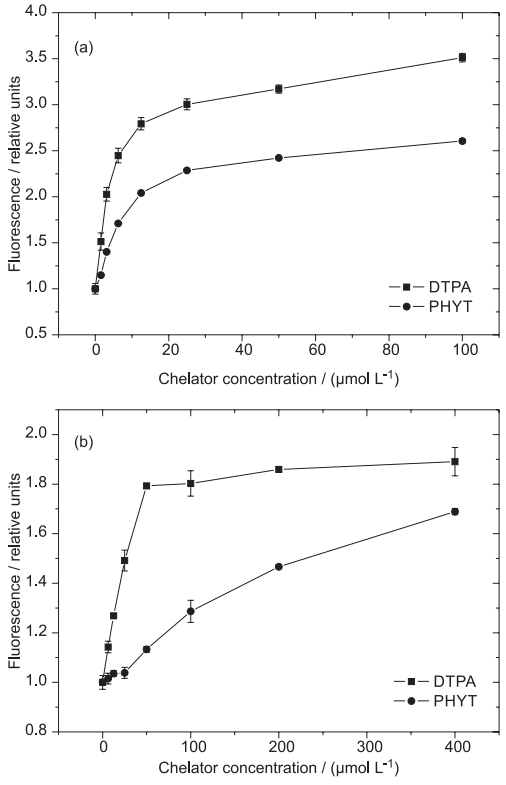

Figure 2. Comparison of the iron chelation strength of DTPA and PHYT against iron-loaded (a) calcein or (b) transferrin.

Antioxidant activity

Phillippy *et al.* showed that only three adjacent phosphate binding moieties are required for phosphorylated inositols to block iron-catalysed oxidation of biomolecules, due to the fact that the six coordination positions of the metal are fully occupied in this situation.[3] Anti-hydroxyl (but not anti-superoxide) activities in solution were demonstrated by Rimbach and Pallauf, although the antioxidant effects of PHYT were observed only at PHYT:Fe molar ratios > 5, probably because of differences in the sensitivity of the methods (ESR as opposed to photometric titrations).[16] In our results of antioxidant study, PHYT was reacted with the pre-formed iron complex Fe(NTA), a model of the stable, redox-active iron forms that might occur in iron-overloaded plasma (Figure 3a).[7]

Stability constants (pK_f) for Fe(NTA) and Fe(PHYT) have been determined as 16.26 and 18.87, respectively.[17,18] Therefore, the low effect of PHYT on the oxidation rate observed in Figure 3 even at high PHYT:Fe molar ratios reflects the equilibrium between a redox-active and a redox-inactive species, or even reflects the possibility of formation of ternary NTA-Fe-PHYT systems similar to others observed for NTA complexes.[17] In the other hand, the pro-oxidant activity of free iron (FAS) is completely quenched by PHYT (Figure 3b).

This experiment was repeated in real IO serum samples (Figure 3c), where "free" iron must probably include iron-citrate and/or iron-albumin species[19,20] which are less stable than the Fe-NTA system. This time PHYT produced a marked decrease in the oxidation rate of DHR, comparable to that induced by the strong chelator DTPA. This indicates that circulatory PHYT might be helpful on lessening the noxious effects promoted by excess iron in iron-overloaded patients.

Similarly, iron in the cytosol is expected to integrate a "labile iron pool" where the metal is bound to low molecular weight components such as citrate, aminoacids, ATP and ADP.[20] Calcein is a suited probe for the quantification of iron by means of stoichiometric fluorescence quenching, as discussed above. Since calcein is not cell permeant, HeLa cells were treated with the CAL-AM derivative, a non-fluorescent molecule that, upon internalization and cleaving of the ester bond, renders the molecule fluorescent again. In our experimental setting, just after loading the cells with the

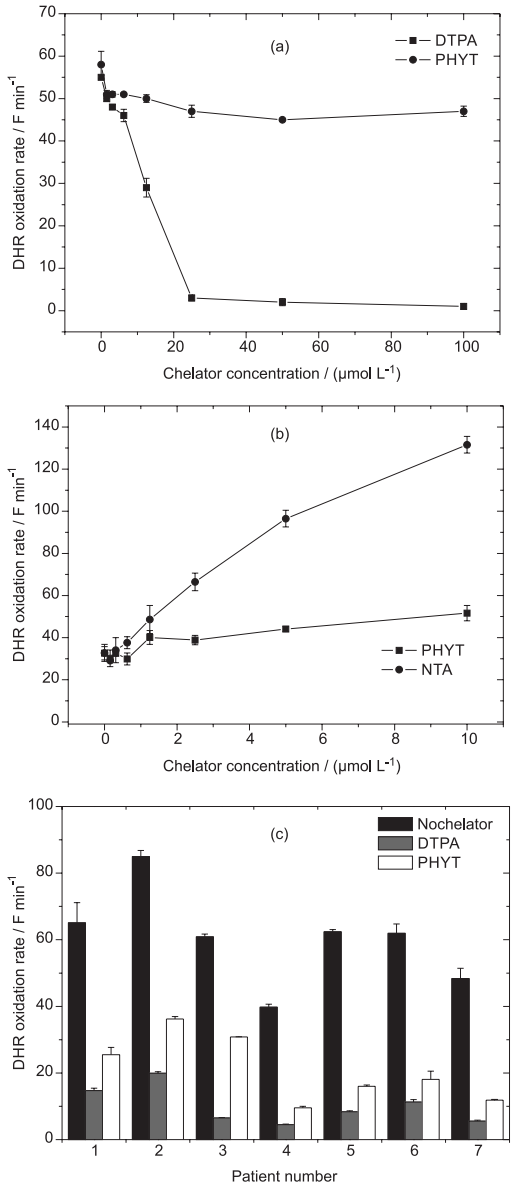

Figure 3. (a) Effect of DTPA or PHYT on the rate of Fe(NTA)-catalyzed oxidation of DHR ([Fe(NTA)] = 10 μmol L^{-1}); (b) effect of NTA or PHYT on the rate of "free" iron (as FAS) - catalyzed oxidation of DHR ([chelator] = 10 × [Fe]); (c) effect of DTPA or PHYT (100 μmol L^{-1}) on the rate of oxidation of DHR in samples from iron-overloaded patients.

probe, we added iron to quench its fluorescence. Therefore, upon further addition of chelators (DTPA or PHYT), cell fluorescence should be restored if these compounds were both cell permeant and able to compete with cytosolic ligands. By means of *z*-stacking in confocal microscopy, it is possible to take pictures of slices of the cell in order to determine that the fluorescence recovery originates within the cell rather than from membrane-bound fluorescent molecules. It was determined that PHYT enters the cell and after 8 minutes sequesters enough iron from calcein as to be detected (Figure 4a) and that the fluorescence recover originates within the cell (Figure 4b), indicating that PHYT entered the cells. Longer times of exposure are not desired for a variety of reasons that include probe leakage from the cells, probe photobleaching and cell death in the buffer. It should be noted that internalization plus iron removal was observed for the relatively low PHYT concentration of 20 μmol L^{-1}. Results for the DTPA treatments are not shown since no fluorescence recovery was observed, as this chelator is not cell permeant.[21]

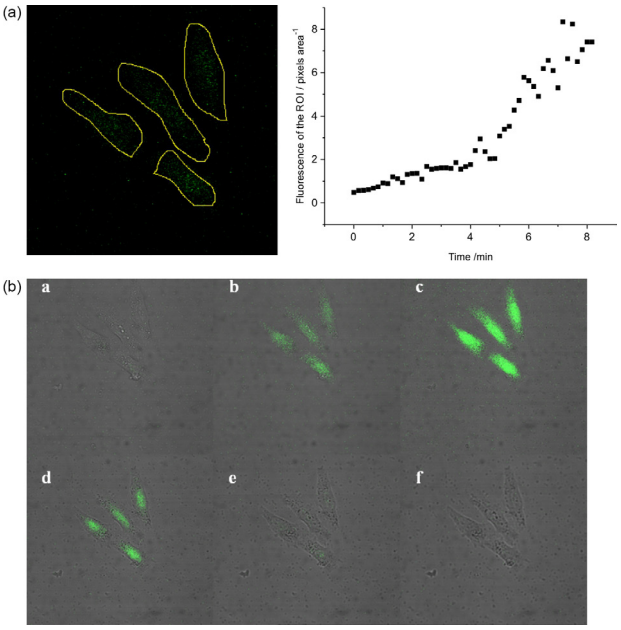

Figure 4. (a) Definition of the region of interest (ROI; left) to determine the kinetics of fluorescence recover in iron-overloaded HeLa cells treated with 20 μmol L^{-1} PHYT at 10 s intervals (right); (b) *z*-stack of HeLa cells incubated with PHYT 20 μmol L^{-1} for 8 min (*a* → *f*: bottom to top; slices of 13 μm).

Finally, PHYT loading into iron-overloaded cells was shown to halt oxidative damage mediated by peroxide and iron (Figure 5), as probed by the redox-sensitive molecule DCFDA (which fluoresces only in ROS-enriched environments). PHYT-treated cells did not revert to control-like oxidative status, since the chelator was added after incubation with iron and peroxide. Since the overload was induced by the addition of a salt (ferrous sulfate), the antioxidant effect here is more pronounced than when compared to Fe(NTA) (Figure 3a).

In physiological medium, when challenged with relatively stable Fe forms, the protective effect of PHYT appears to be marginal, at least in the concentration range used in this study. However, higher PHYT concentrations will start to disrupt Ca^{2+} metabolism and add stress to the cells.[1] Beneficial antioxidant effects of the intake of PHYT for both humans and animals is a notion still under debate, since PHYT is also involved in the metabolism of other minerals *in vivo* (for a review, see reference 22), although

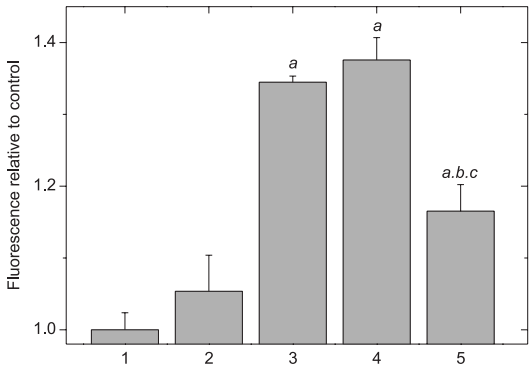

Figure 5. Fluorescence of HeLa cells incubated with DCFDA after several treatments. Fluorescence data were normalized against the control. 1: control; 2: H_2O_2 35 μmol L^{-1}; 3: FAS/ascorbic acid 10/100 μmol L^{-1}; 4: H_2O_2 35 μmol L^{-1} + FAS/ascorbic acid 10/100 μmol L^{-1}; 5: H_2O_2 35 μmol L^{-1} + FAS/ascorbic acid 10/100 μmol L^{-1} + PHYT 20 μmol L^{-1}. *a*, different from the control ($p < 0.05$); *b*, different from the Fe/ascorbic acid treatment ($p < 0.05$); *c*, different from the Fe/ascorbic acid/H_2O_2 system ($p < 0.05$).

antioxidant effects of PHYT on a mice model of genetic IO have been preliminarily reported.[23] Given the ubiquity of PHYT in food items it may be of importance as one of the front defenses against the early onset of oncogenesis or free radical damage to living tissue induced by iron overload (without PHYT, it could be argued that humans would be afflicted by those maladies earlier in life, and more often). In the other hand, PHYT probably is not available as such in the bloodstream, being instead hydrolyzed to less substituted polyphosphates after consumption.[1] Therefore, PHYT as a chelator for iron-overload conditions (e.g., thalassemia or hereditary hemochromatosis) would probably be most effective only if intravenously applied.

In connection with this, PHYT could be an interesting chelator for iron redistribution treatments, which are relevant whenever the metal is accumulated in specific compartments rather than over the whole system.[12,13] In buffered aqueous solutions, PHYT was less able than the potent chelator DTPA to remove iron from high affinity models of iron binding (calcein and transferrin, Figure 2). It stands to reason then that endogenous PHYT would be able to bind iron in a less redox-active form and release the metal whenever confronted with higher affinity chelators.

Conclusions

In conclusion, PHYT displayed a measurable ability to bind labile, well-defined forms of iron which are thought to compose endogenous pools of labile iron, and to decrease their oxidant (and putatively deleterious) effects. Whether or not this effect would provide a benefit for iron-overloaded patients will depend on the forms of phytate that are available after metabolization.

Acknowledgments

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