Inositol Phosphates Influence Iron Uptake in Caco-2 Cells

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Phytate, inositol hexaphosphate ($InsP_6$), may be hydrolyzed to inositol phosphates with lower degree of phosphorylation, i.e., inositol penta- to monophosphates ($InsP_5$−$InsP_1$), during food processing. Each of these lower inositol phosphates exists in different isomeric forms. The objective of this study was to determine if different isomers of $InsP_3$−$InsP_5$ ($Ins(1,2,4)P_3$, $Ins(1,2,3)P_3$, $Ins(1,2,6)P_3$, $Ins(1,3,4)P_3$, $Ins(1,2,3,4)P_4$, $Ins(1,2,5,6)P_4$, $Ins(1,2,4,5,6)P_5$, and $Ins(1,3,4,5,6)P_5$) and $InsP_6$ affect the uptake of iron. We studied the iron absorption in vitro using the human intestinal epithelial cell line, Caco-2. Addition of a 2-fold molar excess of $InsP_6$ or $InsP_5$ in proportion to Fe (1 h incubation at 37 °C) reduced iron uptake by 46−52% ($p < 0.001$). Neither $InsP_4$ isomers nor $InsP_3$ isomers affected iron uptake significantly at 1 h incubation with a molar InsP:Fe level of 2:1. Iron uptake was shown to not be a function of the isomeric form of inositol phosphates. The inositol phosphate isomers did not seem likely to interact with each other through iron to form more stable iron complexes. At a molar InsP:Fe level of 20:1 an inhibitory effect of $InsP_4$ was found, while $InsP_3$ did not affect the iron absorption even at a 20-fold molar excess.

Keywords: *Inositol phosphate isomers; phytate; iron absorption; Caco-2 cells*

Phytate (*myo*-inositol hexaphosphate, $InsP_6$) is present in plants, where it represents the major storage form of phosphorus. In humans and animals, $InsP_6$, due to its strong chelating properties, interferes with mineral absorption by forming insoluble complexes with nutritionally important minerals such as Fe, Zn, and Ca (Cheryan, 1980; Cosgrove, 1966; Reddy et al., 1982). During processing of foods containing phytate, inositol phosphates, with lower number of phosphate groups bound to the inositol ring, are formed.

Complexation studies have shown that a reduction in the number of phosphate groups results in increased mineral solubility and decreased ability of inositol phosphates to form complexes (Jackman and Black, 1951; Kaufman and Kleinberg, 1971). The effects of inositol phosphates with three to six phosphate groups on Fe, Zn, and Ca absorption have been examined in vitro (Han et al., 1994; Sandberg et al., 1989), as well as in vivo (Lönnerdal et al., 1989; Sandberg et al., 1993; Sandström and Sandberg, 1992). All of the mentioned studies showed inhibitory effects of added $InsP_5$ and $InsP_6$ on mineral absorption. While Lönnerdal et al. (1989), Sandberg et al. (1989), and Sandström and Sandberg (1992) found that inositol phosphates with less than five phosphate groups had no effect on the absorption of Fe, Zn, and Ca, Han et al. (1994) reported that $InsP_3$ and $InsP_4$ had a negative effect on mineral absorption in vitro using Caco-2 cells. Brune et al. (1992) and Rossander et al. (1992) found a strong negative correlation between Fe and Zn absorption and the sum of $InsP_3$−$InsP_6$ in processed foods, suggesting a contributing negative effect of the less phosphorylated inositol phosphates on mineral absorption. The binding affinity of cations (Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}) to inositol phosphates has been shown to be affected by the orientation of the phosphate groups (Hawkins et al., 1993; Mernissi-Arifi et al., 1994). Since the positions of the phosphate groups on the inositol ring plays an important role in the binding of minerals, this might affect mineral absorption as well. Interactions between various inositol phosphate isomers might further influence their mineral binding capacity. However, no study has so far been designed to compare the effects of different isomers of inositol phosphates on the absorption of minerals.

The objective of the present investigation was to study the effects of different isomeric forms of $InsP_3$−$InsP_5$ and $InsP_6$ on Fe absorption in an in vitro model. We used the human colon carcinoma cell line, Caco-2, which is known to terminally differentiate and show characteristics of the brush-border (Hidalgo et al., 1989; Pinto et al., 1983). Several studies have shown that the Caco-2 cell culture system is a useful in vitro model to study food Fe availability (Garcia et al., 1996; Glahn et al., 1996; Han et al., 1994). The effects of the inositol phosphate isomers ($Ins(1,2,4)P_3$, $Ins(1,2,3)P_3$, $Ins(1,2,6)P_3$, $Ins(1,3,4)P_3$, $Ins(1,2,3,4)P_4$, $Ins(1,2,5,6)P_4$, $Ins(1,2,4,5,6)P_5$, and $Ins(1,3,4,5,6)P_5$) and $InsP_6$ on the uptake of Fe, as well as the interaction between some of them, were investigated in the present study. Nonheme Fe was used in this study since the absorption is well-known to be influenced by inhibitors and enhancers.

MATERIALS AND METHODS

Inositol Phosphates. $Ins(1,2,3)P_3$, $Ins(1,2,4)P_3$, $Ins(1,2,6)P_3$, $Ins(1,3,4)P_3$, $Ins(1,2,3,4)P_4$, $Ins(1,2,5,6)P_4$, $Ins(1,3,4,5,6)P_5$, and $Ins(1,2,4,5,6)P_5$ were received as a gift from Perstorp Pharma (Perstorp, Sweden). Sodium phytate was obtained from BDH Chemical Ltd. (Poole, England). Inositol phosphates

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10.1021/jf980745c CCC: $18.00
Published on Web 02/19/1999

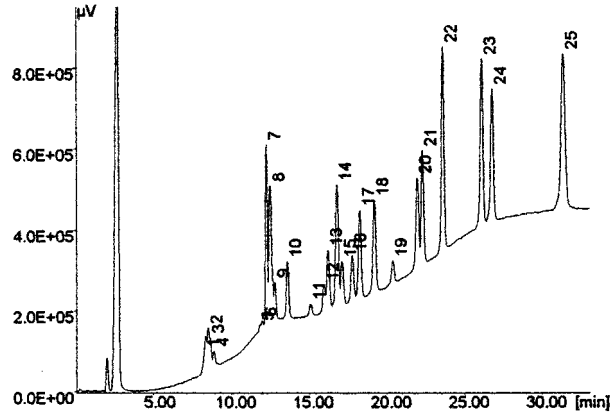

Figure 1. Chromatographic profile of $InsP_6$ hydrolyzed by HCl and analyzed according to the HPIC method of Skoglund and co-workers (1997, 1998). Unidentified peaks are assigned a star (*). Peaks: (1) DL-Ins(1,6)P_2; (2) DL-Ins(1,2)P_2; (3) DL-Ins(1,4)P_2, DL-Ins(2,4)P_2; (4) DL-Ins(4,5)P_2; (5) *; (6) *; (7) DL-Ins(1,2,4)P_3, DL-Ins(1,3,4)P_3, (Ins(1,2,3)P_3); (8) DL-Ins(1,2,6)P_3, Ins(1,2,3)P_3; (9) DL-Ins(1,4,5)P_3; (10) DL-Ins(1,5,6)P_3; (11) DL-Ins(4,5,6)P_3; (12) Ins(1,2,3,5)P_4; (13) DL-Ins(1,2,4,6)P_4; (14) DL-Ins(1,2,3,4)P_4; (15) Ins(1,3,4,6)P_4; (16) DL-Ins(1,2,4,5)P_4; (17) DL-Ins(1,3,4,5)P_4; (18) DL-Ins(1,2,5,6)P_4; (19) Ins(2,4,5,6)P_4; (20) DL-Ins(1,4,5,6)P_4; (21) Ins(1,2,3,4,6)P_5; (22) DL-Ins(1,2,3,4,5)-P_5; (23) DL-Ins(1,2,4,5,6)P_5; and (24) Ins(1,3,4,5,6)P_5; (25) $InsP_6$.

were solubilized in water and the concentration of each inositol phosphate was determined by high-performance ion chromatographic (HPIC) analysis as described by Skoglund et al. (1997b) with some improvements (Skoglund et al., 1998). Samples were stored at 4 °C. A chromatographic profile of sodium phytate hydrolyzed with diluted HCl shows in Figure 1 the separation of inositol phosphate isomers when analyzed using the HPIC method of Skoglund and co-workers (1997 and 1998).

Cells. Caco-2 cells obtained from American Type Tissue Culture HTB 37 (Rockville, MD) were maintained in minimum essential medium with Earle's salts, L-glutamine, and nonessential amino acids (Life Technologies, Inc., Grand Island, NY), pH 7.3, supplemented with 1% penicillin−streptomycin solution (Sigma Chemical Co., St. Louis, MO), and 10% fetal bovine serum, which was not heat-treated (Gemini Bio Products, Inc., Calabasas, CA). Cells were grown in 75 cm^2 flasks at 37 °C, under 5% CO_2−95% air atmosphere. Medium was changed every other day. The confluent cells were washed with phosphate-buffered saline (PBS, pH 7.2; Sigma Chemical Co., St. Louis, MO) and rinsed off the bottom of the flask with nonenzymatic cell dissociation solution (Sigma Chemical Co., St. Louis, MO). Cells were centrifuged to pellets at 1200 rpm, 4 °C, and reseeded at a density of 1×10^5 cells/cm^2. Experiments were performed with cultures between passage 29 and 35. Cells for the assay were seeded in 24-well culture plates (Costar, Cambridge, MA) at 10^5 cells per well. Confluency was assessed by formation of domes, as monitored by inverted microscopy.

Iron Uptake. Metal chelate solution of $FeSO_4 \cdot 7H_2O$ was added to serum-free uptake medium at a concentration of 10 μmol/L. ^{59}Fe (specific radioactivity 496 MBq/mg; DuPont NEN, Boston, MA) in the form of $^{59}FeCl_3$ was added for uptake, to provide 1.85 kBq per well. Ins(1,2,3)P_3, Ins(1,2,6)P_3, Ins(1,3,4)-P_3, Ins(1,2,4)P_3, Ins(1,2,3,4)P_4, Ins(1,2,5,6)P_4, $InsP_5$ (containing equal amounts of Ins(1,2,4,5,6)P_5 and Ins(1,3,4,5,6)P_5), $InsP_6$, or equivalent amounts of Ins(1,2,3)P_3 and Ins(1,2,6)P_3, or Ins-(1,2,3,4)P_4 and Ins(1,2,5,6)P_4, were added to the uptake medium, at a concentration of 20 μmol/L. In control wells, $FeSO_4 \cdot 7H_2O$ (10 μmol/L) and ^{59}Fe (1.85 kBq) were added to the uptake medium, without addition of inositol phosphates. The plates were incubated for 1, 2, and 4 h at 37 °C, and the unbound label was recovered. Further, Ins(1,2,6)P_3 and Ins-(1,2,5,6)P_4, respectively, were added to the uptake medium at concentrations of 0, 20, 40, 60, 100, and 200 μmol/L together with 10 μmol/L of $FeSO_4 \cdot 7H_2O$ and ^{59}Fe (1.85 kBq per well). The plates were incubated for 1 h at 37 °C, and the unbound label was recovered. Cells were washed twice with ice-cold PBS to remove nonspecifically bound label. The washes were removed, and cells lysed with 1% SDS, collected and counted in a γ scintillation counter (Beckman Instruments, Fullerton, CA). Protein content of the cell pellet was estimated by the modified Lowry assay (Lowry et al., 1951) from three wells on each plate. The quantity of extracellular Fe taken up by cells was calculated from the specific radioactivity of the test solutions and is presented as nanomoles per milligram of cell protein.

Data Analysis. All variables were tested in triplicate or quadruplicate for each experiment. Data were analyzed using multiple linear regression analysis (Carlson, 1992) of the computer program Modde 3.0 (Umetri AB, Umeå, Sweden), and evaluated by analysis of variance. To test for differences between the means of two groups a *t*-test (Box et al., 1978) was performed. Results are presented in absolute, as well as fractional figures; variation is presented as standard deviation (SD). Means were considered significantly different if $p \leq 0.05$.

RESULTS

Effects of Inositol Penta- and Hexaphosphates on Iron Absorption. Differentiated cultures of Caco-2 monolayers accumulated 10.6 nmol Fe per mg of cell protein, after incubation at 37 °C for 1 h (Figure 2). Addition of a 2-fold molar excess of $InsP_5$ or $InsP_6$ (20 μmol/L) relative to Fe reduced Fe uptake significantly by 46−52% ($p < 0.001$). There was no significant difference between the inhibitory effects of $InsP_5$ and $InsP_6$ on Fe absorption. As shown in Figure 2, longer incubation times (2 and 4 h, respectively) with a molar $InsP_5$ or $InsP_6$ to Fe ratio of 2:1 reduced Fe absorption by 59% and 77%, respectively, as compared to Fe uptake in the control samples. The time effects were statistically significant.

Effects of Inositol Tri- and Tetraphosphates on Iron Absorption. None of the individual $InsP_3$ isomers or the individual $InsP_4$ isomers inhibited the uptake of Fe by Caco-2 cells after incubation at 37 °C for 1 h at an InsP:Fe molar ratio of 2:1 (Figure 2). There was no significant difference in Fe uptake among various isomers, versus Fe uptake in the control sample. Addition of a mixture of $InsP_3$ isomers (Ins(1,2,3)P_3 and Ins-(1,2,6)P_3) or $InsP_4$ isomers (Ins(1,2,3,4)P_4 and Ins-(1,2,5,6)P_4) did not significantly affect Fe uptake, i.e., stronger complexes due to interactions between the isomers through Fe was not demonstrated. After 2 h of incubation, $InsP_3$ or $InsP_4$ isomers had not significantly affected Fe absorption, while at 4 h of incubation a slight reduction in average Fe absorption by 18% ($p < 0.001$) was shown when adding a 2-fold molar excess of $InsP_3$ or $InsP_4$ (Figure 2). However, no significant difference in inhibition of Fe absorption among the isomers was shown, as compared to Fe uptake in the control sample.

The inhibitory effect of $InsP_3$ (Ins(1,2,6)P_3) and $InsP_4$ (Ins(1,2,5,6)P_4) on Fe absorption at different molar inositol phosphate-to-Fe ratios is depicted in Figure 3. Adding 2, 4, 6, 10, or 20 times molar excess of $InsP_3$ to Fe to the uptake medium did not significantly influence Fe absorption after 1 h of incubation of the cells. When $InsP_4$ was added in a 20-fold molar excess to Fe, however, Fe absorption was inhibited by approximately 37% ($p < 0.001$).

DISCUSSION

We used the Caco-2 human cell line to examine the inhibitory effect of various inositol phosphate isomers

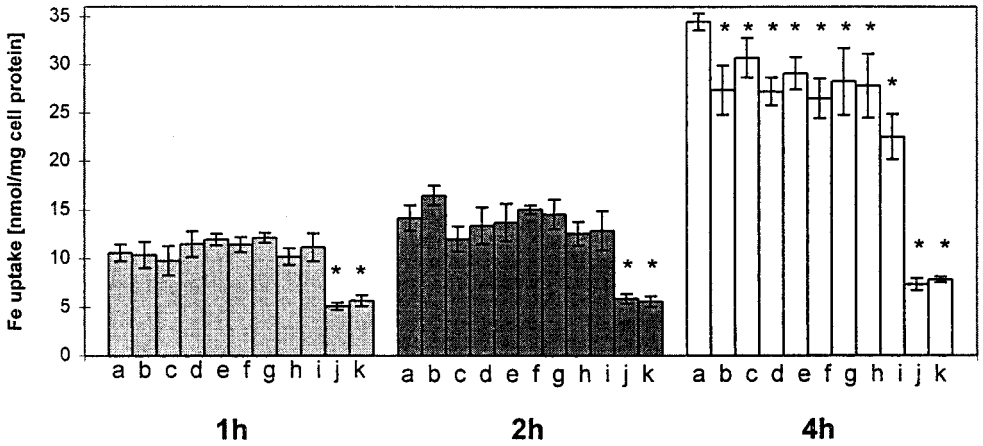

Figure 2. Fe absorption (expressed as nanomoles of bound ^{59}Fe per milligram of cell protein) as function of indicated inositol phosphate (a−k) at InsP:Fe molar ratio of 2:1. Incubation times of 1, 2, and 4 h are shown. Values are means ± SD, $n = 4$. Asterisk indicates significant difference ($p < 0.05$) vs control (no added inositol phosphate) for each incubation time of uptake solution. Inositol phosphates: (a) control, (b) Ins(1,2,4)P_3, (c) Ins(1,3,4)P_3, (d) Ins(1,2,3)P_3, (e) Ins(1,2,6)P_3, (f) Ins(1,2,3,4)P_4, (g) Ins(1,2,5,6)P_4, (h) Ins(1,2,3)P_3 and Ins(1,2,6)P_3, (i) Ins(1,2,3,4)P_4 and Ins(1,2,5,6)P_4, (j) InsP_5, and (k) InsP_6.

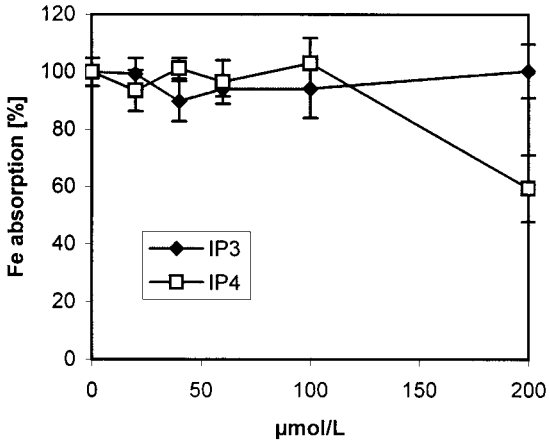

Figure 3. In vitro Fe absorption (% of control) when 0, 20, 40, 60, 100, and 200 μmol/L InsP_3 or InsP_4 and 10 μmol/L Fe were added to each well. The plates were incubated for 1 h. Values are means ± SD, $n = 3$.

on the absorption of Fe. These cells are derived from a human colonic adenocarcinoma and undergo enterocytic differentiation at confluency into a phenotype that possesses tight junctions and forms many domes, characteristic of functionally polarized, transporting epithelial cells (Hidalgo et al., 1989, Pinto et al., 1983). The Caco-2 cell line has previously been proven useful in Fe absorption studies (Glahn et al., 1996; Halleux and Schneider, 1991, 1994; Han et al., 1994, 1995).

Different isomers of inositol phosphates are formed during enzymatic phytate degradation, depending on the origin of the phytase enzyme (Skoglund et al., 1997a). Some of the isomers have important functions in the body, such as antiinflammatory and as secondary messengers (Sirén et al., 1991; Streb et al., 1983), while the effects of various isomers on mineral absorption have not been evaluated. In the present study we used a 2:1 molar ratio of inositol phosphates to the trace metal to study the difference between and interaction of various isomers on Fe absorption. This phytate to Fe relationship is similar to that present in typical diets in industrialized countries (Wisker et al., 1991). We found that InsP_6 and InsP_5 had negative effects on Fe uptake, while InsP_4 and InsP_3 had no significant effect, at an incubation time of 1 h at 37 °C. These results are in conflict with those of Han et al. (1994) who found that the inhibition of Fe absorption was related to InsP_3 and InsP_4, as well as InsP_5 and InsP_6. Brune et al. (1992) found a negative correlation between the sum of InsP_3 to InsP_6 in processed food and Fe absorption. One reason for the contradictory results could be that we studied purified isomers of InsP_3 and InsP_4, while in the study of Han et al. (1994) a mixture of InsP_3 and InsP_4 isomers was used to study Fe absorption. Various isomers might interact with each other through Fe and thereby improve their mineral binding capacity. Depending on the positions of the phosphate groups on the inositol ring such interactions could be more or less probable. However, when mixing equal amounts of the InsP_3 isomers Ins(1,2,3)P_3 and Ins(1,2,6)P_3 or the InsP_4 isomers Ins(1,2,3,4)P_4 and Ins(1,2,5,6)P_4, we found no significant effect on Fe absorption after incubation for 1 h. Thus it is not likely that inositol phosphate isomers interact with each other to form more stable complexes. In this study we only investigated possible interactions between Fe and inositol phosphate isomers with the same degree of phosphorylation.

Another plausible explanation to the diverse results concerning the inhibitory effects of InsP_3 and InsP_4 might be that different inositol phosphate isomers bind Fe with varying strength and therefore affect absorption to different extent. The 1,2,3 (equatorial−axial−equatorial) triphosphate grouping has been shown to be the orientation forming the strongest complex with Fe^{3+} (Hawkins et al., 1993). Stronger binding of cations (Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}) was shown for InsP_3 with three vicinical phosphates (Ins(1,2,6)P_3) than three alternated phosphates (Ins(1,3,5)P_3 or Ins(2,4,6)P_3) (Mernissi-Arifi et al., 1994). In the case of three alternated phosphate groups, one group in axial position (Ins(2,4,6)P_3) largely contributed to complex stabilization. However, there was no significant difference between controls and various isomers of InsP_3 or InsP_4, in their effects on Fe absorption in the present study.

The previous studies on Fe absorption as a function of inositol phosphates differ in the molar ratios of the two components used. When an inhibitory effect of InsP_3 and InsP_4 was found, the InsP:Fe molar ratio used was 10:1 (Han et al., 1994). At lower molar ratios of InsP:Fe neither InsP_3 nor InsP_4 contributed to the inhibition of Fe availability estimated in vitro (Sandberg et al., 1989). We therefore investigated the inhibitory effects

of $InsP_3$ (Ins(1,2,6)P_3) and $InsP_4$ (Ins(1,2,5,6)P_4) at different InsP to Fe molar ratios (2:1, 4:1, 6:1, 10:1, and 20:1). At the highest level of $InsP_4$ (200 μmol/L), Fe absorption was significantly reduced by 37%, while no effect was found for $InsP_3$ at any investigated molar ratio. It is therefore likely that conditions other than InsP:Fe molar ratio, as well, are affecting the influence of $InsP_3$ and $InsP_4$ on Fe absorption. Examples of such conditions are discussed below.

An important factor to consider when it comes to mineral absorption is that minerals added to foods containing inositol phosphates may affect the potential availability of other essential minerals in the diet. Platt and Clydesdale (1987) investigated the effects of Cu, Zn, and Ca on Fe solubility in a simulated gastrointestinal pH treatment. When Zn or Ca were added to a protein and fiber-rich fraction of wheat bran in the presence of Fe, they were found to negatively affect both $InsP_6$ and Fe solubility. Another important factor affecting the solubility of inositol phosphate complexes is pH, which ranges from about 3 in the stomach to 6−8 in the small intestine where minerals are mainly absorbed. In a study on in vitro estimation of Fe availability, increasing the pH from 6.0 to 7.0, decreased the solubility of Fe when $InsP_3$ or $InsP_4$ was added, while the solubility of Fe was not affected by pH when $InsP_5$ or $InsP_6$ was added (Sandberg et al., 1989). Further studies of the effect of inositol phosphates on Fe absorption are needed to evaluate the effects of other minerals, as well as the pH dependence.

CONCLUSION

Addition of a 2-fold molar excess of $InsP_6$ or $InsP_5$ (20 μmol/L) in proportion to Fe reduced Fe uptake by 46−52% ($p < 0.001$), whereas the $InsP_4$ and $InsP_3$ isomers did not affect Fe uptake at the 2:1 molar ratio. No significant variation among isomers was shown. The inositol phosphate isomers did not seem likely to interact with each other through iron to form more stable Fe complexes. At an InsP:Fe level of 20:1 an inhibitory effect of $InsP_4$ was found, while $InsP_3$ did not influence Fe absorption even at a 20-fold molar excess.

ABBREVIATIONS USED

InsP, inositol phosphate; $InsP_1$−$InsP_6$, inositol mono- to hexaphosphate; Ins, an accepted NC-IUB abbreviation for *myo*-inositol with the numbering of the D configuration unless the prefix L is explicitly added.

ACKNOWLEDGMENT

The assistance of Shannon Kelleher in performing the Caco-2 studies is much appreciated.

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Received for review July 13, 1998. Revised manuscript received December 28, 1998. Accepted December 29, 1998. The work is supported by grants from the Swedish Council for Forestry and Agricultural Research 50.0023/94 and from the Swedish Nutrition Foundation.

JF980745C