Effects of Amino Acids and Dipeptides on the Uptake and Transport of Iron by Caco-2 Cells Cultured in Serum-Free Medium

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This study was conducted to investigate the effect of five amino acids, aspartic acid, glutamic acid, histidine, cysteine and phenylalanine, and of two dipeptides, anserine and carnosine, on the uptake and transport of iron across monolayers of Caco-2 cells. Caco-2 cells were grown on polycarbonate membrane inserts in serum-free medium [DMEM plus ITS (5 mg/l insulin, 5 mg/l transferrin and 5 µg/l selenious acid)]. The anserine, carnosine, glutamic acid, histidine and aspartic acid, except for cysteine and phenylalanine, at 20 µM significantly increased the uptake and transport of iron. Iron uptake and rate of iron transport in the presence of aspartic acid at 20 µM were significantly greater than those of other test solutions. When the concentration of added carnosine, anserine, glutamic acid, histidine and phenylalanine was increased from 20 µM to 1 mM in the test solution, the uptake and transport of iron was further increased. The results of the present study indicated that aspartic acid, glutamic acid, histidine, and the dipeptides, anserine and carnosine, enhanced the uptake and transport of iron by Caco-2 cells.

Keywords: amino acid, Caco-2 cells, iron uptake, transport, carnosine, anserine

Iron deficiency anemia is widely prevalent around the world especially in developing countries (Southeast Asia and Africa) (Paracha *et al.*, 1993). The problem of iron deficiency anemia consequently leads to low immunity function, impaired work performance and mental development in children, and increased maternal mortality (FAO/WHO, 1992). Iron deficiency is primarily prevalent in areas where heme iron, in the form of meat, is less consumed (Flynn *et al.*, 1984). There are several important factors which directly affect the bioavailability of dietary iron. For example, the sources of iron in food, solubility, valence state and complexation behavior (Clydesdale, 1982). It has been suggested that low molecular weight ligands are essential to enhance iron bioavailability because they have the ability to form complexes with iron and retain iron in solution (Flynn *et al.*, 1984). However, amino acids are of special biological interest because they constitute a large ligand pool in the intestinal lumen (Van Campen, 1973). On the other hand, certain amino acids have been found to enhance the iron absorption (Martinez-Torres *et al.*, 1981; Flynn *et al.*, 1984). The ability of amino acids to enhance iron absorption is not yet clear. Some studies have reported that certain amino acids increased iron absorption (Van Campen, 1973; Martinez-Torres *et al.*, 1981; Taylor *et al.*, 1986); whereas, the study of Layrisse *et al.* (1984) has shown no effect. Previous studies have shown that the animal tissues (meat) and peptides enhance iron absorption and iron uptake (Slatkavitz & Clydesdale, 1988; Gordon & Godber, 1989; Kapsokefalou & Miller, 1991; Garcia *et al.*, 1996; Glahn *et al.*, 1996). Animal muscles are good sources of anserine and carnosine (Susan *et al.*, 1989). Previous reports showed that

carnosine was completely hydrolyzed to β -alanine and histidine, except for anserine which was hydrolyzed by about 86% (Hama *et al.*, 1976; Ganapathy & Leibach, 1982). Moreover, we have studied the effects of sugars and aspartame on the uptake and transport of iron by Caco-2 cells, and the results revealed that aspartame enhanced the uptake and transport of iron by Caco-2 cells. Aspartame is completely hydrolyzed and metabolized in the body to aspartic acid, phenylalanine and methanol, all of which are further metabolized to provide energy in the cells (Ranney & Oppermann, 1979). Therefore, in this study, we used a cell culture system (Caco-2 cells) to clarify the role of the hydrolysis of aspartame (aspartic acid and phenylalanine), glutamic acid, histidine, cysteine, and dipeptides (anserine and carnosine) on iron uptake and transport by Caco-2 cells.

Materials and Methods

Cell culture Caco-2 cells, obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 18, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals, Inc., OH, USA), 1% nonessential amino acids (NEAA; Dainippon Pharmaceutical Co., Ltd., Osaka), and 50 U/ml penicillin-G, 50 µg/ml streptomycin (Dainippon Pharmaceutical Co., Ltd.) and 50 µg/ml gentamycin (Sigma Chemical Co., St. Louis, MO, USA). Serum-free medium consisted of DMEM plus ITS (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid), 2 nM triiodothyronine and 100 nM hydrocortisone (Sigma Chemical Co.). Stock cultures of Caco-2 cells were seeded at a density 6.7×10^4 cell/cm² in petri dishes and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every

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other day. At 70–80% confluence, generally 6–7 days after seeding, cells were rinsed in Dulbecco's phosphate buffer without calcium and magnesium (PBS(–); ICN Biomedicals, Inc.) and then treated for 20 min with PBS(–) containing 0.25% trypsin and 0.54 mM Na₂EDTA. Cells were counted under a light microscope after trypsinization.

For cells cultured under low-iron conditions Caco-2 cells (1×10^5 cell/cm²) were initially seeded on collagen-treated polycarbonate membrane inserts (each insert had an area of 4.65 cm² and a pore diameter of 3 μ m; Nippon Becton Dickinson Co., Ltd., Tokyo) and cultured with DMEM that contained 10% FBS for the first 48 h. This procedure allows cells to firmly attach to the membrane insert via fibronectin, which is present in FBS (Hashimoto & Shimizu, 1993). After 2 days, DMEM plus FBS was replaced by serum-free medium. Caco-2 cells were incubated under these conditions for 12–14 days to allow cells to proliferate and mature. Experiments were conducted on day 14. By this time, Caco-2 cells have consistently been shown to have undergone complete enterocytic differentiation and the polarization of cells monolayers (Hidalgo *et al.*, 1989) and that was also confirmed using scanning electron microscopy (S-4100; Hitachi, Tokyo).

Iron uptake Metal chelate solutions were freshly prepared before each experiment. Stock solutions containing 10 mM FeCl₃·6H₂O plus 10 mM sodium nitrilotriacetate (NTA) were freshly prepared for each experiment. Stock solutions were diluted 10-fold with sterile deionized water before transfer of an appropriate volume to the test solutions (serum-free medium). Ferric nitrilotriacetate (Fe³⁺: NTA) at 10 μ M was transferred to test solutions containing either an amino acid (glutamic acid, aspartic acid, histidine, cysteine or phenylalanine) or a dipeptide; anserine or carnosine, at final concentrations of 20 μ M or 1 mM. The control test solution contained certain amino acids as contaminants due to the amino acids present in serum-free medium. Each test solution was mixed well and then the pH was adjusted to 7.0–7.4 with sodium bicarbonate. ⁵⁹FeCl₃ (Amersham International, Buckinghamshire, UK) was immediately added to the test solutions to give 1.67 kBq/insert. Before the start of the experiment, each test solution was incubated at 37°C for at least 30 min.

Assays of iron uptake Monolayers of Caco-2 cells grown on polycarbonate membrane inserts were washed twice on both sides of the membrane inserts (apical and basal chambers) with prewarmed serum-free medium. Then 1.5 ml of a test solution was added to the upper compartment, and 2.5 ml of serum-free medium was added to the basal compartment. Cells were incubated at 37°C. At the end of the incubation, the test solution was removed by an aspirator, and the monolayer of cells was washed three times with 2 ml each of ice-cold buffer that contained 150 mM NaCl, 1 mM EDTA, 10 mM HEPES, pH 7, to remove nonspecifically bound ⁵⁹FeCl₃ (Han *et al.*, 1995). The filter with the cell monolayer was then carefully removed from the plastic support, placed in a plastic tube, and 1.0 ml of harvest buffer, which contained 1.7 mM SDS and 1 mM EDTA, pH 7.0, was added. After sonication, the radioactivity in the harvest buffer with cells was determined with a gamma counter (MINAXI

Auto Gamma 5000; Packard, Meriden, CT, USA). The quantity of iron taken up by cells was calculated from the specific radioactivity of the test solution and is presented as pmol/insert (Halleux & Schneider, 1994; Han *et al.*, 1995).

To avoid formation of ferric hydroxide polymers which are insoluble in aqueous medium, NTA, amino acids or dipeptides were added in the test solution, and the solubility of iron in test solutions was examined by the method of Han *et al.* (1994). The molar ratios of Fe³⁺:NTA:amino acid or dipeptide tested were 1:2:2, 1:1:2 and 1:0:2. The mixtures (1:2:2, 1:1:2 and 1:0:2) left at room temperature for 3 h were clearly indicating that iron in the test solutions containing NTA plus amino acid or dipeptide remained soluble. Without NTA (molar ratio of 1:0:2), iron was rapidly precipitated in the test solution. We determined the effect of different concentrations of nitrilotriacetate (NTA) which complexed with FeCl₃ at a molar ratio 1:2 and 1:1 by measuring the iron uptake in Caco-2 cells. Results showed that the iron uptake increased by more than 50% when the molar ratio of Fe³⁺:NTA was decreased from 1:2 to 1:1. It is possible that NTA and amino acid competed in binding with iron or that NTA at a higher concentration inhibited the iron uptake (Raja *et al.*, 1987).

Iron transport The transport assays were initiated in a manner similar to that described for the uptake study. The test solution (1.5 ml) was added to the apical chamber, and 2.5 ml of fresh serum-free medium was added to the basal chamber. The rate of iron transport from the apical to the basal chamber was determined by removing an aliquot (200 μ l) of the test solution from the basal chamber after 20, 40, and 60 min. An equivalent aliquot of fresh medium was added to the basal chamber after each sampling to maintain the original volume. Iron transport across the monolayer was assessed by determining the radioactivity in the aliquots from the basal chamber with a gamma counter. The quantity of iron transferred from the apical to the basal chamber (Fig. 3 and summarized in Table 1) was estimated from the specific radioactivity of the test solution added to the apical chamber and is presented as pmol/cm²/min. The rate of iron transport

Table 1. Rates of iron transport by Caco-2 cells in the presence or absence of amino acids and dipeptides.

Test solution	Transport (pmol/cm ² /min)	
	20 μ M	1 mM
Control	0.84±0.01 ^a	
Aspartic acid	2.09±0.09 ^d	1.90±0.05 ^d
Glutamic acid	1.07±0.05 ^b	1.35±0.06 ^c
Histidine	0.92±0.06 ^a	1.17±0.05 ^c
Phenylalanine	0.80±0.15 ^a	0.87±0.02 ^a
Cysteine	0.73±0.09 ^a	0.84±0.05 ^a
Anserine	1.02±0.06 ^b	1.39±0.10 ^c
Carnosine	0.99±0.08 ^b	1.06±0.15 ^b

Each value represents mean±SEM of two experiments with five replicates each. Significant differences between groups are indicated by different superscript at $p < 0.05$. The test solution (1.5 ml) which contained 10 μ M of ferric chloride complexed with NTA at a molar ratio of 1:1 and 20 μ M or 1 mM of amino acid, dipeptides plus 1.67 kBq ⁵⁹FeCl₃/insert was added in the apical chamber and 2.5 ml of medium in the basal chamber was incubated at 37°C. The amount of radioisotope in the basal chamber was determined at 20, 40, and 60 min as described in Materials and Methods.

(Fig. 3 and summarized in Table 1) was calculated by linear regression analysis of data from 20 to 60 min (Halleux & Schneider, 1994; Han *et al.*, 1995).

Chemicals All amino acids were purchased from Nacalai Tesque Co., Ltd. (Kyoto). Dipeptides were obtained from Sigma Chemical Co.

Statistical analysis All variables were tested using at least six culture inserts for each experiment and each experiment was repeated twice. The iron uptake and transport data were compared using a one-way ANOVA with post hoc comparison by Scheffe's test (Abacus Concepts, Inc., Berkeley, CA, USA).

Results and Discussion

A human colon adenocarcinoma cell line (Caco-2 cells) has the unique property of spontaneously differentiating on reaching confluence. Caco-2 cells exhibited many of the structural and functional properties of mature human small intestinal enterocytes such as formation of a brush border membrane with apical microvilli and tight junctions (Hidalgo *et al.*, 1989; Qian & Tang, 1995). Additionally, they express enterocyte-specific enzymes such as sucrase-isomaltase, aminopeptidase or alkaline phosphatase in their microvilli membrane and hormone receptors (Pinto *et al.*, 1983; Hidalgo *et al.*, 1989; Jumarie & Malo, 1991). Caco-2 cells grown on a microporous membrane insert have been found to be a useful model for the study on mechanism of uptake and transport of nutrients and drugs (Quick & Ong, 1990; Artursson & Karlsson, 1991; Nicklin *et al.*, 1995; Ekmekcioglu *et al.*, 1996; Glahn *et al.*, 1996; Sanchez *et al.*, 1996).

For the above-mentioned characteristics, it is speculated that Caco-2 cells may be a suitable model for studying the influence of food components on the intestinal uptake and absorption of nutrients. In this study, we evaluated the effects

of amino acids and dipeptides on the uptake and transport of iron in Caco-2 cells.

Effects of aspartic acid and glutamic acid on uptake and transport of iron It is apparent from Figs. 1, 3 and Table 1 that the addition of aspartic acid at 20 μ M to the test solution significantly enhanced the uptake and increased the transport of iron when compared with other test solutions ($p < 0.05$). However, significant ($p < 0.05$) reduction was observed in the iron uptake when the concentration of aspartic acid in the test solution was increased from 20 μ M to 1 mM (Fig. 2). At a high concentration of aspartic acid (1 mM), the iron uptake decreased significantly ($p < 0.05$), perhaps due to an inhibition effect of aspartic acid. Of all the amino acids used in this experiment, aspartic acid had the greatest promoting effect on iron uptake and transport by Caco-2 cells. The mechanism by which aspartic acid enhances the uptake and transport of iron is not clear. It may be that aspartic acid binds with iron and carries it towards the cell membrane receptors responsible for iron uptake and transport (Kroe *et al.*, 1966; Cox & Peters, 1980; Stefanini *et al.*, 1989). In the previous study, aspartame (a dipeptide) enhanced iron uptake and transport by Caco-2 cells. It has been reported that aspartame is completely hydrolyzed or metabolized to aspartic acid and phenylalanine (Ranney & Oppermann, 1979; Bertorelli *et al.*, 1990), and therefore, the effect of aspartame observed in our study may be through aspartic acid, one of the components of aspartame.

The effects of glutamic acid on iron uptake and transport of iron by Caco-2 cells The iron uptake and transport of iron by Caco-2 cells were also significantly increased by the addition of 20 μ M of glutamic acid at $p < 0.05$ (Figs. 1, 3 and Table 1). When the concentration of glutamic acid added to the test solution was increased from 20 μ M to 1 mM only iron transport was significantly increased at $p < 0.05$ (Table 1).

Fig. 1. Effect of aspartic acid, glutamic acid, histidine, phenylalanine, cysteine and dipeptides (anserine and carnosine) on iron uptake by Caco-2 cells. Caco-2 cells were cultured in serum-free medium for 14–18 days post confluence culture. Caco-2 cells were incubated with 1.5 ml of test solutions (which contained 10 μ M of FeCl_3 complexed with 10 μ M of nitrilotriacetic acid and 20 μ M of aspartic acid, glutamic acid, histidine, phenylalanine, cysteine, dipeptides (anserine and carnosine) and 1.67 kBq $^{59}\text{FeCl}_3$ /insert) in the apical chamber and 2.5 ml of serum-free medium in the basal chamber. Cells were incubated at 37°C for 1 h. Each value represents mean $\pm$ SEM of two experiments with five replicates each. The different letters above the error bars indicate that values are significantly different at $p < 0.05$.

Fig. 2. Effect of aspartic acid, glutamic acid, histidine, phenylalanine, cysteine and dipeptides (anserine and carnosine) on iron uptake by Caco-2 cells. Caco-2 cells were cultured in serum-free medium for 14–18 days post confluence culture. Caco-2 cells were incubated with 1.5 ml of test solutions (which contained 10 μ M of FeCl_3 complexed with 10 μ M of nitrilotriacetic acid and 1 mM of aspartic acid, glutamic acid, histidine, phenylalanine, cysteine, dipeptides (anserine and carnosine) and 1.67 kBq $^{59}\text{FeCl}_3$ /insert) in the apical chamber and 2.5 ml of serum-free medium in the basal chamber. Cells were incubated at 37°C for 1 h. Each value represents mean $\pm$ SEM of two experiments with five replicates each. The different letters above the error bars indicate that values are significantly different at $p < 0.05$.

Fig. 3. Effect of aspartic acid, glutamic acid, histidine, phenylalanine, cysteine and dipeptides (anserine and carnosine) on iron transport across monolayers of Caco-2 cells. Test solution (1.5 ml) containing $10 \mu\text{M}$ of FeCl_3 complexed with $10 \mu\text{M}$ of nitrilotriacetic acid and either $20 \mu\text{M}$ or 1 mM of aspartic acid, glutamic acid, histidine, phenylalanine, cysteine, dipeptides (anserine and carnosine) and $1.67 \text{ kBq } ^{59}\text{FeCl}_3/\text{insert}$ was added to the apical chamber of 14–18 days post confluent cultures of Caco-2 grown on microporous inserts and 2.5 ml of serum-free medium was added to the basal chamber. Cells were incubated at 37°C , and the quantity of ^{59}Fe transferred into serum-free medium (2.5 ml) in the basal chamber was monitored periodically for up to 60 min. Symbols are mean values of two experiments with five replicates each.

The effects of histidine on iron uptake and transport of iron by Caco-2 cells As shown in Figs. 1, 2, 3 and Table 1, the addition of $20 \mu\text{M}$ of histidine to the test solution significantly increased iron uptake over that of the control. When the concentration of histidine in the test solution was increased from $20 \mu\text{M}$ to 1 mM , the iron uptake was slightly increased, but the rate of iron transport was significantly increased (Fig. 3 and Table 1). The results of this study were

consistent with the observation of Martinez-Torres *et al.* (1981) who suggested that basic amino acids, aromatic and aliphatic amino acids increased the utilization of nonheme iron. Van Campen in 1973 observed the same findings with histidine and lysine and further suggested that the enhancing effect of histidine and lysine is due to their ability to form tridentate chelates and stable complexes of octahedral configuration of ferric ion and retain iron in soluble form.

However, these findings differ from those of Layrisse *et al.* (1984) who found that increasing the doses of histidine from 416 to 2080 mg did not enhance the absorption of corn iron. The differences in observation among these studies may be related to the differences in the sources of iron and the method used.

Effects of cysteine on uptake and transport of iron by Caco-2 cells Previous reports showed that cysteine (a sulfur-containing amino acid) effectively enhances nonheme and heme iron absorption (Layrisse *et al.*, 1984; Kapsokefalou & Miller, 1991). However, the results of our study showed that both the concentrations (20 μ M and 1 mM) in the test solution did not influence the iron uptake or transport of iron by Caco-2 cells (Figs. 1, 2, 3 and Table 1). These results agree with Politz and Clydesdale (1988) and Wien and Van Campen (1991) who found that cysteine did not enhance iron absorption. This result was probably due to the unstable nature of cysteine at neutral or alkaline pH, which is rapidly oxidized to cystine. It has been reported that cystine by nature does not enhance iron absorption (Layrisse *et al.*, 1984). Taylor *et al.* (1986) and Wien and Van Campen (1991) also suggested that the enhancing effect of cysteine on nonheme iron absorption is due to cysteine-containing peptides, which play a role in reducing Fe(III) to Fe(II) in the intestinal lumen.

Effects of phenylalanine on uptake and transport of iron Figures 1, 3 and Table 1 showed that no significant increase was observed in the iron uptake and rate of iron transport by Caco-2 cells at 20 μ M of phenylalanine in the test solution. When the concentration of phenylalanine in the test solution was increased from 20 μ M to 1 mM, the iron uptake was significantly increased (Fig. 2). However, no significant stimulation was observed in the iron transport (Fig. 3 and Table 1). Phenylalanine is an aromatic amino acid which has no electron charge. Politz and Clydesdale (1988) suggested that the formation of chelates in which the iron combines with electron-donating ligands can solubilize the iron and could prevent or delay hydrolysis and polymerization reactions which would otherwise occur. For this reason, phenylalanine showed a low ability to enhance the iron uptake and transport of iron by Caco-2 cells.

Effects of anserine and carnosine on uptake and transport of iron by Caco-2 cells We observed that addition of anserine or carnosine at 20 μ M to the test solution significantly increased the iron uptake and stimulated the rate of iron transport at $p < 0.05$ (Figs. 1, 2, 3 and Table 1). When the concentrations of anserine or carnosine in the test solution were increased from 20 μ M to 1 mM, only the transportation of iron was further significantly increased ($p < 0.05$) in the case of anserine (Figs. 2, 3 and Table 1). Meat muscles are good sources of carnosine and anserine (Susan *et al.*, 1989). Previous investigators have suggested that amino acids or peptides which are released during meat digestion may be involved in enhancing iron absorption (Kane & Miller, 1984; Slatkavitz & Clydesdale, 1988). Our findings suggested that carnosine or anserine may be involved in stimulating iron uptake and rate of iron transport in Caco-2 cells. As far as we know, no study has been conducted on the effects of carnosine or anserine on iron absorption in humans or animals. The

results presented in this study conclude that the supplementation of certain amino acids and dipeptides in foods may improve the absorption and utilization of iron. Further investigation is needed to reveal the effects of carnosine and anserine in the diet on iron absorption in humans.

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