EFFECTS OF DIETARY AND PHYSIOLOGICAL FACTORS ON FE TRANSPORT AND METABOLISM BY HUMAN INTESTINAL CACO-2 CELLS

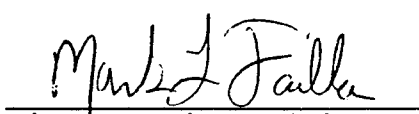
by

Okhee Han

A Dissertation Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro
1995

Approved by



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APPROVAL PAGE

This dissertation has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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December 19, 1994
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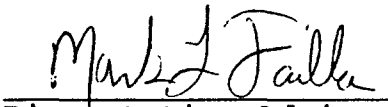

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The Caco-2 human intestinal cell line was selected as a potential model for the study of iron bioavailability and absorption because confluent cultures spontaneously differentiate into polarized, enterocyte-like cells. Initially, the influence of several dietary factors on Fe uptake and transport by Caco-2 intestinal cells were examined. Both the uptake and transport of nonheme Fe were decreased by inositol hexaphosphate and its lesser phosphorylated metabolites. In contrast, ascorbic acid enhanced the cellular acquisition and transport of this micronutrient, even in the presence of inositol phosphates. These and other data support the usefulness of the Caco-2 cell line as an appropriate model for investigating qualitative and quantitative effects of various dietary factors on iron bioavailability for humans.

Next, I examined the mechanism of the stimulatory influence of ascorbic acid on iron uptake across the apical surface of Caco-2 cells. Ascorbic acid-enhanced cellular uptake of iron was inhibited by ascorbate oxidase and chelators of ferrous ion. Ferrireductase activity was also present on the brush border membrane and this activity accounted for the majority of iron taken up by cells in the absence of an exogenous reductant. Thus, ferric ion must be

reduced to its ferrous state for transport across the intestinal brush border membrane.

Specific factors responsible for the regulation of Fe absorption largely remain unknown. Because both iron deficiency and infection of the gastrointestinal tract are prevalent in developing countries, I speculated that chronic activation of gut immune system may decrease the absorption of dietary Fe. Thus, Caco-2 cells were pre-treated with the monokines and iron transport and metabolism was examined. Monokine-treatment significantly ($p < 0.05$) increased both cellular accumulation of Fe from diferric-transferrin and cellular content of ferritin protein. These changes significantly ($p < 0.05$) impaired apical to basolateral transport of nonheme iron. These data suggest that chronic stimulation of the gut immune cells contributes to the high global incidence of iron deficiency.

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The Department of Food, Nutrition and Food Service Management, The School of Human Environmental Sciences, The Graduate School and The North Carolina Institute of

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DEDICATION

I would like to dedicate this work to my beloved family. I express my deepest gratitude and appreciation to my family for their endless encouragement and unconditional love and support.

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CHAPTER I

INTRODUCTION

OVERVIEW

Fe deficiency is the most common human nutritional deficiency, globally afflicting more than 500 million individuals. The incidence of iron deficiency is particularly prevalent in infants, pre-school children, adolescents and women in their child-bearing years. Iron deficiency adversely influences numerous functional processes, including growth, cognitive development and exploratory activities of children, resistance to infection, and the capacity for physical work and play. Detailed discussion of the consequences of Fe deficiency have been the subject of several recent reviews (Scrimshaw 1991; Pollitt 1993). Therefore, effective strategies to reduce the incidence of this nutritional deficiency are needed. Fe deficiency is due not only to a low intake of this essential trace metal, but also to poor bioavailability of dietary Fe and pathophysiological factors that may decrease its absorption. Consequently, numerous investigators continue to study various dietary, physiological and pathological

factors that influence the efficiency of Fe absorption.

Despite extensive study, the mechanism of iron absorption is not clear. Fe homeostasis is maintained primarily by controlling absorption in the proximal intestine, since excessive endogenous metal can only be eliminated by loss of blood. Distinct pathways exist for the uptake and at least the initial steps in the processing of dietary heme and non-heme Fe. The uptake of non-heme Fe across the brush border membrane is affected by dietary components (Conrad 1987; Faiweather-Tait 1992). Dietary factors such as phytic acid, polyphenols, phosvitin, calcium and some trace minerals are known inhibitors of Fe absorption. In contrast, ascorbic acid, citric acid, animal protein, certain amino acids and fructose are enhancers of the absorption of dietary Fe (Linder 1991). Transfer of Fe acquired from the lumen across the basolateral membrane and into plasma is influenced by various conditions, including whole body Fe stores, growth, pregnancy, lactation, and infection/inflammation (Conrad 1987). Specific physiological factors responsible for regulating the transfer of intracellular Fe across the basolateral membrane are unknown. Detailed studies have eliminated key roles for erythropoietin, transferrin (Tf), and the degree of Fe saturation of Tf in the control of this process. Several recent findings suggest that humoral factors produced by

immune cells during infection and inflammatory episodes may affect the cellular Fe homeostasis (see below). The potential influence of these cytokines on Fe absorption has not been examined.

While most investigators have used intact laboratory rodents, tissue segments and isolated cells from such animals to examine factors influencing iron bioavailability, Reddy and Cook (1991) have challenged the validity of extrapolating findings from animals to humans.

Recently, numerous investigators have used the Caco-2 human intestinal cell line to study the characteristics of the transport and metabolism of nutrients and drugs. The rationale for choosing the Caco-2 cell line as a model is that it spontaneously differentiates at confluency to a phenotype with ultrastructural and biochemical properties similar to polarized, absorptive intestinal epithelial cells. Therefore, I decided initially to evaluate the feasibility of using Caco-2 cells to study matters related to the bioavailability of Fe and Zn. Once this objective was successively completed (Chapters II & III), I proceeded to critically examine the mechanism of ascorbate-enhanced Fe transport across the brush border surface (Chapter IV). Finally, I used the Caco-2 cell model to begin to investigate the possibility that the widespread Fe deficiency common in developing countries may result in part

from chronic infection of the gastrointestinal tract, a condition that is endemic in areas with inadequate public health programs. More specifically, I examined the impact of monokines on the characteristics of Fe transport across the basolateral and brush border membranes and the cellular levels of the Fe storage protein ferritin (Chapter V).

THE ROLES OF DIETARY FACTORS ON NON-HEME FE ABSORPTION

The absorption of Fe from a meal is influenced by types and relative amounts of dietary ligands, the chemical form of iron (i.e., heme vs non-heme Fe and Fe^{3+} vs Fe^{2+}), and a variety of physiological factors (Fairweather-Tait 1992). While the absorption of heme Fe is highly efficient and relatively independent of other components in a meal, the efficiency of non-heme Fe absorption is low and markedly influenced by the composition of the meal (Conrad 1987). The absorptive process for non-heme Fe involves three distinct processes, viz., uptake of Fe across the brush border membrane, intracellular transport, and transfer across the basolateral membrane into plasma. It has been suggested that uptake of Fe from the intestinal lumen by enterocytes is the rate limiting step for absorption of this essential mineral. This process is markedly affected by a variety of dietary, physiological, and pathological conditions.

Specific dietary components that promote or inhibit the cellular uptake of non-heme Fe have been identified. For example, relatively high concentration of inositol hexaphosphate (also referred to as IP6 or phytic acid) are present in cereal grains and legumes and can significantly decrease Fe absorption by humans (Hallberg et al. 1987; Hurrell et al. 1992). Because phosphate groups can be cleaved from the inositol ring by both endogenous phytase activity and during the processing of foods (Reddy et al. 1989; Sandberg and Svanberg 1991; Sandberg et al. 1987), investigation of the impact of lesser phosphorylated derivatives of inositol, viz., IP3, IP4 and IP5, on the absorption of minerals is relevant (Lonnerdal et al. 1989; Sandstrom and Sandberg 1992). In contrast to phytic acid, ascorbic acid is recognized as a potent enhancer of non-heme Fe absorption in humans and animals (Cook and Monsen 1977; Hunt et al. 1990; Hallberg 1987). Almost 20 years ago, Cook and Monsen (1977) demonstrated that the efficiency of Fe absorption from a semisynthetic meal was directly proportional to the amount of ascorbic acid added over the range of 25-1,000 mg. However, the mechanism responsible for this phenomenon has remained controversial for decades. One argument is that the stimulatory influence of ascorbic acid on Fe absorption is due to its ability to chelate Fe(III) ion, thereby preventing the formation of insoluble

polynuclear complexes at the slightly alkaline pH of the intestine. In contrast, others argue that ascorbate-mediated reduction of Fe(III) to Fe(II) not only increases the solubility of the trace metal, but also facilities its interaction with a Fe(II) transporter on the brush border surface (Dorey et al. 1993; Gorman and Clydesdale 1983). The possibility that reduction of non-heme Fe is the key factor influencing intestinal Fe uptake is supported by several recent studies with laboratory animals (Raja et al. 1992; Wien and Van Campen 1991). Because chronic supplementation with ascorbic acid does not cause Fe overload (Cook et al. 1984; Hunt et al. 1994), it is evident that the actual absorption of dietary Fe also depends on non-dietary factors.

TRANSFERRIN RECEPTOR-MEDIATED FE TRANSPORT

Homeostatic regulation of Fe metabolism is controlled by modulating the absorption of dietary Fe. Although it has received minimal consideration, it is likely that endogenous Fe also enters mucosal cells from the circulation. Review of the literature indicates that Fe absorption is blocked by transfer of Fe from plasma to the intestinal cells in response to Fe overload due to transfusions (Conrad 1987; Linder and Munro 1977). The delivery of Fe to the cells from

the blood is mediated primarily by the transferrin receptor (TfR) pathway (Aisen 1990).

The TfR, a dimeric glycoprotein composed of two 94-kDa subunits, is involved in the uptake of Fe via the binding and endocytosis of di- and mono- ferric Tf (Aisen 1990). In order to function, TfR must be present on the cell surface (Aisen 1990; Williams and Enns 1991). The mechanism of TfR-mediated Fe transport into the cells and the regulation of TfR gene expression have been investigated extensively (Ende et al. 1987; Klausner et al. 1983, 1983; Richardson and Backer 1992). Studies with normal and transformed cells have shown that diferric Tf binds to unoccupied surface TfR and enters the internal compartment by an endocytic process. Subsequent acidification of the vesicles is associated with release of the metal from the polypeptide. Fe is then transferred across the endosomal membrane for utilization, storage, or efflux. The Tf-TfR complex is recycled to the cell surface where apo-Tf is released (Klausner et al. 1983, 1993; Rhyner et al. 1985). Numerous experimental studies and clinical observations have shown that cellular levels of TfR vary inversely with Fe status (Carriaga et al. 1991; Muller et al. 1988; Rhyner et al. 1985; Sipe and Murphy 1991; Skikne et al. 1990). Likewise, the expression of TfR is inversely correlated with the cellular level of ferritin.

While several investigators have reported the presence of TfR on the basolateral membranes of the intestinal epithelial cells, it is not clear whether TfR is involved in endogenous Fe uptake by mucosal cells and/or required for the absorption from the intestinal lumen (Anderson et al. 1990, 1991).

ENDOCRINE CONTROL OF FE ABSORPTION

Other than Fe status, several physiological conditions that affect Fe absorption are associated with changes in the plasma levels of hormones such as estrogen and thyroxine. These include growth, pregnancy and lactation.

Numerous studies have suggested that increased mineral absorption during pregnancy may be related to elevated estrogen levels which are characteristic of pregnancy. Clinical reports have indicated that women using oral contraceptives containing estrogen for extended periods have increased Fe stores. Likewise, studies with laboratory rodents and domestic animals suggest that estrogen treatment increases Fe absorption (Clermont and Schraer 1979; Frassinelli-Gunderson et al. 1985; Saiz et al. 1980). It is not clear whether this stimulatory influence of estrogen is due to a direct action of the hormone on the enterocyte. Recently, estrogen receptors (ER) have been identified in

IEC-6 cells, a rat cell line possessing a crypt-like phenotype, and in intestinal epithelial cells isolated from the duodenal, jejunal, ileal, and colonic segments of the rat gut (Arjmandi et al. 1993; Tomas et al. 1993). Furthermore, Arjmandi et al. (1993) found that estrogen treatment directly enhanced uptake of Ca by isolated rat duodenal cells. Together, these data support the possibility that estrogen directly affects intestinal Fe absorption during pregnancy.

In a comprehensive review of gut and thyroid hormone interrelationships, Miller et al. (1978) postulated that the thyroxine affects gastrointestinal structure and function, including gastric secretion, gastrointestinal motility, ion excretion and the absorption of nutrients (e.g., fat and glucose). The intestine, like the liver, is a major site for the metabolism of thyroid hormones. It has been demonstrated that Caco-2 cells metabolize thyroid hormones (Lee et al. 1989). Thyroid hormones have been suggested to be involved in Fe homeostasis. A number of clinical observations and rodent studies have shown that chronic changes in thyroid hormone status affect the levels of ferritin in serum, liver and kidney (Takamatsu et al. 1985; Winkelmann et al. 1981). The presence of T_3 receptors in the rat small intestinal mucosa (Hodin et al 1992) support the possibility that thyroid hormones also may directly influence Fe absorption.

THE EFFECTS OF CYTOKINES ON FE TRANSPORT AND METABOLISM

Localized and circulating immune cells secrete a family of regulatory proteins referred to as cytokines in response to inflammatory stimuli. Cytokines have been shown to have autocrine, paracrine or endocrine influences in diverse types of cells and tissues. The secretory products of activated phagocytic cells are collectively referred to as monokines. These include interleukin (IL)-1, IL-6, tumor necrosis factor (TNF) and α-interferons (INF).

A number of clinical observations and experimental studies have clearly demonstrated a regulatory role of monokines on Fe and Zn homeostasis during episodes of inflammation and infection (Elin et a. 1977; Gordeuk et al. 1988; Klasing 1984; Oppenheimer 1989; Potter et al. 1989). Rapid reduction in plasma levels of Fe and Zn and increased hepatic synthesis of their respective storage protein, viz., ferritin and metallothionein, respectively, are recognized as early host responses to infection. Several in vitro studies have demonstrated that monokines affect cellular Fe metabolism (Johnson et al. 1989; Rogers et al. 1990; Torti et al. 1988; Tsuji et al. 1991). Tsuji et al. (1991) reported that treatment with IL-1 and TNF induced ferritin mRNA and subsequently increased the TfR mRNA level in MRC5 human lung diploid fibroblasts. Other studies using TA1

mouse adipocytes, human muscle cells, and HepG2 cells have shown that treatment with IL-1 and TNF induce the synthesis of ferritin mRNA and ferritin protein (Rogers et al. 1990; Torti et al. 1988).

Recently, Molmenti et al. (1993) reported that IL-1, IL-6, TNF, and INF regulated the expression of several "acute phase plasma proteins" in the Caco-2 and T84 cell lines. Changes in plasma levels of these proteins are normally associated with hepatocyte activity during inflammatory episodes. This discovery suggests that localized acute phase responses may also be an important component in the host's defense. Together, these observations support the possibility that cytokines modulate Fe metabolism and transport in the intestinal epithelial cells during chronic infection and inflammation.

CACO-2 SYSTEM AS A MODEL SYSTEM FOR INVESTIGATION OF INTESTINAL FE TRANSPORT AND METABOLISM

Most studies of Fe bioavailability and absorption have used laboratory animals, isolated segments or cells from rodent intestine and non-differentiated cell lines derived from rodent intestine. However, the relevance of data from such studies for humans has been criticized because of recognized differences in the impact of dietary factors on

Fe absorption in humans and animals (Reddy and Cook 1991), the rapid loss of function and viability of freshly isolated cells and the undifferentiated status of cell lines. Therefore, the need to select the appropriate model for defining qualitative and quantitative effects of dietary and physiological factors on intestinal Fe uptake, metabolism and absorption in the human is apparent.

The Caco-2 cell line is derived from a human colonic adenocarcinoma. During the past several years, this cell line has been widely used for investigating the transport and metabolism of various drugs and nutrients. Monolayers of Caco-2 cells spontaneously differentiate into highly polarized cells with well developed brush border microvilli that contain hydrolytic enzymes, such as sucrase-isomaltase, lactase, and aminopeptidase, and basolateral membranes possessing a Na^+, K^+-ATPase and hormone and cytokine receptors (Giuliano et al. 1991; Hidalgo et al. 1989; Pinto et al 1983; Varilek et al. 1993). Caco-2 cells grown on microporous membrane inserts allow the investigation of vectorial transport of nutrients and drugs. The characteristics of the transport of diverse nutrients, including, Fe, Zn, Ca, monosaccharides, amino acids, and vitamins, by Caco-2 cells have been recently reported (Alvarez-Hernandez et al. 1991; Blais et al. 1987; Dix et al. 1990; Ellwood et al. 1993; Fleet et al. 1993; Giuliano

and Wood 1991; Halleux and Schneider 1991; Han et al 1994; Hidalgo and Borchardt 1990; Mohrmann et al. 1986; Raffaniello et al. 1991, 1992; Souba and Copeland 1992).

Several preliminary reports on Fe transport and metabolism by Caco-2 cells were available when this project was initiated (Alvarez-Hernandez et al. 1991; Halleux and Schneider 1991). Demonstration that the cells contained ferritin protein and probably expressed the TfR supported its potential usefulness as a model. With this background, I defined the Specific Aims outlined in the following section.

SPECIFIC AIMS

The overall goal of this project has been to develop the Caco-2 human cell line as a useful model system for investigating matters related to a) The bioavailability of Fe and Zn, and b) the physiological regulation of Fe absorption. To achieve this goal, the specific aims outlined below have been systematically addressed.

1. To examine the influence of phytic acid (inositol hexaphosphate) and its lesser phosphorylated metabolites on the apical uptake and transepithelial flux of non-heme Fe and Zn by differentiated cultures of Caco-2 cells (Chapter II).

2. To assess the influence of combinations of inhibitors and enhancers of dietary Fe absorption on Fe uptake and transport by Caco-2 cells (Chapter III).
3. To define the mechanism by which ascorbate enhances Fe uptake cross the apical surface of Caco-2 cells (Chapter IV).
4. To characterize the basolateral uptake and the possible storage of Fe from Fe-transferrin (Chapter V).
5. To examine the impact of monokines on Fe transport and metabolism by Caco-2 cells (Chapter V).

CHAPTER II

INOSITOL PHOSPHATES INHIBIT UPTAKE AND TRANSPORT OF IRON AND ZINC BY A HUMAN INTESTINAL CELL LINE

ABSTRACT

To examine the influence of inositol phosphates on the uptake and absorption of Fe and Zn, Caco-2 cells were grown on either plastic (uptake studies) or porous membranes in bicameral chambers (transport/absorption studies). Caco-2, a human colon adenocarcinoma cell line, was selected as the test cell because it spontaneously differentiates into polarized enterocyte-like cells at confluency. Uptake of Fe (added as Fe-nitrilotriacetate complex) from a calcium-free solution by fully differentiated cells was 37 pmol/cm^2. Addition of 10-fold molar excess of individual inositol phosphates (IP_3, IP_4, IP_5 or IP_6) decreased Fe solubility by 13 to 25% and reduced Fe uptake by 50 to 65%. The rate of transport of Fe from the apical solution into the basolateral chamber (1.4 ± 0.1 pmol/(h·cm^2)) decreased (34-96%) in proportion to the degree of phosphorylation of the inositol derivative in the apical compartment. Uptake and transepithelial transport of Zn were 200 pmol/cm^2 and 23

pmol/(h·cm^2), respectively. The solubility, uptake and rate of transport of Zn also decreased in proportion to the degree of phosphorylation of inositol. These results demonstrate the inhibitory influence of IP_3-IP_6 on the uptake and transport of Fe and Zn and support the usefulness of Caco-2 human cell line as an appropriate model for evaluating the effects of specific dietary factors on trace metal bioavailability.

INTRODUCTION

The absorption of iron (Fe) and zinc (Zn) from a meal is influenced by the types and relative amounts of dietary ligands, the chemical form of iron (i.e., heme vs. non-heme Fe and Fe^{3+} vs. Fe^{2+}), and a variety of physiological factors (Fairweather-Tait 1992). Numerous dietary enhancers and inhibitors of the absorption of these essential trace metals have been defined. Inositol hexa-phosphate (IP_6) or phytate is present in cereal grains and legumes and has been implicated as a significant inhibitor of Fe (Hallberg et al. 1987; Hurrell et al. 1992) and Zn (Fairweather-Tait et al. 1992; Navert et al. 1985) absorption by humans. During food processing, IP_6 may be hydrolyzed to lower phosphorylated derivatives of inositol, e.g., IP_3, IP_4 and IP_5 (Reddy et al. 1982; Sandberg et al. 1987; Sandberg and Svanberg 1991). Several studies suggest that these hydrolysis products may have a lesser inhibitory effect on dietary mineral utilization than IP_6 (Lonnerdal et al. 1989; Sandstrom and Sandberg 1992). A more quantitative examination of the relationship between the degree of phosphorylation of inositol and the uptake and transepithelial transport of Fe and Zn by the human intestine would be facilitated by the availability of an appropriate cell model.

The transformed human colonic cell line Caco-2 is a useful in vitro model for investigating nutrient and drug transport and metabolism by the intestinal absorptive epithelium. Monolayers of Caco-2 cells spontaneously differentiate into highly polarized cells with well developed brush border microvilli that possess many of the properties of mature intestinal absorptive epithelial cells (Hidalgo et al. 1990; Pinto et al. 1983). Vectorial transport of nutrients and drugs from the apical to the basolateral compartments can be examined by culturing cells on microporous membrane inserts. Using such approaches, the characteristics of Fe (Alvarez-Hernandez et al. 1991; Halleaux and Schneider 1991), Zn (Raffaniello and Wapnir 1991; Raffaniello et al. 1992), and calcium (Giuliano and Wood 1991) transport by Caco-2 cells have been reported recently.

The objectives of this study were to use the Caco-2 cell model to study the effects of different isoforms of inositol phosphates present in plant-derived foods on the uptake and transcellular transport of Fe and Zn.

MATERIALS AND METHODS

Purification of Inositol Phosphates Aliquots (25 μL) of phytic acid (40 g/100g, Aldrich Chemical Co., Milwaukee, WI) were added to 20 mL deionized water and applied to silica-based anion exchange (SAX) columns (20 x 10 cm, Analytical Instrument Inc., Harbor City, CA) mounted in a vacuum manifold (Supelco Inc., Bellefonte, PA). After washing with 10 mL deionized water, samples were eluted in stepwise manner with 100 mL volumes of 150 mmol/L HCl (fractions 1-10), 225 mmol/L HCl (fractions 11-20), and 350 mmol/L HCl (fractions 21-30) and 20 mL volumes of 2 mol/L HCl (fractions 31-32). The profile of inositol phosphates in each fraction was examined by reverse phase HPLC using a Hamilton PRP-1 column (5 μm; 150 x 4.1 mm) essentially as described by Lehrfeld (1989). The solvent system was composed of 50% methanol: 50% formic acid (35mmol/L adjusted to pH 4.3 with H_2SO_4): 1% tetrabutylammonium hydroxide (1.54mol/L). HPLC separation was isocratic at a column temperature of 50°C and a flow rate of 1 mL/min. Inositol phosphates were detected by refractive index (Waters 410 Differential Refractometer; Milford,MA). Based on this analysis, fractions 5 and 6 were combined for IP_3, fractions 12-15 for IP_4, fractions 22-26 for IP_5 and fractions 29-31 for IP_6. After repeating this procedure for 12 separate

aliquots of phytic acid, all fractions containing each inositol phosphate were pooled and concentrated to 5 mL by rotary evaporation (Buchi, Inc., Switzerland). Remaining volume was transferred to scintillation vials and taken to dryness with a centrifugal vacuum concentrator (Jouan, Inc., Winchester, VA). Residues were dissolved in deionized water and aliquots were reassessed by HPLC to determine purity. Analysis of IP_4, IP_5 and IP_6 indicated that purity exceeded 98%. The concentration of each inositol phosphate was determined by phosphate analysis after wet ashing with a mixture of nitric acid and hydrogen peroxide (Fiske and Subbarow, 1925).

The commercial solution of phytic acid did not yield sufficient quantities of IP_3. Therefore, 10 mL sodium phytate (Sigma Chemical Co., St. Louis, MO) solution (35g/L) was added to 40 mL sodium acetate (100 μmol/L) with magnesium acetate (2 mmol/L)) and pH was adjusted to 5.15 with concentrated glacial acetic acid. Wheat phytase (final conc., 800 U/L; Sigma Chemical Co., St. Louis, MO) was added to initiate hydrolysis. Samples were shaken at room temperature for 20 hours, boiled 2 min and filtered to remove particulate matter. Filtrate (3 mL) was diluted, applied to SAX column and processed as above. The product was free of IP_4, IP_5 and IP_6 as determined by HPLC.

Purified inositol phosphates were solubilized in water and filter sterilized (0.2 μm pore syringe filter; Nagle Company, Rochester, NY) for use in experiments. Samples were stored at 4°C for a maximum of two weeks. HPLC analysis showed that purified inositol phosphates were stable during this period.

Cells The human colon carcinoma derived cell line, Caco-2, was purchased from American Type Culture Collection (Rockville, MD) at passage 17 and experiments were performed with cultures between passages 22 and 40. Cells were grown in 75 cm^2 plastic flasks in high glucose Dulbecco's modified Eagle's medium (DMEM) containing heat inactivated fetal calf sera (15% v/v), $NaHCO_3$ (44 mmol/L), non-essential amino acids (1%), L-glutamine (4 mmol/L), Hepes (15 mmol/L) and antibiotics (50 mg gentamicin and 0.5 mg fungizone/mL) in an atmosphere of air-CO_2 (95:5) at 37°C and 90% humidity.

Stock cultures were seeded at a density of 6700 cells/cm^2 and medium was changed every second day. At 70% confluency, cells were collected by washing 3x with Hank's balanced salts solution (HBSS) without Ca^{2+} and Mg^{2+} followed by treatment with the same buffer containing trypsin (0.25%) and 2 mmol/L EDTA. Suspended cells were transferred to multiwell cluster dishes (9.6 cm^2/well; 25,000 cells/cm^2) to examine the effects of inositol phosphates on ^{55}Fe and ^{65}Zn

uptake (Figure 2.1). Likewise, cells (100,000 cells/cm^2) were transferred to transparent membrane filters (Falcon, microporous inserts area=4.9 cm^2; Becton Dickinson & Company, Lincoln Park, NJ) to examine the effects of inositol phosphates on the transport of the trace metals from the apical to the basolateral compartment (Figure 2.1). Inserts were placed in six well plates with 2.5 mL medium in the well and 1.5 mL medium in the insert. Medium was changed every second day and the day before cultures were used for uptake and transport studies. All cultures were used 10-12 d after reaching confluency, i.e., when cells were fully differentiated (Ellwood et al., 1993; Hidalgo et al., 1989).

The integrity of tight junctions between cells on inserts for 10-12 days after the monolayer become confluent was assessed using D-(1-^{14}C) mannitol (sp. act. 2.04 GBq/mmol, Dupont NEN, Boston, MA). This sugar alcohol is primarily absorbed by the paracellular route.

The rate of ^{14}C-mannitol transport from the apical to the basolateral compartment was determined using the identical procedure as that for trace metal transport. In the absence and presence of Caco-2 cells the rate of ^{14}C-mannitol transfer across the membrane was 2.1%/(hr·cm^2), and 0.06%/(h·cm^2), respectively. The latter value is similar to that reported by other investigators (Ranaldi et al. 1992).

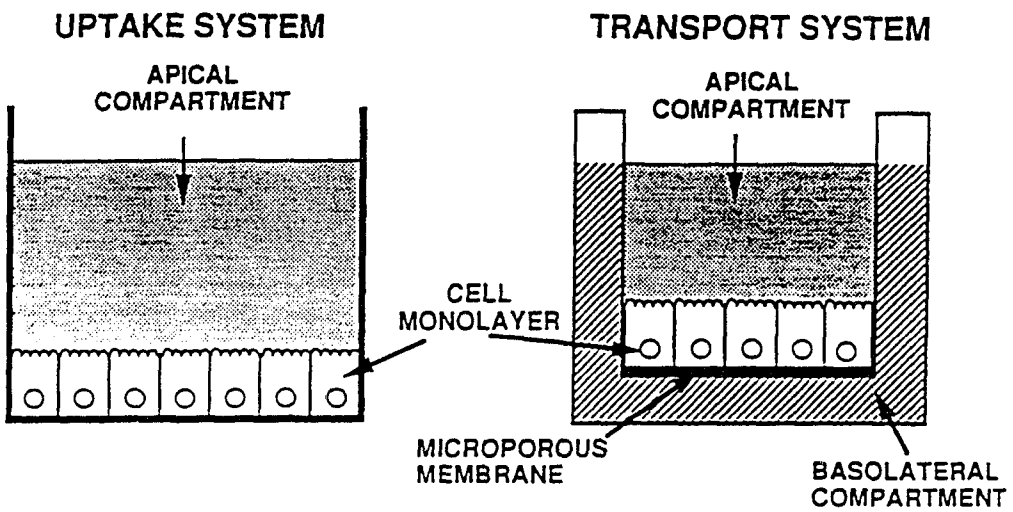


Figure 2.1. Schematic representation of uptake and transport models using Caco-2 cells.

Cellular uptake of Fe and Zn Metal chelate solutions were prepared fresh before each experiment. Stock solutions contained either 10 mmol/L $FeCl_3 \bullet 6H_2O$ and 20 mmol/L nitrilotriacetic acid (NTA) or 10 mmol/L $ZnSO_4 \bullet 7H_2O$ and 20 mmol/L sodium citrate in 1 mmol/L HCl. Stocks were diluted 10-fold in sterile, deionized water before transfer of appropriate volume (1% v/v) to uptake buffer. Immediately after addition of the metal chelate solution to uptake buffer, either ^{55}Fe (sp. act. = 132 GBq/mmol; Dupont NEN, Boston, MA) or ^{65}Zn (sp. act. = 7.2 GBq/mmol; Dupont NEN) was added to provide 37 or 74 kBq per well for uptake and transport studies, respectively. Uptake buffer contained 130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose and 50 mmol/L Hepes, pH 7.0. Background concentrations of Fe and Zn in uptake buffer were less than 0.3 μmol/L before addition of metal stock as determined by flame atomic absorption spectrophotometry. Following the additions of metal chelates and radioisotopes to uptake buffer, solutions were mixed well prior to adding test inositol phosphate. Complete uptake buffer was incubated at 37°C until initiation of experiments. The effect of inositol phosphates on solubility of Fe and Zn in the uptake buffer was determined by measuring the level of radioactivity in vortexed aliquots before and after centrifugation at 8,160xg for 10 min. at 20°C.

Spent medium was removed from multiwell dishes and monolayers were washed three times with Ca^{2+}, Mg^{2+}-free HBSS at 37°C. Test uptake buffer (1.2 mL) was added and dishes were incubated at 37°C in 95% humidified air:5% CO_2 atmosphere for 1h. Then, radioactive medium was removed and the cell surface was washed three times with ice-cold buffer containing 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Hepes, pH 7.0, to remove residual medium and non-specifically bound metal. Cellular material was collected after adding 1mL solution containing 3.5 mmol/L SDS with 1 mmol/L EDTA, pH 7.0, to each well and scraping the surface of the dish with a rubber policeman. Following sonication, aliquots were transferred to minivials containing 4 mL scintillation cocktail (Bio-Safe II, RPI, Mount Prospect, IL) and the amount of radioactivity measured (Beckman model LS 6000 SE Palo Alto, CA). The quantity of extracellular Fe and Zn taken up by cells was calculated from the specific activity of the test solution and is presented as pmol/well.

Transport of Fe and Zn from apical to basolateral compartment Spent medium was removed from upper (apical) and lower (basolateral) chambers. The apical and basolateral surfaces of the monolayer were washed three and one time(s), respectively, with Ca^{2+}, Mg^{2+}-free HBSS at 37°C. Complete DMEM (2.5 mL) was added to the lower chamber. Test uptake buffer

(1.5 mL) was added to the insert and cultures were incubated at 37°C as described above. To determine the rate of trace metal transport from the apical to the basolateral compartment, aliquots (0.20 mL) were removed hourly for up to 5h. An equivalent aliquot of fresh, complete DMEM was added to the basolateral compartment after each sampling to maintain volume. After correcting data to account for sample replacement, the rate of transport was calculated by linear regression analysis using results from 1-5 h. In some experiments the amount of radioactivity in cells was determined at 5 h by washing the monolayer three times with ice-cold saline-EDTA and harvesting cells from the insert membrane as described for uptake studies. The quantity of Fe and Zn transferred from the apical to the basolateral compartment was estimated from the specific activity of the test solution added to the apical chamber and is presented as $pmol/(h \cdot cm^2)$.

Analysis of data All variables were tested in at least triplicate wells for each experiment and experiments were repeated two to four times. Results are presented from a representative experiment, since variation between absolute values for replicate experiments using cells at different passages was 10-20%. Data are means ± SE. Data were analyzed with the SAS System for Personal Computers, Version 6.04

(SAS Institute, Cary, NC). Uptake data were analyzed by one-way ANOVA (Snedecor & Cochran, 1980). The effects of time and treatment on transport of Fe or Zn from the apical to the basolateral compartment of cultures were compared using repeated measures analysis. Transport rates were calculated using linear regression analysis of data from 1-5 h. Calculated rates were compared using General Linear Model. Tukey's multiple range test was used to determine significant differences among means ($P < 0.05$) for uptake and transport data.

RESULTS

Uptake and transport of Fe Introduction of Fe^{3+} - NTA (1:2) complex to a final concentration of 10 μmol/L into Ca^{2+} - free uptake buffer appeared to facilitate solubility of the metal ion at neutral pH, since the quantity of Fe in aliquots before and after centrifugation was identical for as long as six hours. The solubility of Fe decreased slightly when 10-fold molar excess of inositol phosphate was also added to the uptake buffer. The amount of Fe in supernate of samples containing IP_3, IP_4, IP_5 and IP_6 were 13, 16, 20 and 25%, respectively, less than that in the control solutions (data not shown).

Differentiated cultures of Caco-2 monolayers accumulated approximately 300 pmol Fe/well (equivalent to 35-40 pmol/cm^2 or 100-110 pmol Fe/mg cell protein) after incubation at 37°C for 1h (Fig. 2.2). Addition of a 10-fold excess of either IP_3, IP_4, IP_5, or IP_6 (100 μmol/L) decreased Fe uptake by 50-65%. Unexpectedly, the inhibitory effect of IP_6 on Fe uptake routinely was 10-20% less than that of IP_4 and IP_5. Neither inositol nor phosphate nor inositol plus phosphate inhibited Fe uptake by Caco-2 cells, demonstrating the specificity of the inhibitory effects of inositol phosphates (Table 2.1).

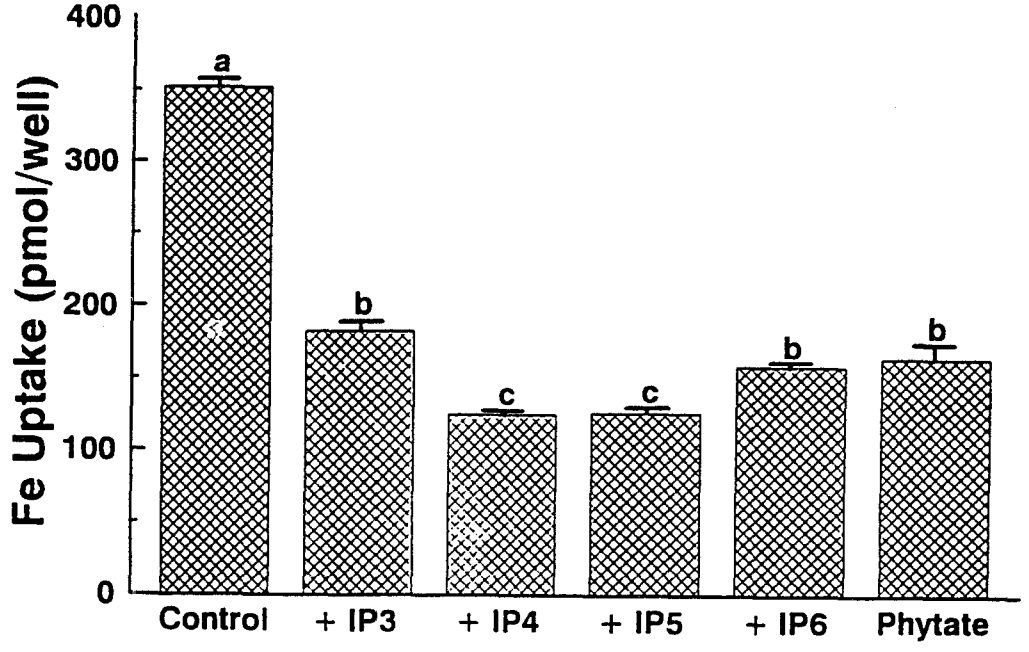


Figure 2.2. Inositol phosphates inhibit Fe uptake by Caco-2 cells.

Uptake buffer (1.2 mL) containing 37 kBq ^{55}Fe (added as 10 μmol/L Fe complexed with 20 μmol/L NTA) and either 0 or 100 μmol/L indicated inositol phosphate was added to wells containing 10-12d post-confluent cultures of Caco-2 cells. Cultures were incubated for 1 h at 37°C prior to removal of uptake buffer and loosely bound ^{55}Fe was removed by multiple washes with ice cold buffered saline containing 1 mmol/L EDTA. Values are means ± SE for representative experiments (total of 3) with 3 wells for each test variable. The presence of different letters above the error bar indicates that values are significantly different ($p < 0.05$).

TABLE 2.1. Uptake of Fe and Zn by Caco-2 cells is not altered by inositol, phosphate or inositol plus phosphate.[1,2]

Uptake Solution	Uptake (pmol/well) Fe	Zn
Metal only	282 ± 19[a]	1873 ± 11[a]
" + inositol	317 ± 14[a]	2158 ± 19[a]
" + phosphate	305 ± 7[a]	2032 ± 138[a]
" + inositol and phosphate	285 ± 2[a]	2032 ± 28[a]
" + IP_6	153 ± 20[b]	960 ± 59[b]

[1]Data are mean ± SE for at least 3 replicate wells from a representative experiment (N=3). Significant differences between groups are indicated by the presence of different letters as superscripts within a column ($p < 0.05$).

[2]The influences of inositol (100 μmol/L), phosphate (600 μmol/L added as NaH_2PO_4) and both inositol (100 μmol/L) and phosphate (600 μmol/L) on cellular uptake of ^{55}Fe (as 10 μmol/L Fe complexed with 20 μmol/L NTA) and ^{65}Zn (as 10 μmol Zn with 20 μmol/L citrate) were compared to the inhibition of metal uptake by IP_6 (100 μmol/L). Post confluent (12d) cultures were exposed to test compounds at 37°C. After 1h, uptake buffer was removed and loosely bound metal was removed from the surface of the monolayer as described in the legend for Figure 2.2.

The quantity of Fe transferred from the apical to the basolateral compartment of cultures containing 10-12d postconfluent cultures of Caco-2 was linear between 1 and 5 h of incubation (Figure 2.3) and had a mean rate of 1.4 pmol/(h.cm^2) (Table 2.2). Addition of inositol phosphates to the uptake buffer significantly decreased both the quantity and the linear rate of Fe transport across the cell monolayer (Figure 2.3 and Table 2.2). The relative degree of inhibition of Fe transport by inositol phosphates was proportional to the level of phosphorylation of inositol at all sampling times. The rate of Fe transport into the basolateral compartment declined by 34, 56, 71 and 96% when IP_3, IP_4, IP_5, and IP_6, respectively, was present in the uptake buffer.

<u>Uptake and transport of Zn</u> Zn (final concentration of 10 μmol/L) was soluble in Ca^{2+}-free uptake buffer for as long as 6 h when the metal ion was added as the Zn-citrate complex (1:2). The addition of 10-fold molar excess of IP_3, IP_4, IP_5 and IP_6 to uptake buffer decreased Zn solubility by 7, 10, 25 and 92%, respectively (data not shown).

Uptake of Zn by differentiated cultures of Caco-2 cells was 6-7 fold greater than that of Fe at 37°C (Figures 2.2 and 2.4). Cells took up 1800-2000 pmol Zn/well (equivalent

TABLE 2.2. Rates of Fe and Zn transport by Caco-2 cells in absence and presence of inositol phosphates.[1,2]

Apical Solution	Transport Rate (pmol/h.cm^2) Fe	Zn
Metal only	1.39 ± 0.12^a	22.8 ± 1.0^a
" + IP_3	0.92 ± 0.04^b	12.8 ± 0.4^b
" + IP_4	0.61 ± 0.02^c	6.7 ± 0.3^c
" + IP_5	0.40 ± 0.01$^{c\,d}$	2.5 ± 0.2^d
" + IP_6	0.06 ± 0.02^d	0.9 ± 0.1^e

[1]Data are mean ± SE for at least 3 replicate wells from a representative experiment (N=4). Significant differences between groups are indicated by the presence of different letters as superscripts within a column ($p < 0.05$).

[2] ^{55}Fe and ^{65}Zn were added to apical solution as 10 μmol/L Fe chelated with 20 μmol/L NTA and 10 μmol/L Zn chelated with 20 μmol/L citrate, respectively, with or without 100 μmol/L given inositol phosphate. The quantity of radioisotope present in the basolateral compartment was measured hourly as described in Materials and Methods. Transport rate was calculated using linear regression analysis of data from 1-5 hours (see Figures 2.3 and 2.5).

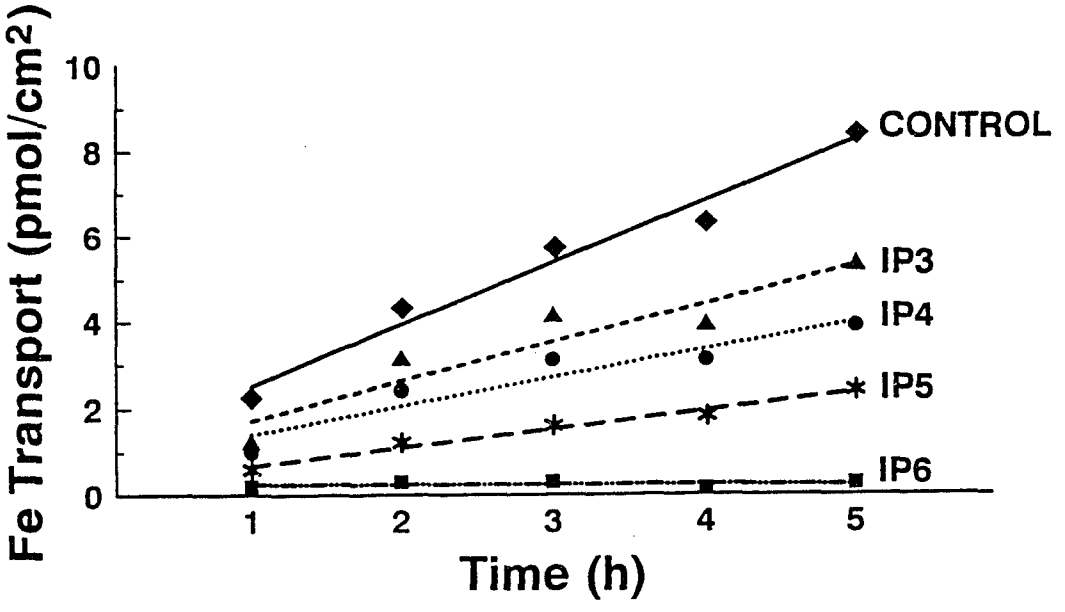


Figure 2.3. Extent of inhibition of Fe transport across Caco-2 monolayer by inositol phosphates is proportional to the level of phosphorylation of inositol.

Uptake buffer (1.5 mL) containing 37kBq ^{55}Fe as 10 μmol/L Fe complexed with 20 μmol/L NTA and either 0 or 150 μmol/L indicated inositol phosphate was added to the apical compartment of 10-12d post-confluent cultures of Caco-2 grown or microporous inserts. Cultures were incubated at 37°C and the quantity of ^{55}Fe transferred into complete medium (2.5 mL) in the basolateral compartment was monitored periodically for up to 5 h. Symbols are mean values for 3 wells from a representative experiment (N=3). Standard errors for all values are less than 10% of the means.

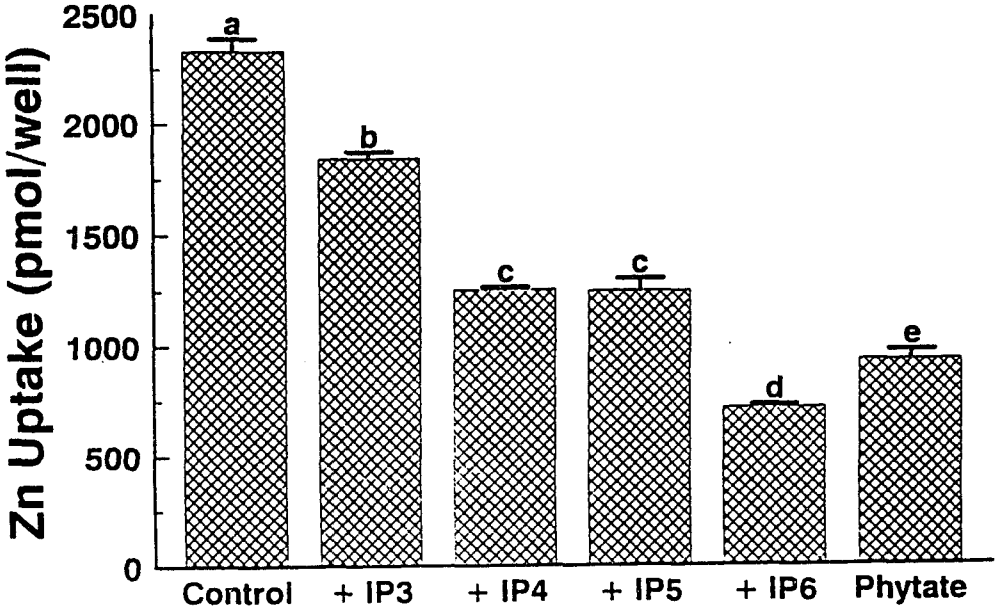


Figure 2.4. Inositol phosphates inhibit Zn uptake by Caco-2 cells.

Details are identical to those in the legend of Figure 2.2, except that the uptake buffer contained ^{65}Zn and Zn:citrate (1:2) instead of ^{55}Fe and Fe:NTA (1:2).

to approx. 200 pmol Zn/cm^2 or 600 pmol Zn/mg protein) during a 1h incubation (Figure 2.4). The quantity of Zn accumulated from medium during a 1h incubation was reduced 22, 47, 48 and 70% when 100 μmol/L of IP_3, IP_4, IP_5 or IP_6, respectively, was present (Figure 2.4). Neither inositol nor phosphate nor inositol plus phosphate affected the ability of Caco-2 cells to take up Zn (Table 2.1).

As observed for Fe, the quantity of Zn transferred across the Caco-2 monolayer increased linearly from 1 to 5 h (Figure 2.5). The quantity of Zn and the rate of Zn transported from the apical to the basolateral compartment were significantly decreased by the addition of inositol phosphates to the upper compartment (Figure 2.5 and Table 2.2). The degree to which inositol phosphates inhibited the rate of Zn transport was proportional to the extent of phosphorylation of inositol. The rate of Zn transport was reduced by 44, 71, 89 and 96% when 100 μmol/L IP_3, IP_4, IP_5 or IP_6, respectively, was added to the apical compartment.

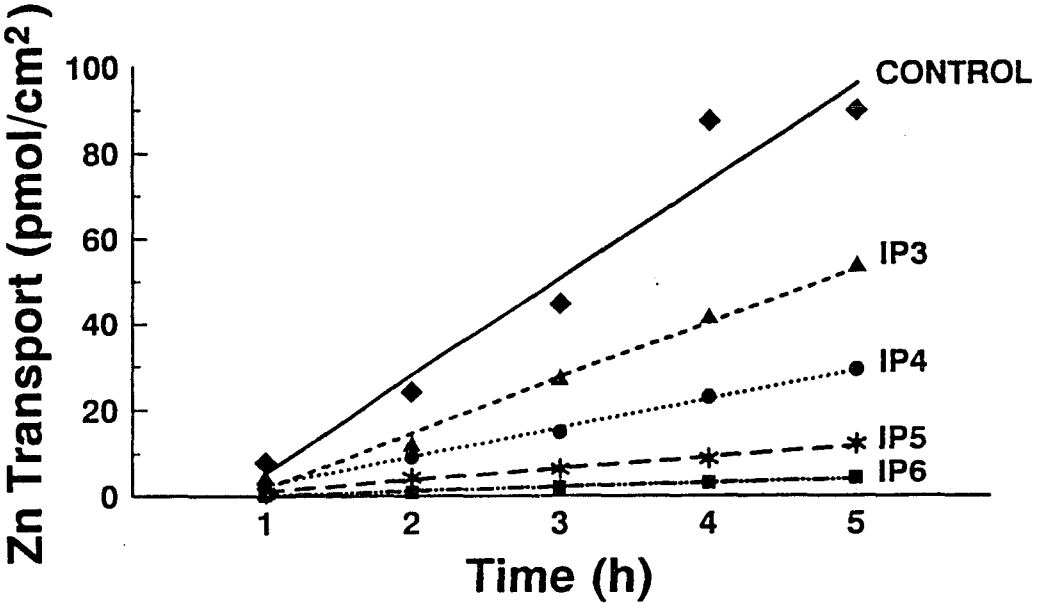


Figure 2.5. Degree of inhibition of Zn transport across Caco-2 monolayer by inositol phosphates is proportional to the level of phosphorylation of inositol.

Details are identical to those in legend of Figure 2.3, except that uptake buffer contained ^{65}Zn and Zn:citrate (1:2) instead of ^{55}Fe and Fe:NTA (1:2).

DISCUSSION

The Caco-2 cell line, which was derived from a human colonic adenocarcinoma, has the unique property of spontaneously differentiating at confluency into cells exhibiting many of the morphological and functional characteristics of mature enterocytes. Confluent monolayers are characterized by polarized cells with brush border membranes containing hydrolytic enzymes, such as sucrase-isomaltase and aminopeptidase, and basolateral membranes possessing a Na^+, K^+ -ATPase and hormone receptors (Hidalgo et al. 1989; Pinto et al. 1983). Growth on porous membrane inserts facilitates the investigation of the mechanism of transfer of substances from the apical to the basolateral compartments. Because of the many similarities of differentiated Caco-2 cells to enterocytes, the cell line has been used to study the transport and metabolism of a variety of nutrients. These include monosaccharides (Blais et al. 1987; Ellwood et al. 1993), amino acids (Hidalgo and Borchardt 1990; Souba and Copeland 1992), vitamins (Dix et al. 1990; Giuliano et al. 1991), calcium (Giuliano and Wood 1991), phosphate (Mohrmann et al. 1986), iron (Alvarez-Hernandez et al. 1991; Halleux and Schneider 1991), and zinc (Raffaniello and Wapnir 1991; Raffaniello et al. 1992). Caco-2 cells have also served as a model for evaluating the

relative bioavailability and transport mechanisms of diverse drugs (Artursson 1990; Ranaldi et al. 1992).

With the above as background, we postulated that Caco-2 cells might provide a useful tool for studying the influence of dietary factors on the intestinal uptake and absorption of nutrients. This hypothesis has been evaluated by testing the effect of various isoforms of IP on the absorption of Fe and Zn. In our studies, the ratio of phytate and its lesser phosphorylated derivatives to the trace metals was 10; this is within the range found in typical western diets and particularly that by vegetarians (e.g., Ellis et al. 1987; Wise et al. 1987). IP_6 significantly inhibited the uptake and transport of these micronutrients by Caco-2 cells. The specificity of this inhibitory action was demonstrated by the lack of any effect of equimolar levels of either inositol or phosphate or inositol plus phosphate on the cellular uptake of Fe and Zn.

Endogenous phytase activity, fermentation and other types of food processing, as well as digestive enzymes in some species, can cleave phosphate groups from inositol phosphates present in plant foods (Reddy et al. 1989; Sandberg et al. 1987; Sandberg and Svanberg 1991; Zhou et al. 1992). The possible influence of lesser phosphorylated

derivatives of inositol, viz., IP_3, IP_4 and IP_5, on mineral bioavailability has received recent attention. Brune et al. (1992) reported that either IP_3, IP_4, IP_5 or IP_6 all inhibited the absorption of dietary iron by adult humans. In contrast, investigations using suckling rats (Lonnerdal et al. 1989) and adult human subjects (Sandstrom and Sandberg 1992) suggest that Zn absorption is selectively impaired by IP_5 and IP_6, but not IP_3 and IP_4. We found that IP_3-IP_6 all attenuated both the uptake and rate of transport of Fe and Zn by Caco-2 cells. While the degree of inhibition of Fe uptake (50-65%) was relatively independent of the extent of phosphorylation of inositol, the rate of Fe transport from the apical to the basolateral compartment decreased in proportion to the number of phosphate groups linked to inositol. Similarly, the rate of Zn transport by Caco-2 cells declined in direct proportion to the level of inositol phosphorylation. Unlike the situation in vivo, other dietary, pancreatic and biliary ligands were not present in the cellular uptake buffer. Some of these ligands probably enhance the uptake of Fe and Zn at the brush border surface by competing with inositol phosphates for the trace metals. It is feasible that the relative proportions of the various inositol phosphates affect the ability of competing dietary and endogenous chelators to present the metal to the enterocyte in a more readily available form. In this regard,

we have demonstrated that ascorbate effectively eliminates the inhibitory influence of equimolar levels of IP_3, IP_4 and IP_5, but not IP_6, on Fe uptake and transport by Caco-2 cells (unpublished data).

It is generally accepted that phytate inhibits the absorption of Fe and Zn by reducing their solubility in the lumen of the small intestine (Hallberg et al. 1987; Sandberg et al. 1989). As expected, the ability of Caco-2 cells to transfer these trace metals from the apical compartment containing test inositol phosphates to the basolateral compartment was correlated with the relative solubility of Fe and Zn. However, our data clearly demonstrate that the inhibitory effect of inositol phosphates on Fe uptake or accumulation was independent of the degree of phosphorylation of inositol and the solubility of the trace metal. While there was a better correlation among the levels of phosphorylation of inositol, Zn solubility and the quantity of Zn taken up by Caco-2 cells during a 60 minute incubation, the amount of Zn accumulated also was higher than that predicted solely by solubility. The concentration of the trace metals in uptake buffer (10 μmol/L), the presence of excess EDTA (1 mmol/L) in wash solution and the inability of Caco-2 cells to secrete mucin suggest that Fe and Zn accumulated by Caco-2 cells was not simply "trapped"

at the cell surface, but was probably bound to high affinity sites on the cell surface and/or transferred into the cell. The possibility that Caco-2 cells may accumulate insoluble inositol phosphate-trace metal complexes, perhaps by pinocytosis, warrants consideration. It was recently demonstrated that orally administered IP_6 is taken up rapidly and then slowly degraded by epithelial cells lining the stomach and small intestine of the rat (Sakamoto et al. 1993). Moreover, IP_5 and IP_6 are present in all mammalian cells where they represent the bulk of IPs and are relatively resistant to turnover (Berridge and Irvine 1989). Thus, if accumulated within the cell as the inositol phosphate-metal complex, the availability of Fe and Zn for subsequent transfer to the basolateral surface appears to require cleavage of phosphate groups from IP_6 and IP_5.

In summary, the results from these initial series of studies support the usefulness of Caco-2 cells for screening the effects of dietary and endogenous factors on the cellular uptake and subsequent transport of trace minerals across polarized epithelial cells. The data also clearly demonstrate that the efficiency of Fe and Zn transfer across the epithelial barrier can be decreased by lesser phosphorylated derivatives of IP_6. This effect is largely dependent on the degree of phosphorylation of inositol.

Finally, the results raise the possibility that intestinal cells may take up inositol phosphate-metal complexes that require cellular metabolism before the micronutrients can be transferred across the basolateral surface to complete the process of absorption.

CHAPTER III

INFLUENCES OF INOSITOL PHOSPHATES, ASCORBATE AND AMINO ACIDS ON APICAL UPTAKE AND TRANSPORT OF NON-HEME FE BY CACO-2 HUMAN INTESTINAL CELL LINE

ABSTRACT

Differentiated cultures of Caco-2 cells were used as a model to examine the interactions between various dietary factors on apical uptake of nonheme Fe and its transfer across the basolateral surface. Caco-2 cells accumulated 91-98 pmol Fe/mg protein from uptake buffer containing 12 nmoles of Fe(III)-nitrilotriacetate during 1 h incubation at 37°C. Addition of a 10-fold molar excess of phytic acid (IP6) and its lesser phosphorylated derivatives (IPs) decreased cellular uptake and transport of Fe. Ascorbic acid (AA) stimulated Fe uptake and transport from a solution containing IPs and was dependent upon the ratio of AA to IP and inversely proportional to the degree of phosphorylation of inositol, i.e., IP3 > IP4 > IP5 > IP6. An essential amino acid mixture had minimal impact on Fe uptake in either the

absence or presence of IPs. However, simultaneous addition of both essential amino acid mixture and AA synergistically stimulated cellular acquisition of Fe from uptake buffer with or without IPs. These data further support the usefulness of Caco-2 cells as a model for investigating the effects of various dietary factors on mineral bioavailability in human enterocytes.

INTRODUCTION

Various dietary components are known to enhance or inhibit apical uptake of non-heme iron by enterocytes. In contrast, physiological factors such as iron status, chronic infection and age influence relatively undefined processes responsible for regulating the transfer of Fe from within the intestinal cell to the portal circulation (Conrad 1987; Cook et al. 1991; Layrisse et al. 1990). Phytate, i.e., inositol hexaphosphate (IP6), is present in high concentrations in cereal grains and legumes and can significantly inhibit absorption of non-heme Fe (Hallberg et al. 1987; Hurrell et al. 1992). Lesser phosphorylated derivatives of inositol, viz., IP3, IP4, and IP5, also have been reported to limit dietary Fe acquisition in vivo (Brune et al. 1992) and in vitro (Han et al. 1994). In contrast, ascorbic acid can be a potent enhancer of non-heme Fe absorption in humans and animals (Cook and Monsen 1977; Hunt et al. 1990; Van Campen 1972). The extent to which ascorbic acid stimulates the absorption of dietary Fe largely depends on both an individual's iron status and diet composition. For example, supplementation of humans with ascorbic acid offsets the inhibitory influence of phytic acid and polyphenols on Fe absorption (Hallberg et al. 1986 and 1989; Siegenberg et al. 1991). In contrast, long term

supplementation with ascorbic acid does not increase iron stores in normal adults (Cook et al. 1984; Hunt et al. 1994). Such observations suggest that the primary effect of ascorbic acid is to enhance Fe uptake across the brush border membrane surface of the enterocyte, whereas the subsequent absorption of this newly acquired Fe is primarily controlled by iron status.

We have adopted the human Caco-2 cell line as a model to investigate the characteristics and regulation of intestinal Fe uptake and transport. Previous studies (Chapter II) revealed that IP6 and its lesser phosphorylated derivatives, viz., IP3, IP4 and IP5, inhibit uptake of Fe from the apical compartment and its transport across the basolateral surface (Han et al. 1994). In contrast, ascorbic acid enhanced apical uptake and transport of non-heme Fe by Caco-2 cells by reducing Fe(III) to Fe(II) (Chapter IV).

In the present study, the effect of addition of different inositol phosphates combined with various concentrations of ascorbic acid on Fe uptake and transport by Caco-2 cells has been examined. In addition, the influence of a mixture of amino acids alone or in combination with inositol phosphates and ascorbic acid was also investigated, since the literature contains

contradictory reports on the effects of amino acids and peptides on the absorption of dietary non-heme Fe (e.g., Layrisse et al. 1984; Van Campen 1973).

MATERIALS AND METHODS

Reagents Dulbecco's modified minimal essential medium (DMEM) with 4.5 g/L glucose, fetal calf serum, 100x stock solution of nonessential amino acids and L-glutamine, and phytic acid were purchased from Sigma Chemical Company (St. Louis, MO). Gentamicin, fungizone and 100x stock solution of essential amino acid mixture for Eagle's Basal Medium were purchased from GIBCO BRL (Grand Island, NY). ^{55}Fe (132 GBq/mmol $FeCl_3$) and D-[1-^{14}C] mannitol (2.04 GBq/mmol) were obtained from Dupont New England Nuclear (Boston, MA). The procedures for purification of inositol triphosphate (IP3), inositol tetraphosphate (IP4), inositol pentaphosphate (IP5) and inositol hexaphosphate (IP6) have been described in Chapter II and published elsewhere.

Cells Caco-2 cells were obtained from ATCC (Rockville, MD), and were used between passages 22 and 40. Details concerning the growth and maintenance of the stock and experimental cultures are outlined in Chapter II. Cultures were used to study Fe uptake and transport 10-12d after reaching confluency. Cells were fully differentiated as evidenced by maximal activities of sucrase and alkaline phosphatase (Pinto et al. 1983) and minimal rate (0.06%/h·cm^2) of paracellular flux of ^{14}C-mannitol from the apical to the

basolateral chamber (Chapter IV).

Fe uptake and transport Measurement of cellular uptake of ^{55}Fe and transport of the metal from the apical to the basolateral chambers containing confluent monolayers of Caco-2 cells on microporous membrane inserts (3 μmeter pores) were described in Chapter II.

Analysis of data Presented data (means ± SE) represent results from at least two separate experiments using three wells for each parameter. Data were analyzed by the General Linear Model procedure using the SAS System for Personal Computers, Verson 6.04 (SAS Institute, Cary, NC). Tukey's multiple range test was used to determine significant differences among means ($p < 0.05$) for uptake and transport data (Snedecor and Cochran 1980).

RESULTS

Differentiated cultures of Caco-2 cells accumulated 255-275 pmol Fe/well (equivalent to 27-29 pmol/ cm^2 and 91-98 pmol/mg protein) from uptake buffer containing 12 nmoles of Fe(III)-NTA during 1 h incubation at 37°C. Addition of a 10-fold molar excess of phytic acid (i.e., 100 μmol/L IP6) decreased Fe uptake by 50%, which was similar to that reported in Chapter II. Because ascorbic acid (≥ 8 μmol/L) significantly enhances uptake of Fe across the apical surface of Caco-2 cells in a dose-dependent manner (see Chapter IV), the impact of simultaneous addition of both the enhancer (ascorbic acid) and the inhibitor (phytic acid) on Fe uptake was tested. When the molar ratio of ascorbic acid to phytic acid was ≤ 0.6, Fe uptake remained 50-60% as high as that in cultures containing only uptake buffer (Figure 3.1). At a molar ratio of ascorbic acid to phytic acid of 1.25, cellular uptake of Fe was restored to the control level. Further increases in the ratio of ascorbic acid to phytic acid in the uptake solution (i.e., 2.5-10) were associated with a proportional elevation of Fe uptake by the cells.

Fe uptake by Caco-2 cells was also attenuated by the presence of a 10-fold molar excess of lesser phosphorylated

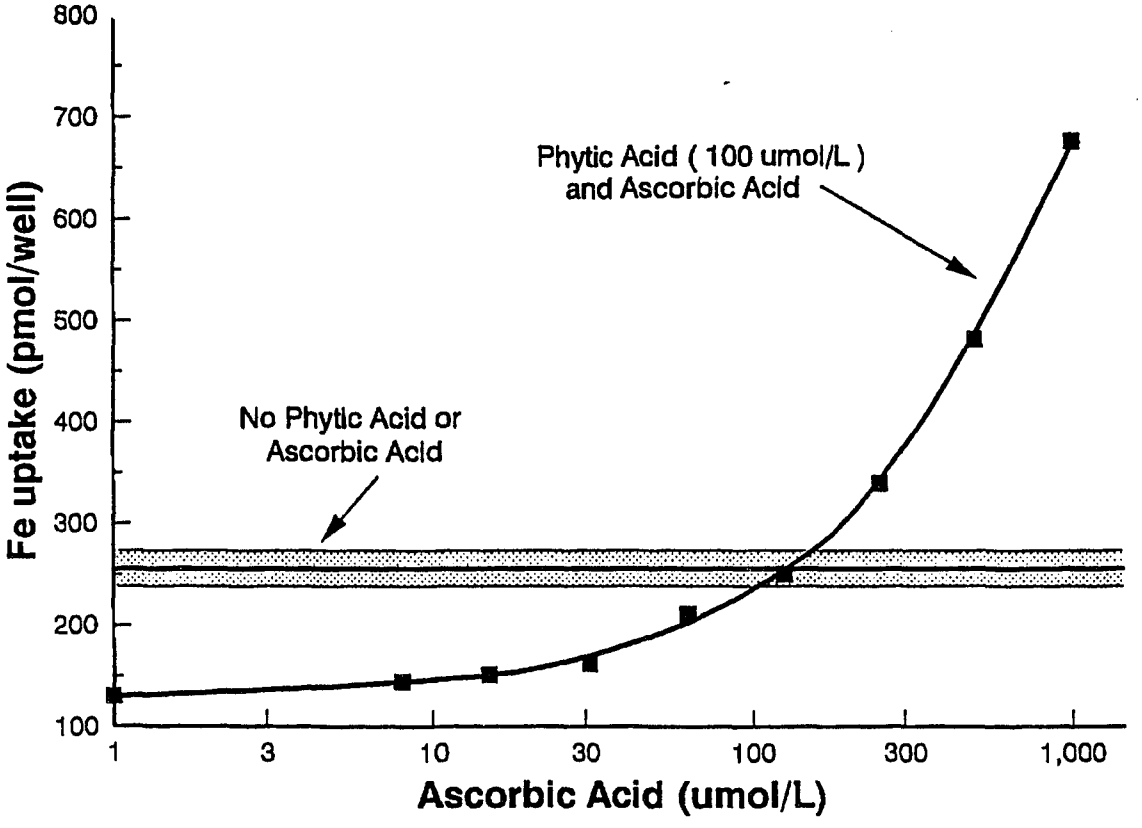


Figure 3.1. Effect of the concentration of ascorbic acid on phytic acid-mediated inhibition of Fe acquisition by Caco-2 cells.

Differentiated cultures of Caco-2 cells were incubated for 1 h at 37°C with uptake buffer (1.2 mL) containing 37 kBq of ^{55}Fe as 10 μmol/L $Fe^{3+}(NTA)_2$, 100 μmol/L phytic acid (IP6), and 0-1000 μmol/L ascorbic acid. Fe uptake was measured after removing loosely bound ^{55}Fe from the cell surface by washing with ice-cold saline containing 1 mmol/L EDTA, pH 7.0. Values are means ± SE from two separate experiments each using three replicate wells for a test parameter. In the absence of phytic acid, iron uptake was 255 ± 17 as indicated by the shaded region. Cellular acquisition of Fe in the presence of phytate was enhanced significantly ($p < 0.05$) by addition of ≥ 64 μmol/L ascorbic acid.

derivatives of phytic acid, viz., IP3, IP4 and IP5 (Table 3.1). This inhibitory influence of IPs on Fe uptake was offset by addition of 1000 μmol/L ascorbic acid to uptake buffer (Table 3.1). Ascorbic acid stimulated Fe uptake from solutions containing IPs from 1.8 to 8.2-fold above that of cultures containing Fe(III)-NTA alone. The degree to which ascorbic acid enhanced Fe uptake was inversely proportional to the degree of phosphorylation of inositol, i.e., IP3 > IP4 > IP5, IP6 ($p < 0.05$).

Fe transport from the apical to the basolateral compartment by differentiated cultures of Caco-2 cells was linear between 1-5 h of incubation (data not shown) with a mean rate of 1.45 pmol/(h·cm^2). Addition of IPs to the uptake buffer decreased the rate of Fe transport across the cell monolayer (Table 3.2). The degree of inhibition of Fe transport by IPs was proportional to the extent of phosphorylation of inositol. Addition of ascorbic acid to the apical compartment increased the rate of Fe transport between the two compartments in a dose-dependent manner. Similarly, addition of an equimolar quantity of ascorbic acid to the solution containing IPs (100 μmol/L) significantly increased Fe transport across the Caco-2 monolayer. As observed for uptake, the extent of the increase was inversely proportional to the level of

TABLE 3.1. Ascorbate-mediated stimulation of Fe uptake by Caco-2 cells is inversely proportional to the degree of phosphorylation of inositol.

Apical solution	Fe uptake	
	No ascorbic acid	Ascorbic acid
	pmol/well	
Fe only	256 ± 10[a]	2813 ± 58[a]
Fe + IP3	147 ± 10[b]	2111 ± 26[b]
Fe + IP4	109 ± 5[c]	684 ± 27[c]
Fe + IP5	100 ± 1[c]	480 ± 23[d]
Fe + IP6	158 ± 3[b]	466 ± 18[d]

Uptake buffer containing 37 kBq ^{55}Fe, 10 μmol/L Fe^{3+}, 20 μmol/L NTA and 100 μmol/L of the indicated inositol phosphate and either 0 or 1000 μmol/L ascorbic acid was added to 10-12d post-confluent cultures of Caco-2. Cultures were incubated with test compounds for 1 h at 37°C. Fe uptake was assessed as described in Materials and Methods. Values are means ± SE for two separate experiments each using 3 wells per test variable. Significant differences ($p < 0.05$) within cultures incubated in the presence or absence of ascorbic acid are indicated by different letters as superscripts within a column.

TABLE 3.2. Ascorbate enhances Fe transport by Caco-2 cells exposed to inositol phosphates (IPs).

Apical solution	Ascorbic acid		
	None	100 μmol/L	1000 μmol/L
	Apical→Basolateral Transport of Fe pmol/(h·cm²)		
Fe only	1.45 ± 0.14[a]	14.90 ± 1.40[a]	43.7 ± 1.1[a]
Fe + IP3	1.22 ± 0.33[b]	8.61 ± 0.50[b]	34.5 ± 1.7[b]
Fe + IP4	0.66 ± 0.02[c]	2.15 ± 0.16[c]	25.7 ± 0.7[c]
Fe + IP5	0.50 ± 0.02[cd]	1.60 ± 0.02[c]	18.4 ± 0.6[d]
Fe + IP6	0.05 ± 0.03[d]	0.30 ± 0.01[d]	10.1 ± 0.8[e]

Uptake buffer containing 74 kBq of $^{55}Fe^{3+}$ as 10 μmol/L Fe^{3+} complexed with 20 μmol/L NTA, indicated concentrations of ascorbic acid, and either 0 or 100 μmol/L of the specific inositol phosphate was added to the apical compartment of 10-12d post-confluent cultures of Caco-2 grown on microporous membrane inserts. Cultures were incubated for 5 h at 37°C. The rate of Fe transport to the basolateral compartment was assessed as described in Materials and Methods. Data are means ± SE for at least three replicate wells from each of three separate experiments. Significant differences ($p < 0.05$) within cultures exposed to the same level of ascorbic acid (i.e., either 0, 100, or 1000 μmol/L) are indicated by different superscripts within a column. The rate of Fe transport by cultures treated with each IP was significantly different ($p < 0.05$) at each concentration of ascorbic acid.

phosphorylation of inositol, i.e., ascorbate enhanced Fe transport in the presence of IP3 > IP4 > IP5 > IP6. However, Fe transport by cultures treated with equimolar concentrations of IP6 and ascorbic acid was only 20% that observed with Fe alone. Elevating the molar ratio of ascorbic acid to IPs to 10 in the apical compartment markedly increased the rate of Fe transport in all cultures. At the higher concentrations of ascorbic acid, the rate of Fe transport remained inversely proportional to the degree of phosphorylation of the inositol derivative in the apical compartment. However, the relative degree of enhancement of Fe transport associated with the elevation of the concentration of ascorbic acid was the greatest in cultures containing IP6.

Fe uptake was increased about 30% ($p < 0.05$) by the addition of a mixture of essential amino acids to the uptake buffer containing Fe(III)-NTA (Table 3.3). Likewise, the mixture of essential amino acids slightly, but significantly ($p < 0.05$), offset the adverse impact of IPs in cellular acquisition of Fe from uptake buffer. Simultaneous addition of both the mixture of essential amino acids and ascorbic acid (1000 μmol/L) to uptake buffer enhanced Fe uptake 11.5-fold, which was significantly greater ($p < 0.05$) than that in cultures treated with

TABLE 3.3. Essential amino acids (EAA) and ascorbic acid (AA) act synergistically to enhance Fe uptake in the presence or absence of IPs.

Apical solution	Additions to uptake buffer			
	None	+ EAA	+ AA	+ EAA + AA
	pmol Fe/well			
Fe only	258 ± 6[a]	332 ± 2[a]	2588 ± 74[a]	2956 ± 8[b]
Fe + IP3	138 ± 8[b]	180 ± 10[b]	2127 ± 77[b]	3431 ± 66[a]
Fe + IP4	106 ± 7[c]	127 ± 17[c]	1090 ± 68[c]	1695 ± 36[c]
Fe + IP5	102 ± 2[c]	115 ± 7[c]	788 ± 16[d]	1410 ± 44[d]
Fe + IP6	158 ± 3[b]	173 ± 4[b]	743 ± 12[d]	930 ± 21[e]

Uptake was determined as in Table 3.1. The mixture of essential amino acid included 20 μmol/L L-isoleucine, L-leucine, L-lysine·HCl, L-threonine, and L-valine: 10 μmol/L L-arginine·HCl, L-phenylalanine, and L-tyrosine; 5 μmol/L L-cystine, L-histidine and L-methionine; and 2 μmol/L L-tryptophan. Data are means ± SE for at least 3 wells from one representative experiment. Significant differences ($p < 0.05$) within cultures treated with EAA and/or AA are indicated by the presence of different superscripts within a column. The accumulation of Fe within cultures treated with or without indicated IP was significantly different, i.e., EAA + AA > AA > EAA > control (none) ($p < 0.05$).

ascorbic acid only. Finally, the addition of both essential amino acids and ascorbic acid also stimulated cellular acquisition of Fe from uptake buffer containing IPs synergistically, since the degree of enhancement exceeded that predicted from the addition of either amino acids or ascorbic acid.

DISCUSSION

While laboratory rodents and intestinal segments and isolated cells from animals have been used to investigate factors influencing iron bioavailability, the validity of extrapolating these findings from animals to humans has been challenged by Reddy and Cook (1991). Consequently, we and other investigators have used the Caco-2 human cell line as an alterative model to examine matters related to mineral bioavailability (cited in Chapter I and II). These cells are derived from a human colonic adenocarcinoma and have the unique characteristic of spontaneously differentiating at confluency into a phenotype with morphological and functional characteristics quite similar to polarized, absorptive intestinal epithelial cells (Hidalgo et al. 1989; Pinto et al. 1983)

The inhibitory effect of phytic acid (IP6) on nonheme iron absorption has been extensively documented in humans (Hallberg et al. 1987, Hurrell et al. 1992). Phosphate groups can be cleaved from the inositol ring by both endogenous phytase activity and the processing of foods (Reddy et al. 1989; Sandberg and Svanberg 1991; Sandberg et al. 1987). Therefore, there is interest in the impact of lesser phosphorylated derivatives of inositol on mineral

bioavailability (Han et al. 1994; Lonnerdal et al. 1989; Sandstrom and Sandberg 1992). We previously reported that IP3, IP4 and IP5 also inhibit the uptake of Fe across the brush border surface and its transfer across the basolateral surface by differentiated cultures of Caco-2. Moreover, the degree of the inhibitory effects of IPs on Fe transport was correlated with the level of phosphorylation of inositol.

The ability of ascorbic acid to enhance the absorption of dietary nonheme Fe by humans and animals is widely recognized (Cook and Monsen 1977; Hallberg 1987; Monsen 1988). For example, the level of Fe absorption from a semisynthetic meal was directly proportional to the amount of ascorbic acid added over the range of 25-1,000 mg of ascorbic acid (Cook and Monsen 1977). However, the stimulatory influence of ascorbic acid on Fe absorption was attenuated if the diet contained substantial amounts of meats (Cook et al. 1984; Hunt et al. 1994) or if the subjects have adequate stores of Fe (Cook et al. 1991; Gavin et al. 1994). We have demonstrated that ascorbic acid stimulates iron uptake and transport by Caco-2 cell grown and maintained in medium containing standard levels of iron (Chapter IV). The stimulatory effect of ascorbic acid on iron uptake was shown to be due to the reduction of Fe(III) to Fe(II) in the apical compartment.

Hallberg and associates (1986 and 1989) have examined the influence of ascorbic acid supplementation on the absorption of Fe by humans consuming a meal containing relatively high levels of phytic acid. They reported that the absorption of Fe from test meals was dependent on the relative levels of phytic acid and ascorbic acid present. Likewise, the stimulatory effect of ascorbic acid on cellular acquisition and transport of Fe by Caco-2 cells was dependent on both the relative amounts of ascorbic acid and IPs and the degree of phosphorylation of inositol.

The roles of specific amino acids and peptides as enhancers of Fe bioavailability have received considerable attention, because meat and fish enhance the absorption of nonheme Fe (Monsen 1988). Investigations using intact rats (Van Campen 1972) and ligated intestinal segments of the rat (Van Campen 1973) suggested that histidine, lysine and cysteine increase Fe absorption. In human studies, cysteine and glutathione, but not other amino acids, increased the absorption of Fe from black beans (Layrisse et al. 1984; Martinez-Torres and Layrisse 1970). In the present study, we examined the effect of a mixture of amino acids which included cystine, lysine and histidine on acquisition of nonheme Fe from the apical compartment by Caco-2 cells. Alone, the amino acid mixture had minimal impact (10-30%) on

Fe uptake in the absence and presence of IPs. However, the addition of both the amino acid mixture and ascorbic acid increased Fe uptake to a greater extent than the sum of that with amino acids and ascorbic acid alone. This observation is similar to that reported in a study using rats in which Fe absorption was stimulated to a greater extent by the addition of ascorbic acid plus histidine than treatment with ascorbic acid alone (Van Campen and Gross 1969).

It is generally accepted that specific amino acids enhance the absorption of Fe by either chelating Fe(III) and preventing its hydration, polymerization and precipitation (Carpenter and Mahoney 1989), or by reducing Fe(III) to Fe(II) in the gut (Wien and Van Campen 1991). Van Campen and Gross (1969) suggested that cysteine, lysine and histidine enhance Fe absorption in rat by increasing the solubility of ferric ion. In the present study, it is likely that amino acids alone had minimum stimulating effect on Fe uptake because the metal was introduced as a soluble complex with NTA (Chapter II). It is more likely that the synergistic influence of ascorbic acid plus amino acid is related to ascorbate-mediated reduction of Fe(III) to Fe(II) and the higher affinity of many amino acids for Fe(II) than Fe(III) (Albert 1950). Perhaps the presence of amino acids retarded the reoxidation of ascorbate generated Fe(II) by forming a

stable complex with Fe(II).

In summary, the results of this study further support the usefulness of Caco-2 cells as a model for investigating the influence of various dietary factors on mineral bioavailability. The present data also demonstrate that the inhibitory influence of phytic acid and lesser phosphorylated derivatives of inositol on Fe bioavailability can be offset by equimolar or higher levels of ascorbic acid, but not by a mixture of amino acids alone.

CHAPTER IV

REDUCTION OF FE(III) IS REQUIRED FOR UPTAKE OF NON-HEME IRON BY CACO-2 CELLS

ABSTRACT

Differentiated cultures of Caco-2 human colonic cells were used to examine the importance of reduction of non-heme ferric iron, Fe(III), for transport across the brush border surface. Cultures accumulated approximately 100 pmol Fe/h·mg protein when 10 μmol Fe was added as Fe(III)-NTA complex to the apical compartment. Ascorbic acid enhanced cellular acquisition of Fe in a dose dependent manner with as low as 8 μmol/L ascorbate increasing Fe uptake by 50%. Similarly, the rate of Fe transport from the apical to the basolateral compartment increased 5.6- and 30-fold when 100 and 1000 μmol/L ascorbic acid, respectively, were present in the apical chamber. Ascorbate-mediated stimulation of Fe uptake was temperature-dependent and required the reduction of Fe(III) to Fe(II), since it was inhibited by ascorbate oxidase and chelators of Fe(II). Moreover, Caco-2 cells recycled dehydroascorbic acid to ascorbic acid. Ferricyanide and Fe(II) chelators also partially inhibited Fe uptake from

medium devoid of ascorbic acid. Intact Caco-2 cells exhibited a ferrireductase activity on the apical surface that accounted for the majority of Fe accumulated by cells incubated in the absence of exogenous reductant. These data suggest that reduction of Fe(III) within the lumen or at the cell surface is required for transfer of this essential micronutrient across the intestinal brush border surface.

INTRODUCTION

Iron (Fe) deficiency is the most common nutritional deficiency, afflicting an estimated 500 million humans with infants, children and women in their child-bearing years at particular risk (Scrimshaw 1991). Fe deficiency is due not only to a low intake of this essential trace metal, but often to poor bioavailability of dietary Fe as well. Consequently, numerous investigators continue to study various dietary and physiological factors that influence the efficiency of Fe absorption in order to better formulate diets that enhance the availability of this micronutrient.

Despite extensive study, many details of the mechanism of iron absorption remain unknown (Conrad 1987). Fe homeostasis is maintained primarily by controlling absorption in the proximal intestine rather than excretion of the endogenous metal. Distinctly different pathways exist for the uptake and at least the initial steps in the processing of dietary heme and non-heme Fe. The absorptive process for non-heme Fe involves three distinct processes, viz., 1) uptake of the ingested metal across the brush border membrane of the enterocyte, 2) intracellular transport, and 3) transfer across the basolateral membrane into plasma. It has been suggested that uptake of Fe from

the intestinal lumen into enterocytes may be the rate limiting step for absorption of this micronutrient. This step is markedly affected by a variety of dietary, as well as physiological and pathological, factors. Many luminal components of dietary origin are capable of promoting or inhibiting the cellular acquisition of this trace metal. One of the most potent natural promoters of intestinal Fe absorption is ascorbic acid. It has been proposed that the stimulatory influence of ascorbic acid on Fe absorption is due to either its ability to chelate Fe(III), thereby maintaining its solubility and preventing formation of polynuclear complexes, or to reduce Fe(III) to the more soluble Fe(II) form (e.g., Dorey et al. 1993; Gorman and Clydesdale 1983). The possibility that reduction of non-heme Fe is a key factor influencing mucosal uptake of this metal is supported by several recent studies with rodents (Raja et al. 1992; Wien and Van Campen 1991).

While most investigators use intact animals and intestinal segments and cells derived from laboratory rodents to examine problems related to the mechanism of intestinal transport and metabolism of Fe, Reddy and Cook (1991) have challenged the validity of extrapolating results related to the influence of dietary factors on Fe absorption from animal studies to humans. Because differentiated

cultures of the Caco-2 human cell line possess many characteristics of mature enterocytes (Hildago et al. 1989; Pinto et al. 1983), they provide a useful model for investigating diverse problems of nutrient bioavailability and absorption without concern of possible differences between humans and rodents. Caco-2 cells have been used for studying several characteristics of intestinal Fe uptake and metabolism (Alvarez-Hernandez et al. 1992; Halleux and Schneider 1994; Han et al. 1994). The objectives of the present study were to characterize the influence of ascorbic acid on the uptake and transport of Fe by Caco-2 cells, and to examine whether cell-mediated reduction of Fe(III) to Fe(II) is required for acquisition of the metal in the absence of an exogenous reductant.

MATERIALS AND METHODS

Reagents Dulbecco's modified Eagle medium (DMEM), Hanks balanced salts solution (HBSS) with 25 mmol g/L glucose, fetal bovine serum, medium supplements except antibiotics, and test reagents were obtained from Sigma Chemical Company (St. Louis, MO). Gentamicin and fungizone were purchased from GIBCO (Grand Island, NY). Fetal Clone II, a bovine serum product, was obtained from Hyclone (Logan, UT). ^{55}Fe (132 GBq/mmole $FeCl_3$), ^{65}Zn (7.2 GBq/mmol $ZnCl_2$), and D-[1-^{14}C] mannitol (2.04 GBq/mmol) were obtained from Dupont New England Nuclear (Boston, MA).

Caco-2 cell culture Cultures of Caco-2 cells were used between passages 22 and 40. Stock and experimental cultures were maintained as described in Chapter 2 with one exception. Prior to confluency, the medium contained 75 mL/L fetal bovine serum plus 75 mL/L Fetal Clone II bovine serum product and the concentration of Fe in this complete medium was 10 μmol/L. Once monolayers on plastic or inserts reached confluency, the quantities of both fetal bovine serum and Fetal Clone II in complete medium were decreased from 7.5 to 5% (concentration of Fe was 7 μmol/L). This modification was initiated to reduce cost associated with fetal bovine serum. The levels of all other supplements to DMEM were the

same in pre-and post-confluent cultures. It should also be noted that the activities of sucrase and alkaline phosphatase, two brush border enzymes used as markers to assess degree of differentiation (Hidalgo et al. 1989; Pinto et al. 1983), were similar for monolayers attached to plastic and inserts at this time.

Iron uptake and transport Cellular uptake of ^{55}Fe and transfer of the metal from the apical chamber to the basolateral chamber (transport) were determined as described in Chapter II.

Zinc uptake Details related to measuring apical uptake of Zn by differentiated cultures of Caco-2 are given in Chapter II.

^{14}C-mannitol transport The sugar alcohol mannitol is primarily absorbed by the paracellular route. Therefore, the rate of ^{14}C-mannitol transport from the apical to the basolateral compartment was used to evaluate the integrity of tight junctions between cells. The effect of addition of ascorbic acid to uptake buffer containing 10 μmol/L Fe(III) NTA complex on the rate of transfer of ^{14}C-mannitol from the apical to the basolateral compartment by 10 day post confluent cultures of Caco-2 was assessed in an identical

manner to that described above for ^{55}Fe transport.

Determination of Fe(II) The concentration of Fe(II) in uptake buffer was measured spectrophotometrically by monitoring the formation of Fe(II)-ferrozine (FZ) (Stookey 1970) and Fe(II)-bathophenanthroline disulphonate (BPS) (Trinder 1956) complexes at 562 nm and 535 nm, respectively. Chelators were added to a final concentration of 100 μmol/L.

Reductase assays Ferrireductase activity of 10-12-d postconfluent monolayers grown on cluster dishes and inserts was assessed by exposing cells to uptake buffer containing 100 μmol/L FZ and various concentrations of Fe(III) ion (0-200 μmol/L) for 1 hour at 37°C. Preliminary studies showed that the rate of formation of the FZ-Fe(II) complex increased linearly during a 1 h incubation ($y=0.0119X + 0.0047$; $R=0.973$). Uptake buffer was collected and possible cellular debris was removed by centrifugation at 500xg for 10 min at 20°C. Absorbance (562nm) readings were compared to known concentrations of Fe(II)-FZ complex. Standards were prepared by adding 500 μmol/L ascorbate to uptake buffer containing 100 μmol/L Fe(III) and 100 μmol/L FZ and incubating at 37°C for 30 min. Residual ascorbate was oxidized by addition of 1,000 U/L ascorbate oxidase (E.C.1.10.3.3) prior to spectrophotometric analysis.

Absorbance values for specific concentrations of Fe(II)-FZ closely agree (> 95%) with those calculated using the extinction coefficient of 27.9 liters $mmol^{-1} \cdot cm^{-1}$ (Stookey 1970). Cells accumulated minimal levels of ^{55}Fe-FZ (< 30 pmol per mg protein) when 10 μmol/L of the radiolabeled complex was incubated with cultures for 1 h at 37°C. Also, a parallel series of tests were performed using culture dishes without cells to determine the degree of non-specific reduction of Fe(III)-NTA in the presence of FZ. This non-specific activity was very low and was subtracted from that obtained with the same concentration of Fe in cultures with cells to calculate ferrireductase activity at the apical surface.

Dehydroascorbate reductase activity was estimated by immediately determining the level of ascorbate present in uptake buffer containing 1 mmol/L dehydroascorbate after 1 hr incubation with monolayers at 37°C. Ascorbate concentrations were measured spectrophotometrically by determining the reduction of α,α-diphenyl-β-picrylhydrazyl radical at 517 nm as described by Blois (1958). Aliquots of freshly prepared and "conditioned" uptake buffer (i.e., incubated with cells for 1 h at 37°C), freshly prepared uptake buffer with 1 mmol/L dehydroascorbate, and uptake buffer with dehydroascorbate incubated for 1 h at 37°C in

cluster dishes without cells did not reduce the stable free radical. Addition of ascorbate to each of these solutions reduced the free radical in a concentration-dependent manner.

Ferrocyanide production The concentration of ferrocyanide in uptake buffer was determined after a 1 h incubation of cells with 500 μmol/L ferricyanide at 37°C as outlined by Avron and Shavit (1963).

Miscellaneous The level of thiobarbituric acid reactive substances (TBARS) in cultures was quantified according to Buege and Aust (1978). To assess the effect of the addition of ascorbic acid and Fe on cellular integrity, release of cytoplasmic lactate dehydrogenase (LDH; E.C.1.1.1.27) into the medium was determined (Glascott et al. 1992). Cellular protein was assayed as described by Nerurkar et al. (1980) using bovine serum albumin as the standard.

Analysis of data All variables were tested in at least triplicate wells for each experiment, and each experiment was repeated two to four times. Data are means ± SE. Uptake data were analyzed by one-way ANOVA (Snedecor and Cochran 1980) using the Statistical Analysis System (SAS Institute, Cary, NC). Values were considered to be significantly

different at $p < 0.05$. Transport rates were calculated using linear regression analysis of data from 1-4 h and were compared using the General Linear Model. Tukey's multiple range test was used to determine differences among means for uptake and transport data (see Chapter II and III).

RESULTS

Ascorbic acid enhances Fe uptake and transport by reducing Fe(III)

Caco-2 cells attached to plastic accumulated approximately 300 pmol Fe per well (equivalent to 31 pmol per cm^2 and 100 pmol per mg protein) during a 1 h incubation in medium containing 10 μmol/L Fe(III)-NTA without ascorbic acid (Figure 4.1). Fe uptake was temperature dependent, since cultures incubated at 5°C for 1 h accumulated only 20% as much Fe as cultures at 37°C.

Ascorbic acid enhanced Fe uptake in a dose dependent manner (Figure 4.1). Cellular uptake of Fe was increased 0.5 ($p < 0.05$)- and 6.2 ($p < 0.001$)-fold when 8 and 500 μmol/L ascorbic acid, respectively, were added to uptake buffer. Similarly, Fe uptake increased 13-fold ($p < 0.001$)when the concentration of ascorbic acid was 1000 μmol/L (data not shown). Ascorbate-enhanced uptake of Fe was completely blocked when cultures were incubated at 5°C (90 ± 1 pmol Fe per well in presence of 1000 μmol/L ascorbic acid). To examine if the increase in cellular acquisition of Fe uptake from uptake buffer containing both ascorbic acid and Fe was associated with Fe(II)-mediated peroxidative damage, the levels of thiobarbituric acid reactive substances (TBARS)

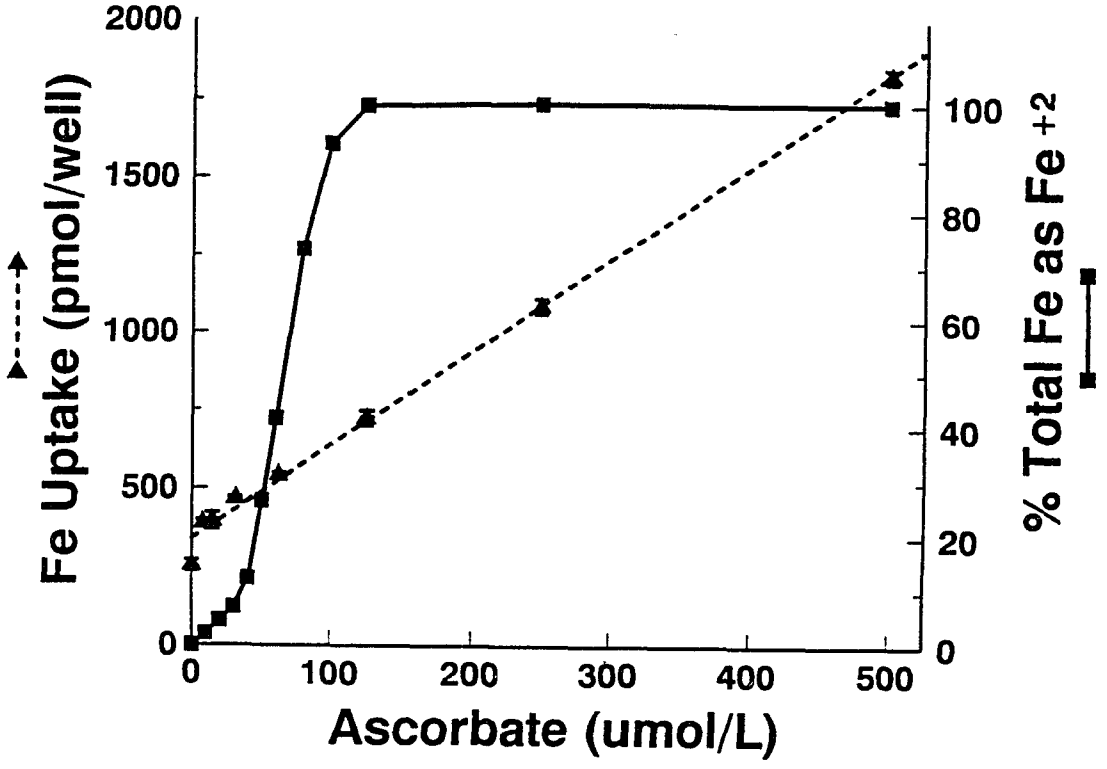


Figure 4.1. Ascorbic acid enhances Fe uptake by Caco-2 cells in a dose-dependent manner.

Post-confluent 10-d cultures of Caco-2 cells attached to the surface of 35 mm multiwell dishes were incubated with uptake buffer (1.2 ml) containing 10 μmol/L ^{55}Fe (37 kBq), 20 μmol/L NTA and 0-500 μmol/L ascorbic acid for 1 h at 37°C. Fe uptake was measured after removing loosely bound ^{55}Fe from the cell surface by multiple washings with ice-cold saline containing 1 mmol/L EDTA, pH 7.0. The percentage of total Fe as Fe(II) in uptake solution containing different concentrations of ascorbic acid was estimated by adding 100 μmol/L ferrozine to aliquots and spectrophotometrically measuring the amount of Fe(II)-ferrozine as described in Materials and Methods. Symbols are means ± SE for a representative experiment with three wells for each test variable. Cellular acquisition of Fe was enhanced ($p < 0.05$) by addition of ≥ 8 μmol/L ascorbic acid.

and medium lactate dehydrogenase (LDH) activity were measured. Neither TBARS nor cellular release of LDH were significantly different ($P > 0.05$) in cultures exposed to 10 μmol/L Fe(III)-NTA in the absence and presence of 1000 μmol/L ascorbic acid (data not shown). Also, the stimulatory influence of ascorbic acid on Fe uptake by Caco-2 cells exhibited specificity, since Zn uptake was similar for cultures incubated in the absence or presence of 1000 μmol/L ascorbic acid (1755 ± 74 vs. 1697 ± 24 pmol Zn per well; $p > 0.05$).

To examine if the increased uptake of Fe from solutions with ascorbic acid was associated with a change in the ratio of Fe(III) to Fe(II), a high affinity chelator of Fe(II), viz., ferrozine (FZ), was added to uptake solutions containing different concentrations of ascorbic acid. Maximal levels of FZ-Fe(II) were present in solutions containing ≥ 100 μmol/L ascorbic acid (Figure 4.1). Since FZ traps and stabilizes Fe(II), its rapid oxidation to Fe(III) in the presence of oxygen at neutral pH is blocked. Our observation suggests that concentrations of ascorbic acid in excess of 100 μmol/L further enhance the uptake of Fe by Caco-2 cells by increasing the probability that Fe is in its reduced state at any time.

The basal rate of Fe transport across the Caco-2 monolayer was 1.5 pmol/(hr•cm^2) from 1-4 hr (see insert, Figure 4.2). Consistent with its stimulatory effect on Fe uptake by Caco-2 cells, ascorbic acid increased the rate of Fe transport in a dose dependent manner (Figure 4.2). Concentrations of 100 and 1000μmol/L ascorbic acid enhanced the rate of Fe transport from the apical to the basolateral compartment 5.6- and 30-fold, respectively, above that in control cultures. In contrast, the rate of ^{14}C-mannitol transport from the apical to the basolateral compartment was not altered significantly ($p > 0.05$) by the addition of 1000 μmol/L ascorbic acid to uptake buffer containing 10 μmol Fe(III)-NTA (0.061 ± 0.001 vs. 0.062 ± 0.001 %/(h•cm^2) in the absence and presence of ascorbic acid, respectively; Figure 4.3). This suggests that ascorbic acid stimulates Fe transport by a transepithelial, rather than a paracellular, route.

Preincubation of uptake buffer containing either 1000 μmol/L ascorbic acid or its synthetic analog isoascorbic acid with ascorbate oxidase (1 U/mL) oxidized greater than 99% of the substrates and eliminated more than 95% of the stimulatory influence of these compounds on Fe uptake (Figure 4.4). Addition of ascorbate oxidase did not decrease Fe solubility in uptake buffer (i.e., solubility >98%).

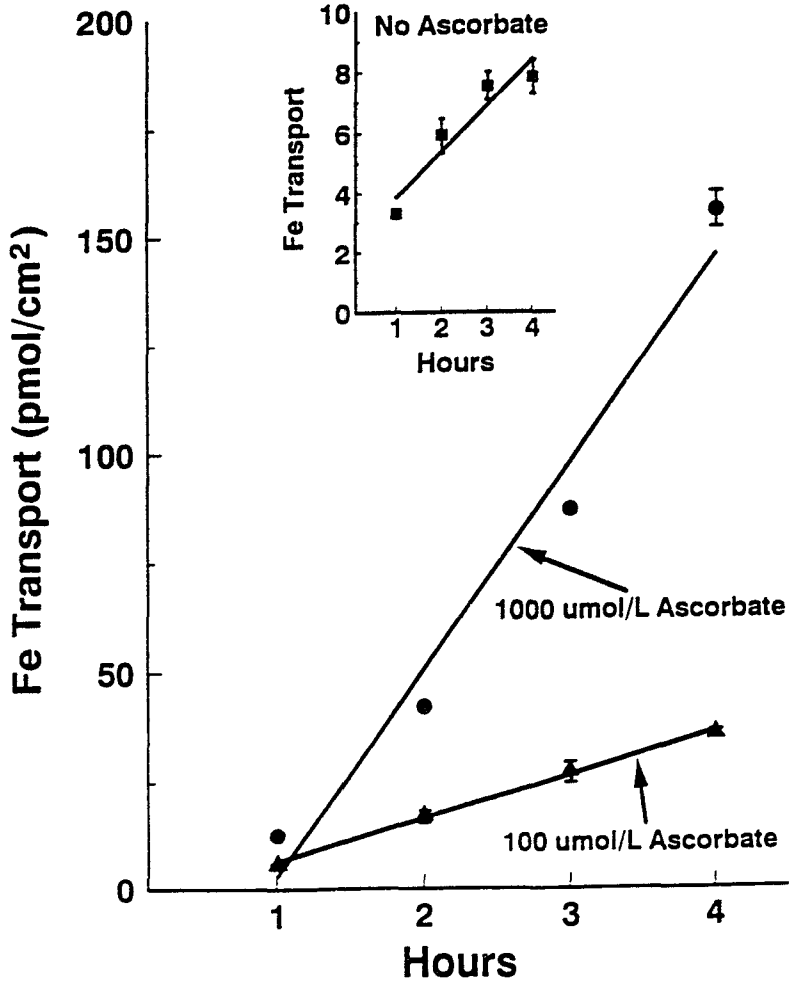


Figure 4.2. Ascorbic acid stimulates Fe transport across Caco-2 monolayer.

Uptake buffer (1.5 mL) containing 10 μmol/L ^{55}Fe and 20 μmol/L NTA and either 0, 100, or 1000 μmol/L ascorbic acid was added to the apical compartment of 12-d post-confluent cultures of Caco-2 grown on microporous inserts. Cultures were incubated at 37°C and the quantity of ^{55}Fe transferred into the basolateral compartment containing DMEM with 5% FCS was monitored hourly for 4 h. Symbols are means ± SE for at least 3 wells from a representative experiment. Plotted lines represent the computer derived line of best fit and the quantity of Fe transported across the monolayer at all times was increased ($p < 0.05$) by ascorbic acid in a concentration-dependent manner.

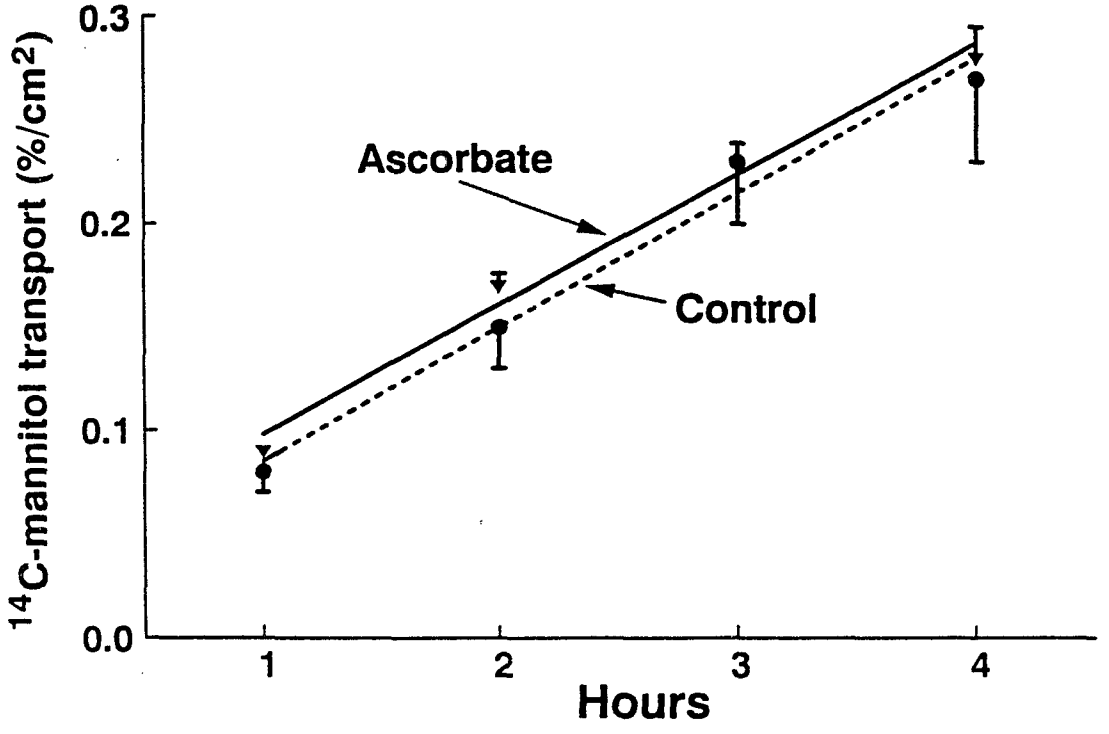


Figure 4.3. Paracellular flux across the Caco-2 monolayer is not altered by ascorbic acid.

Uptake buffer containing 37 kBq D-(1-^{14}C) mannitol, 10 μmol/L Fe(III)-NTA and either 0 or 1000 μmol/L ascorbic acid was added to the apical compartment of 10-12-d post-confluent cultures of Caco-2 cells grown on microporous inserts. The rate of ^{14}C-mannitol transport from the apical to the basolateral compartment was determined by using the identical procedure as that for Fe transport. Symbols are mean ± SE for a representative experiment with at least 3 wells for each treatment. The transport rate of ^{14}C-mannitol was not changed by the presence of ascorbic acid ($P > 0.05$).

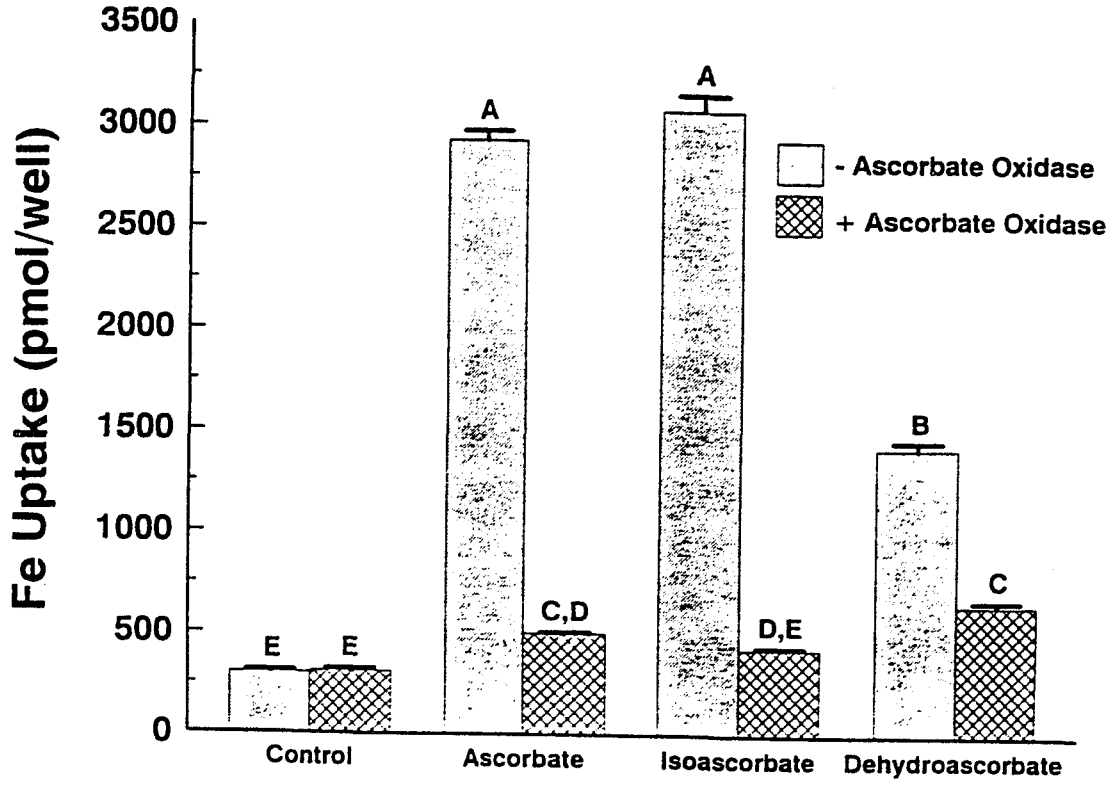


Figure 4.4. Ascorbic acid oxidase blocks the stimulatory effects of ascorbic acid, isoascorbic acid and dehydroascorbic acid on the acquisition of Fe by Caco-2 cells.

Uptake buffer containing ^{55}Fe, NTA and either 1000 μmol/L ascorbic acid, isoascorbic acid or dehydroascorbic acid with or without 1000 U/L ascorbate oxidase was added to wells containing 10-12-d post-confluent cultures of Caco-2. ^{55}Fe uptake was measured as described in the legend to Figure 4.1. Values are means ± SE for at least 3 replicate wells. Different letters above the error bar indicate that values differ significantly ($p < 0.05$).

Unexpectedly, dehydroascorbic acid (1000 μmol/L) also enhanced Fe uptake 3.5-fold. However, this stimulatory influence of dehydroascorbic acid on Fe uptake also was markedly blocked by ascorbate oxidase, suggesting that the cells were capable of reducing dehydroascorbic acid to ascorbic acid. Indeed, 25-30 μmol/L ascorbic acid was detected in medium after incubation of the monolayer for 1 h with uptake buffer that initially contained 1000 μmol/L dehydroascorbic acid, but no ascorbic acid, at 37°C. In contrast, ascorbic acid was not detected in conditioned uptake buffer that did not contain dehydroascorbic acid, indicating that the cells did not secrete either ascorbic acid or substances capable of reducing dehydroascorbic acid. Finally, dehydroascorbic acid was not reduced by cultures incubated at 0°C. These data suggest that Caco-2 cells possess a dehydroascorbate reductase activity.

Cellular acquisition of Fe(II) was inhibited by 97 and 93% when FZ and BPS, respectively, were added to uptake buffer (Table 4.1). Similarly, these chelators effectively blocked cell mediated transfer of Fe(II) from the apical to the basolateral compartment. While ferricyanide did not alter uptake of Fe(II) from ascorbate containing solution (Table 4.1), FZ and ferricyanide decreased ($p < 0.05$) cellular acquisition of Fe(III) from ascorbate-free uptake

Table 4.1. Effects of chelators of Fe(II) and ferricyanide on Fe uptake and transport by Caco-2 cells incubated in medium with and without 1mmol/L ascorbic acid.[1]

Uptake buffer	Uptake	Transport rate
	pmol/well	pmol/(h•cm^2)
Ascorbic acid		
Control	4291 ± 6^a	45.7 ± 2.8^a
plus BPS[2]	310 ± 9^b	0.3 ± 0.1^c
plus FZ[2]	134 ± 1^c	4.8 ± 0.8^b
plus Ferricyanide	4225 ± 32^a	ND[3]
without Ascorbic acid		
Control	300 ± 3^a	1.7 ± 0.1
plus FZ	244 ± 10^b	ND
plus Ferricyanide	214 ± 3^c	ND

[1]Data are means ± SE for at least 3 replicate wells from a representative experiment. Significant differences ($p < 0.05$) within sets of cultures incubated in the present or absence of ascorbic acid are indicated by difference letter as superscripts within a column.

[2]BPS: bathophenanthroline disulfonate; FZ: ferrozine

[3]ND: not determined

buffer.

Fe uptake requires reduction of Fe(III) and transport of Fe(II) Basal Fe uptake in the absence of ascorbic acid was investigated next. We reasoned that Caco-2 cells were capable of either transporting both Fe(III) and Fe(II) ion across the brush border membrane or reducing Fe(III) to Fe(II) prior to uptake. Generation of Fe(II) was monitored by adding either FZ or BPS to uptake buffer containing various concentrations of Fe(III)-$(NTA)_2$. The levels of Fe(II)-FZ (Figure 4.5) and Fe(II)-BPS (data not shown) increased in a curvilinear manner with increasing concentrations of Fe(III)-$(NTA)_2$ in uptake buffer after a 1 h incubation with Caco-2 cells. Cellular activity was required for the reduction, since the complexes were not formed in cultures incubated at 0°C or during incubation at 37°C in wells without cells. Also, conditioned medium had slight, if any, ability to reduce Fe(III). Maximal ferrireductase activity was observed when the concentration of Fe(III) in uptake buffer was ≥ 100 μmol/L (Figure 4.5). Analysis of these data using Lineweaver-Burk double reciprocal plots indicates that the apparent Km and Vmax for ferrireductase activity were 38.5 ± 3.0 μmol/L and 900 ± 2 pmol/(h•mg protein), respectively. The ferrireductase activity appears to be present on the trans face of the

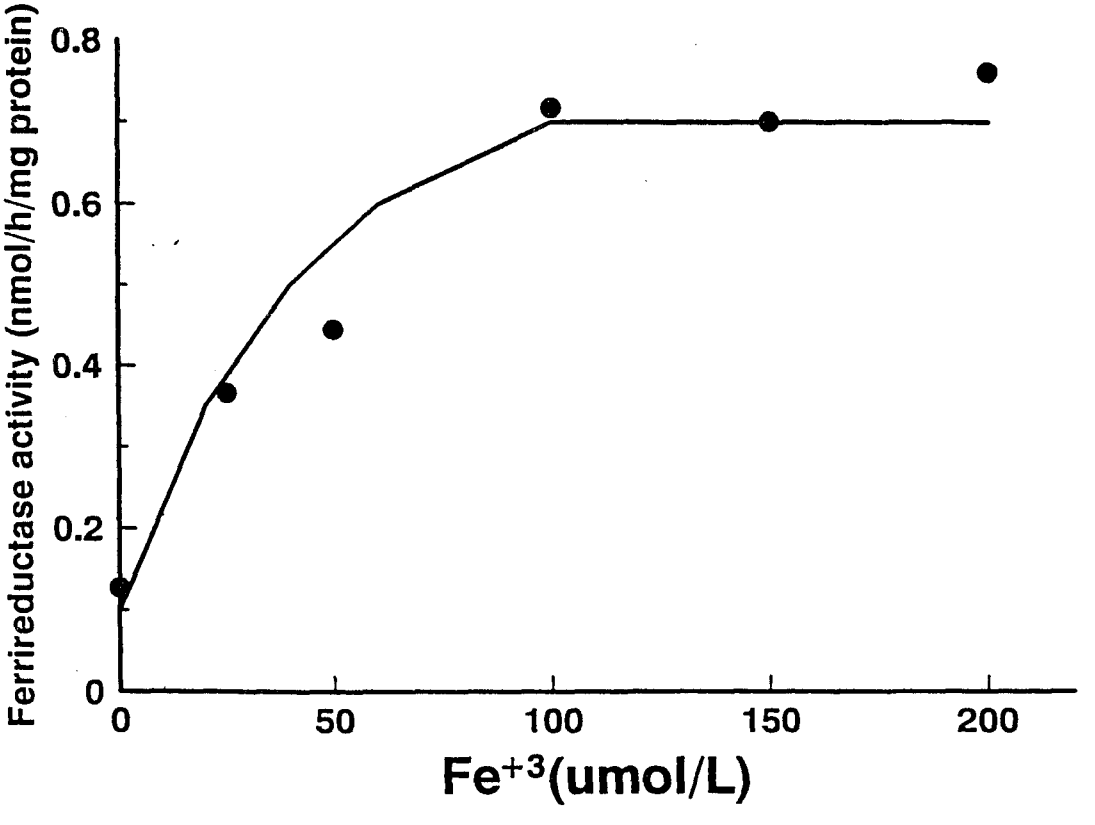


Figure 4.5. Ferrireductase activity of intact Caco-2 cells.

Uptake buffer (1.2 mL) containing 0-200 μmol/L Fe(III) (as 1:2 complex with NTA) and 100 μmol/L ferrozine (FZ) was added to wells containing 12-d post-confluent cultures of Caco-2. Cultures were incubated for 1 h at 37°C. Uptake buffer was collected and centrifuged at 500xg for 10 min at 20°C to remove debris. Cell-mediated reduction of Fe(III) was assessed by measuring the concentration of the Fe(II) -FZ complex as described in Materials and Methods. Test solution incubated in dishes without cells and conditioned uptake buffer to which Fe(III) and FZ were added did not produce Fe(II)-FZ (data not shown). Symbols are mean values for at least 3 wells from a representative experiment. Standard errors for all values are within 5% of the mean.

brush border membrane, since the impermeable electron acceptor, ferricyanide, also was reduced by Caco-2 cells (data not shown). Finally, interpolation of data presented in Figure 4.5 suggests that the ferrireductase activity accounts for 70-80% of basal Fe uptake, i.e., the amount of Fe taken up by Caco-2 cells from uptake buffer containing 10 μmol/L Fe(III)-NTA but no ascorbic acid.

Specificity of Fe(II) uptake process The effects of selected divalent cations on the uptake and transport of Fe(II) were examined to assess the specificity of these processes (Table 4.2). Twenty-fold excesses of Co^{2+} and Mn^{2+} inhibited uptake of Fe(II) by 55-60%, whereas Zn only decreased Fe(II) uptake by 21%. In addition, these three transition metals significantly ($p < 0.05$) reduced the rate of transport of Fe across the cell monolayer in the order of Co > Zn > Mn. Decreased uptake of Fe(II) was not due to reduced solubility in solutions with these metals. Also, cell viability (i.e., trypan blue exclusion) was not decreased by the presence of 200 μmol/L concentrations of these multivalent cations during the test period, suggesting that the observed impairment of Fe uptake and transport did not reflect a general loss of cell integrity.

Table 4.2. Impact of selected divalent cations on uptake and transport of Fe(II).[1,2]

Apical solutio	Uptake	Transport rate
	pmol/well	pmol/(h•cm^2)
Control	3752 ± 27^a	65 ± 3.0^a
+ 200 μmol/L Co^{2+}	1640 ± 15^c	19 ± 0.6^c
+ 200 μmol/L Mn^{2+}	1585 ± 12^c	39 ± 0.7^b
+ 200 μmol/L Zn^{2+}	2952 ± 10^b	33 ± 1.4^b

[1] Data are means ± SE for at least 3 wells from a representative experiment. Significant differences ($p < 0.05$) between groups are indicated by the presence of different letters as superscripts within a column.

[2] When Fe was introduced as Fe(III) without ascorbic acid, the uptake and rate of transport were 294 ± 7 pmol/well and 2.0 ± 0.2 pmol/(h·cm^2), respectively.

DISCUSSION

Heme Fe and non-heme Fe represent two distinct forms of Fe in foods. The absorption of heme Fe is highly efficient and relatively independent of other components in a meal (Conrad 1987). In contrast, the efficiency of non-heme Fe absorption is low and markedly influenced by the composition of the meal. It is well established that ascorbic acid and animal protein are potent enhancers of non-heme Fe absorption in several species, including humans (Hallberg 1981; Lynch and Cook 1980). Similarly, the uptake of non-heme Fe by intestinal brush border membrane vesicles (Muir et al. 1984) and human intestinal cells (Alvarez-Hernandez et al. 1991) is enhanced by ascorbic acid. In this study, we have found that ascorbic acid increases Fe uptake by differentiated cultures of Caco-2 human intestinal cells in a dose-dependent manner. Of particular interest was the observation that Fe uptake was increased significantly when the concentration of ascorbic acid was as low as 8 μmol/L. This suggests that low concentrations of ascorbic acid that enter the lumen of the small intestine from biliary and pancreatic secretions (Conrad and Shade 1968) and, perhaps, secreted across the epithelial cell surface may increase the bioavailability of non-heme Fe in foods. The presence of ascorbic acid in the apical solution also increased the rate

of transport of Fe, but not ^{14}C-mannitol, across the cell monolayer. This suggests that the additional complement of Fe transported from the apical to the basolateral compartment in the presence of ascorbic acid represents increased transepithelial flux of the trace metal. These observations concerning the stimulatory effect of ascorbic acid on Fe uptake and transport support our recent suggestion that Caco-2 cells provide a useful model for investigating the influences of dietary and luminal factors on mineral bioavailability (Han et al. 1994).

The stimulatory influence of ascorbic acid on Fe absorption has been attributed to its ability both to chelate Fe(III) ion thereby preventing the formation of insoluble polynuclear complexes at the slightly alkaline pH of the intestine and to reduce the Fe(III) to the more soluble Fe(II) ion (Dorey et al. 1993; Gorman and Clydesdale 1983). Our results indicate that ascorbate-mediated reduction of Fe(III) is primarily responsible for the increased uptake of the trace metal across the brush border surface of Caco-2 cells. This conclusion is based on the following observations. First, Fe(III) introduced into uptake solution as either the NTA (Han et al. 1994 and this study) or the citrate complex (data not shown) remains soluble for at least 6 hours in the absence of ascorbic

acid. Second, it is unlikely that Fe(III) was complexed to ascorbic acid in the uptake buffer since the stability constant for Fe(III)-NTA (log Keq = 16.3; Sillen and Martell 1971) is much higher than for Fe(III)-ascorbate (log Keq = 3.2-4.2; Gorman and Clydesdale 1983). Third, enzymatic oxidation of ascorbic acid and isoascorbic acid eliminates virtually all of their stimulatory influence on Fe uptake, although the primary oxidation product dehydroascorbic acid and metabolites that result from its spontaneous cleavage at neutral pH (e.g., diketogulonic acid) possess Fe binding ligands. Fourth, while addition of dehydroascorbic acid to uptake buffer also enhanced Fe uptake, this was associated with the reduction of the dehydroascorbic acid to ascorbic acid by Caco-2 cells. This reduction was not associated with a secretory factor, but required the presence of Caco-2 cells. Therefore, dehydroascorbic acid was reduced either at the cell surface or within the cell. Na-independent transport of dehydroascorbic acid across brush border membrane vesicles from guinea pig ileum has been reported (Bianchi et al. 1986). Likewise, NADPH-dependent, dehydroascorbic acid reductase activity has been identified in the soluble fraction of rat colonic mucosa (Choi and Rose 1989). Since ascorbic acid was present in uptake buffer after exposure to solution containing dehydroascorbic acid, it seems likely that if ascorbic acid is regenerated within

Caco-2 cells at least some must be transported back across the brush border membranes. Efflux of ascorbic acid from intact erythrocytes has been demonstrated (Orringer and Roer 1979). Additional studies are required to critically examine the mechanism and functional significance of ascorbic acid recycling in Caco-2 cells and within the human intestine.

Since the stimulatory influence of ascorbic acid on Fe transport appears to be due primarily to reduction rather than chelation of Fe(III), this suggests that Fe(II) is the species efficiently transported across the brush border membrane. The hypothesis is supported by recent observations with yeast (Anderson et al. 1992; Eide et al. 1992), rodent intestine (Barrand et al. 1990; Raja et al. 1992; Wollenberg and Rummel 1987) and the IEC-6 rat intestinal cell line (Nichols et al. 1992). In these models high affinity chelators of Fe(II) blocked Fe uptake. Likewise, FZ and BPS inhibited ascorbate-enhanced acquisition of Fe by Caco-2 cells.

The observation that Caco-2 cells take up a basal level of Fe in the absence of ascorbic acid or in the presence of Fe(II) chelators suggests that there may be separate pathways for the uptake of Fe(III) and Fe(II) and/or the cell itself reduces Fe(III). As mentioned above, conditioned

medium did not contain a detectable level of substance(s) capable of reducing Fe(III). The ability of ferricyanide, an impermeable electron acceptor, to partially block Fe uptake and the finding that ferrocyanide was being produced suggested the presence of a ferrireductase activity on the cell surface. Analyses of intact cells confirmed the presence of a saturable, temperature-dependent activity capable of reducing Fe(III). Moreover, the kinetic properties of the ferrireductase in Caco-2 cells are similar to those recently reported for mouse small intestine (Raja et al. 1992).

The importance of ferrireductase for cellular acquisition of Fe has been demonstrated in an elegant series of studies with a mutant strain of yeast (FRE 1) with minimal ferric reductase activity (Anderson et al. 1992; Eide et al. 1992). Growth of the mutant in medium with Fe(III) is markedly impaired, but reverts to normal when either the medium contains Fe(II) or the yeast is transformed with the wild type gene. While we cannot completely eliminate the possibility that some Fe(III) is transferred across the brush border surface, our data strongly suggest that non-heme Fe is reduced within the lumen by dietary or endogenous factors such as ascorbic acid and glutathione (Wien and Van Campen 1991) or by

ferrireductase at the brush border surface prior to membrane translocation of Fe(II) (Figure 4.6).

Chelators of Fe(II) were weak inhibitors of Fe uptake in the absence of ascorbic acid. We believe this may be due to competition of the water soluble chelator and the Fe transport protein for Fe(II). If the ferrireductase and Fe transport protein are situated near one another in the membrane, availability of Fe(II) to the chelator may be limited. This also suggests that the kinetic properties defined for ferrireductase are underestimates, because formation of the measured product, viz. Fe(II), is inefficient. Conrad and associates (1992 and 1993) have recently identified an integrin in the brush border membrane that binds the cytoplasmic Fe binding protein mobilferrin. The possibility that the integrin also participates in translocation of Fe(II) across the brush border surface has been raised (Figure 4.6).

The final matter that was addressed concerned the effects of selected divalent cations on the uptake of Fe(II) and the subsequent transport of Fe across the cell monolayer. Co, Mn and Zn were selected because they have been reported to antagonize Fe absorption in humans and animals (e.g., Rossander-Hulten et al. 1991; Thomson et al.

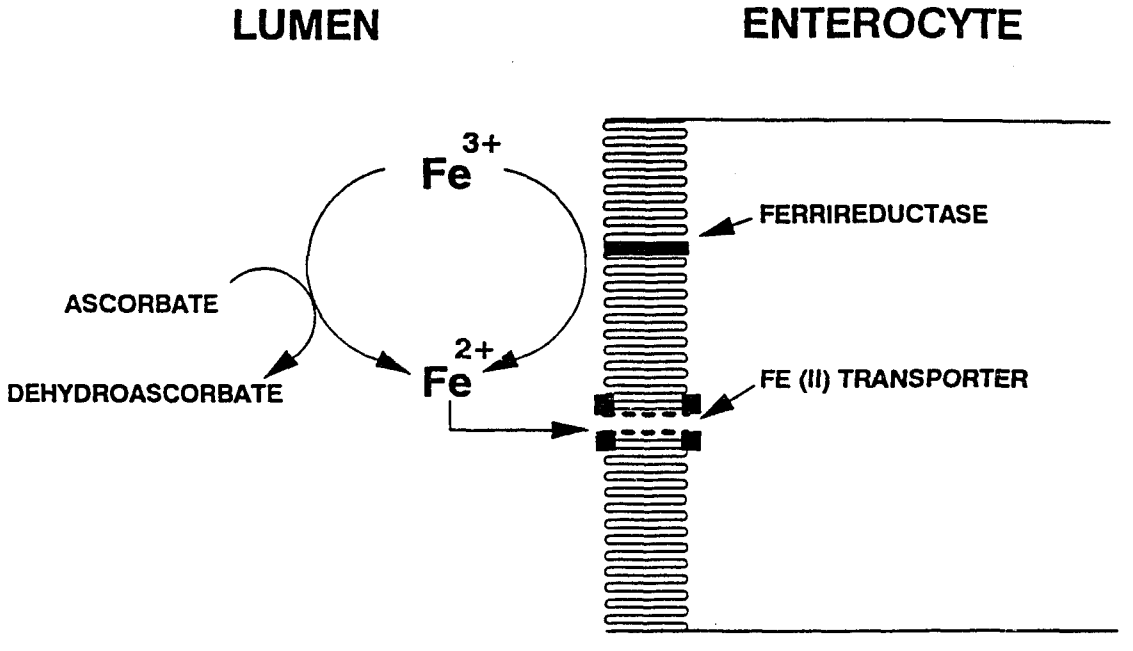


Figure 4.6. Proposed mechanism for uptake of dietary non-heme Fe by Caco-2 cells.

Dietary non-heme Fe must be converted to the ferrous state either by dietary or endogenous reductants, e.g., ascorbic acid, in the lumen or by a ferrireductase activity on the brush border membrane surface prior to transport of this essential trace metal into the cells.

1971). The results demonstrate that molar excesses of each of these divalent metals decreased the efficiency of Fe uptake and transport. Since neither the solubility of Fe(II) nor the general integrity of the cell were altered by the relatively high levels of the divalent cations, they likely compete with Fe(II) for transfer across the brush border and, perhaps, intracellular shuttling to the basolateral surface. The ability of Co and Zn to compete with Fe for binding to mobilferrin is of particular interest in this regard (Conrad et al. 1990 and 1992). The Caco-2 cell model is well suited to more detailed investigation of the biochemical basis for the reported interactions among dietary multivalent cations.

CHAPTER V

MONOKINES ATTENUATE APICAL TO BASOLATERAL FLUX OF FE IN CACO-2 CELL CULTURES

ABSTRACT

Specific physiological factors that regulate the efficiency of Fe absorption remain unknown. Because both Fe deficiency and infections of the gastrointestinal tract are prevalent in developing countries, I speculated that chronic activation of the gut immune system may decrease the absorption of dietary Fe. To test this hypothesis, the monokines interleukin (IL)-1 (25 ng/mL), IL-6 (25 ng/mL) and tumor necrosis factor (25 ng/mL) were added to the basolateral (BL) chamber of Caco-2 cells anchored on membrane inserts. Monokine treatment (24h) increased cellular accumulation of ^{59}Fe from ^{59}diferric-transferrin (Tf). Also, the quantity of cellular ferritin protein was significantly higher in monokine-treated cells than in control cultures (2.1 ± 0.1 vs 1.6 ± 0.2 ng/mg protein, respectively). To evaluate the effect of these changes on the rate of transfer of Fe from the apical (AP) to the basolateral (BL) compartment, monokines and diferric-Tf (10

μM) were added to the BL chamber for 24h. Then, ^{59}Fe-NTA complex (10 μM) was added to the AP compartment. The rate of ^{59}Fe transport from the AP to the BL compartment was 30% lower ($P < 0.05$) in monokine treated cultures than in controls cultures (12.6 $\pm$ 0.6 vs. 17.9 $\pm$ 1.0 pmol/mg.h, respectively). This decrease in Fe transport was inversely related to the increase of cellular ferritin and Fe. Monokines also delayed the reduction of cellular ferritin content after removal of extracellular Fe. These data suggest that increased levels of monokines due to chronic stimulation of the gut immune system may decrease the efficiency of dietary Fe absorption, thereby increasing the risk of Fe deficiency.

INTRODUCTION

Homeostatic control of dietary iron (Fe) in mammals is regulated largely within the proximal intestine. Usually, the absorption of dietary Fe is inversely related to the level of whole body Fe stores. The characteristics of Fe transport at the basolateral surface have not been defined. While cellular to plasma transfer of the metal is evident, the possibility that enterocytes also take up Fe from plasma has not been critically examined. Several observations support this possibility. First, it is well established that Fe absorption is blocked in response to Fe overload due to transfusions (Conrad 1987). Second, several recent studies have identified the transferrin receptor (TfR) on the basolateral membrane of intestinal epithelial cells (Anderson et al. 1990; Banerjee et al. 1986). Delivery of plasma Fe to cells is normally mediated by TfR pathway (Klausner et al. 1983; Rhyner et al. 1985; Sipe and Murphy 1991) and the concentration of TfR on the cell surface is inversely related to cellular Fe status (Anderson et al. 1990; Muller-Eberhard et al. 1988). It is unknown if the intestinal TfR participate in endogenous Fe uptake and/or are required for the transfer of dietary Fe from the enterocyte to the plasma.

Fe deficiency is a common problem in developing countries. Because chronic infections of the gastrointestinal tract are also prevalent in these countries, the possibility that the infectious state impairs the absorption of Fe and, perhaps, other nutrients should be considered. It has been estimated that 40-50% of the immune system is located in the gut. The lamina propria region located beneath the intestinal epithelium in villi contains numerous immune cells including B- and T-lymphocytes, macrophages, mast cells and eosinophils (Kagnoff 1987). Infection of the GI tract increases the activities of these cells, and especially phagocytic cells, causing the secretion of numerous cytokines and other mediators of inflammation (Castro and Arntzen 1993; Cooke et al. 1994; Kagnoff 1987; Perdue and McKay 1994).

Systemic inflammation and infection are characterized by a rapid decrease in the level of plasma Fe and an increase in the level of ferritin in liver (Beisel 1991). Numerous studies have shown that many of the changes in metabolism of nutrients by the infected host are mediated by the monokines (interleukin (IL)-1, IL-6 and tumor necrosis factor) which are synthesized primarily by monocytes and macrophages (Beisel 1991). Furthermore, several in vitro studies have demonstrated that monokines can modulate the synthesis of proteins involved in Fe transport and

metabolism in hepatocytes and macrophages (Hirayama et al. 1993; Rogers et al.1990; Tsuji et al 1991).

In vivo and in vitro studies have shown that the cytokine receptors are present on the basolateral surface of intestinal epithelial cells (Ciacci et al. 1993; McGee et al. 1993; Gustafson-Svard et al. 1993; Madara and Stafford 1989; Perdue and McKay 1994; Varilek et al. 1993). Caco-2 and several other intestinal cell lines also express such receptors (e.g., Andoh et al. 1993; Molmenti et al. 1993; Varilek et al. 1993). Numerous studies during the past decade have focused on the powerful influences of locally produced cytokines on the gut functions including water and electrolyte secretion and motility of smooth muscle in the intestine and colon (Castro and Arntzen 1993; Kagnoff 1987; Perdue and McKay 1994). In contrast, we are only aware of a single report that has considered the impact of cytokines on gut utilization of an essential nutrient, viz., the inhibitory influence of monokines on the transport and metabolism of glutamine by Caco-2 cells (Souba and Copeland 1992). Therefore, it seemed both reasonable and timely to examine the possibility that monokines influenced the characteristics of Fe transport and metabolism by Caco-2 cells. The initial data discussed in this chapter support the hypothesis that chronic elevation of the concentrations of monokines in the basolateral region decreases the ability

of the intestinal epithelial cell to absorb dietary Fe.

MATERIALS AND METHODS

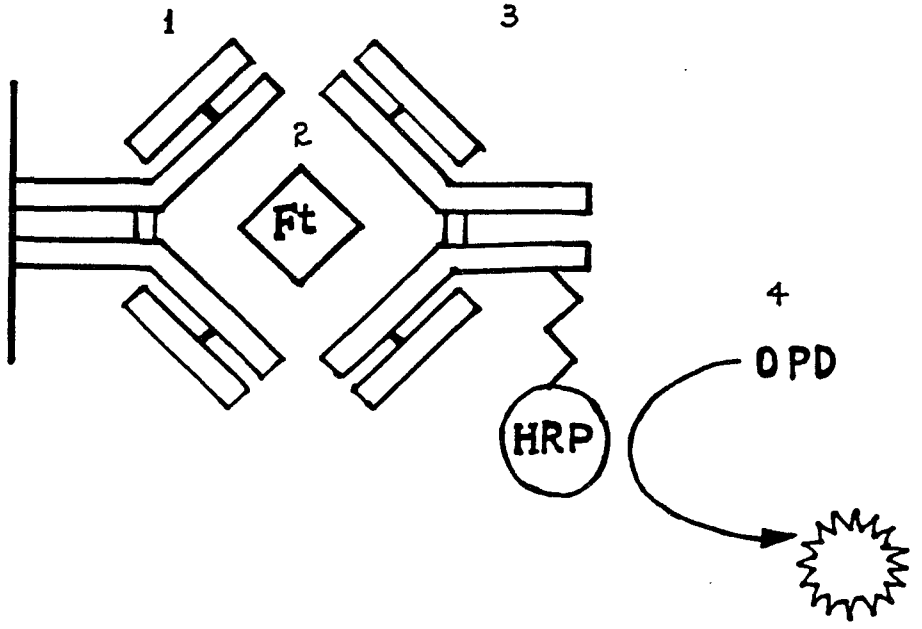
Reagents Reagents used for cell cultures were same as described in Chapter II. ^{59}Fe (132 GBq/mmol $FeCl_3$) and D-[1-^{14}C] mannitol (2.04 GBq/mmol) were obtained from Dupont New England Nuclear (Boston, MA). Human apotransferrin (Tf) and recombinant human interleukin-6 were purchased from Boehringer Mannheim (Indianapolis, IN). Recombinant human IL-1ß was purchased from Genzyme (Cambridge, MA). Recombinant human TNF-α was a generous gift from Dr. Phil Ekala, East Carolina School of Medicine. Human liver ferritin, polyclonal rabbit anti-human ferritin and horse radish peroxidase-labeled rabbit anti-human ferritin were purchased from Dako Corp.(Santa Barbara, CA).

Cells Caco-2 human cells were used between passages 25 and 40. Conditions for the growth and maintenance of the stock and experimental cultures were described in Chapter II. For all the experiments reported in this chapter, monolayers were grown and maintained on membrane inserts (3 μmeter pores). Cultures were used to study Fe uptake and transport 10-15d after reaching confluency. Cells were fully differentiated as evidenced by maximal activities of sucrase and alkaline phosphatase (Pinto et al. 1983) and a minimal rate (0.06%/h.cm^2) of paracellular flux of ^{14}C-mannitol from

the apical to the basolateral chamber (Chapter IV).

Preparation of $^{59}Fe_2$-Tf Apo-Tf (10 mg) was dissolved in 1.0 mL buffer A (150 mmol/L NaCl, 25 mmol/L Hepes, 5 mmol/L $NaHCO_3$, pH 7.4). $^{59}FeCl_3$ (NTA) complex (final Fe concentration was 282 μmol/L with 80 μCi ^{59}Fe and 1128 μmol/L NTA) was added and incubated at 37°C for 30 min. To separate residual ^{59}Fe-NTA complex from ^{59}Fe-Tf, the solution was then applied to a column (2.5 x 60 cm) containing Sephadex G-25 that had been previously equilibrated with buffer A. Sample was eluted with buffer A at a rate 30 mL/hr and aliquots of 1.3 mL were collected. Fractions containing void volume were pooled and the solution was analyzed spectrophotometrically. The ratio of A_{465nm}/A_{280nm} was 0.046 for these preparations indicating complete saturation of the two high affinity binding sites (Klausner et al. 1983) and is referred to as diferric-Tf or Fe_2-Tf.

Quantification of cellular ferritin Cellular ferritin was quantified by a "sandwich" ELISA assay (Figure 5.1). Wells of 96-well flat bottomed microtiter plates were coated by addition of 100 μL of 1% (w/v) polyclonal rabbit anti-human ferritin (IgG fraction) in 0.01 M phosphate buffer (pH 7.2) and incubated overnight at 4°C. After washing the surface of the wells extensively with buffer B (10 mM phosphate with



1. Polyclonal antibody for human ferritin (Ft).
2. Antigen human ferritin or samples.
3. Horseradish Peroxidase (HRP) conjugated polyclonal antibody for human ferritin.
4. Color development by oxidation of O-phenylenediamine dihydrochloride (OPD) by HRP.

Figure 5.1. Antibody sandwich ELISA for human ferritin.

0.1% Tween 20) to remove unbounded materials, the diluted test samples and standards (100 μL) were added to wells. After 2 h at room temperature, wells were again washed 5 times with 100 μL buffer B. Next, 100 μL buffer B containing 130 μg/L horseradish peroxidase (HRP)-labeled rabbit anti-human ferritin (IGg fraction) was added to wells and the plate was incubated for 1 h at room temperature. Reacting was maintained by adding 100 μL 1,2-phenylenediamine dihydrochloride (0.7 mg/mL) as substrate. HRP-dependent product formation was quantified by monitoring A_{492} (TECAN Model ATCC 340 plate reader) after quenching the reaction with 150 μL 1M H_2SO_4. Highly purified human liver ferritin was used as a standard (0-10 ng/mL; 100 μL/well). The limit of detection in this assay was 200 pg human ferritin.

<u>^{59}Fe uptake from $^{59}Fe_2$-Tf</u> Spent medium was removed from the apical (AP) and basolateral (BL) chambers of differentiated cultures and monolayers were washed 3x with DMEM. Fresh DMEM (1.5 mL) containing 0.2% BSA and indicated concentrations of ^{59}Fe-Tf (0.4-1.5 μmol/L) was added to BL chamber. The apical chamber contained 1.0 mL fresh DMEM only. After incubation for 2 h, medium was removed from both chambers and the cell monolayer was washed 3x with ice-cold buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Hepes, pH 7.0). Cells were collected and sonicated as described in Chapter II. ^{59}Fe was

measured in aliquots by gamma ray spectrometry.

Cellular uptake and transport of Fe Methods to assess cellular uptake and transport of ^{59}Fe from the apical to the basolateral chambers were similar to those described in Chapter II. To investigate the effects of monokines on Fe transport and metabolism, IL-1, IL-6 and TNF were added separately or as indicated combinations to complete DMEM containing 0.2% BSA instead of 2% FCS. These solutions (2.5 mL) were added to the BL compartment of differentiated cultures approximately for 24 h before initiating experiments.

Analysis of data Presented data (means ± SE) represent results from at least two separate experiments using three wells for each parameter. Data were analyzed by the General Linear Model or Student's t test as described in previous chapters. Tukey's multiple range test was used to determine significant differences among means ($p < 0.05$) for Fe uptake.

RESULTS

Basolateral uptake of Fe from Fe_2-Tf To examine whether differentiated cultures of Caco-2 have the ability to take up Fe from Fe_2-Tf, $^{59}Fe_2$-Tf (0.45 μmol/L) was added to BL compartment. Caco-2 accumulated 5-7 pmol Fe/mg protein after 2 h incubation at 37°C. Cellular accumulation of Fe from Fe_2-Tf increased linearly ($p < 0.05$) as duration of incubation was extended from 1 to 5 h (Figure 5.2). Also, Fe uptake from Fe_2-Tf was proportional to the concentration of Fe_2-Tf in the BL compartment (Figure 5.3) within the physiological range (plasma level of Tf approx. 10 μmol/L). However, Fe uptake from Fe_2-Tf was significantly decreased when cultures of Caco-2 were incubated at 0°C, indicating that the process was energy dependent (Figure 5.2). Fe uptake from Fe_2-Tf by Caco-2 cells at 37°C was saturable, since ^{59}Fe uptake was blocked (<1% of control level) when 100 μmol/L non-radiolabeled Fe_2-Tf was added to medium containing 0.45 μM $^{59}Fe_2$-Tf. Lesser differentiated cultures of Caco-2 (4d post-confluency) accumulated 2.5-4 fold more ^{59}Fe than fully differentiated (12d post-confluency) cultures. Together, these data suggest that Fe uptake from Fe_2-Tf is mediated by the transferrin receptor (TfR) pathway in Caco-2 cells.

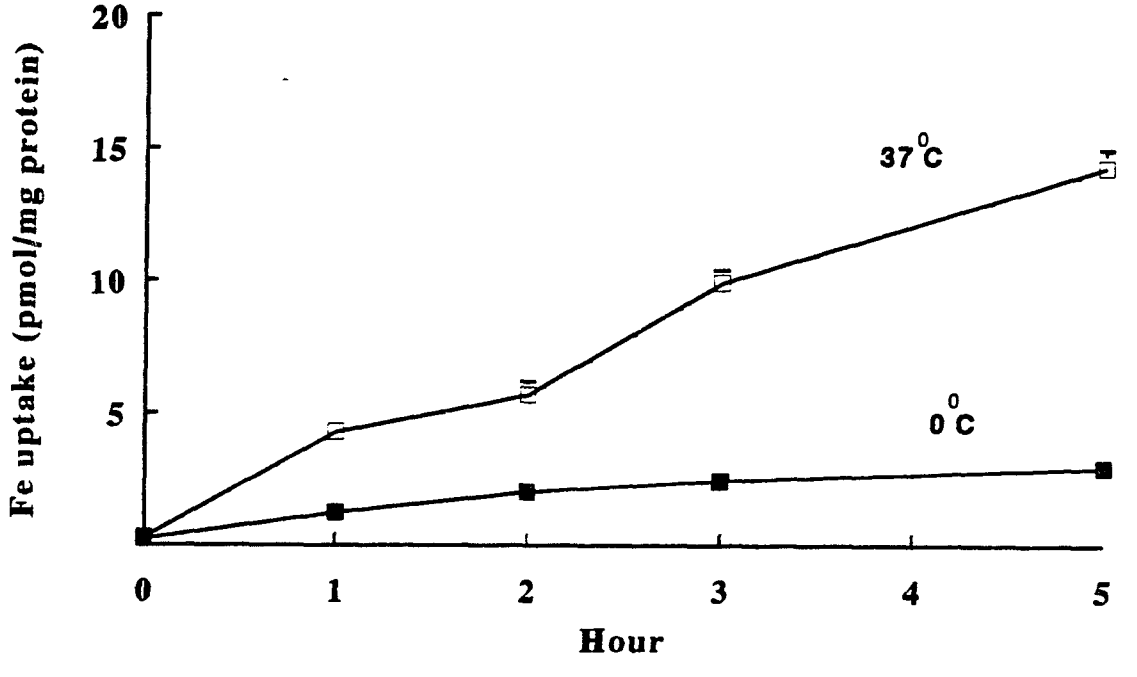


Figure 5.2. Fe uptake from Fe_2-Tf by Caco-2 cells is dependent on temperature and length of incubation.

Values are means ± SE for 3 wells from a representative experiment.

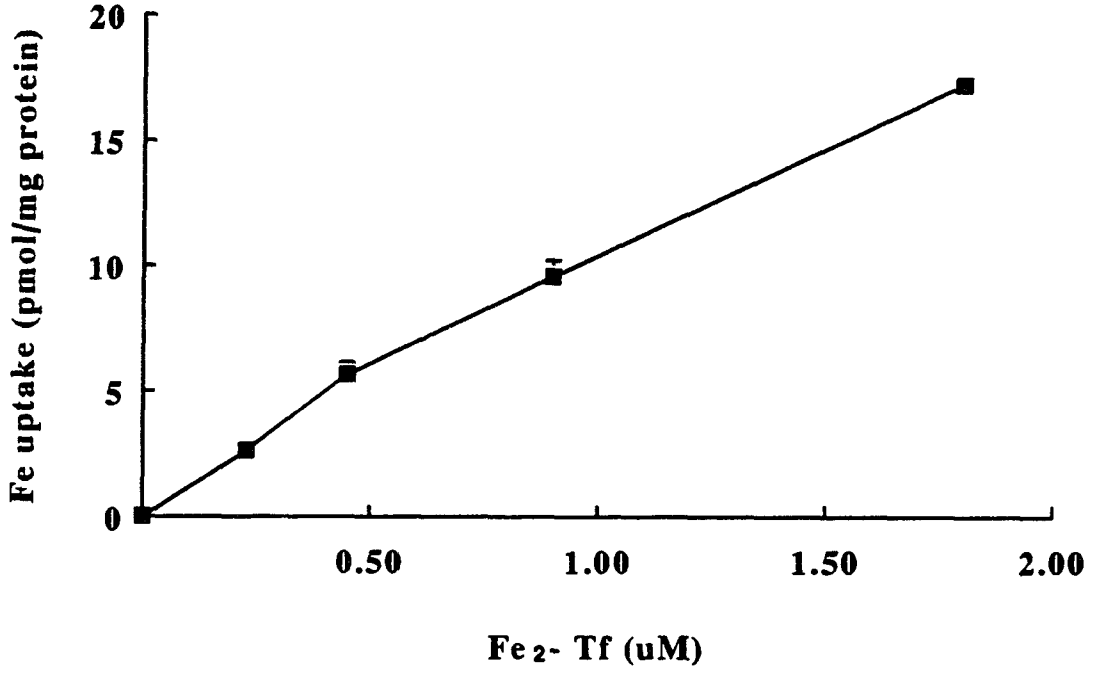


Figure 5.3. Fe uptake from Fe_2-Tf is concentration dependent below the physiological range of transferrin protein.

Cultures were incubated with given concentrations of $^{59}Fe_2$-Tf for 2h at 37°C. Serum levels of transferrin are 10-20 μmol/L. Values are means ± SE from a representative experiment with 3 wells.

Effect of extracellular Fe on cellular ferritin The cellular ferritin level was assessed by the ELISA described in Materials and Methods section (Figure 5.1). The quantity of ferritin in 10-12 d post-confluent cultures of Caco-2 cells maintained in the complete DMEM containing 10% FCS was 2-3 ng/mg protein. Cellular ferritin protein significantly increased when cultures were incubated in medium containing elevated levels of Fe either as the Fe-NTA complex in the AP chamber or as Fe-Tf in the BL chamber. The increase in the level of ferritin in response to Fe(III)-NTA was dependent on duration of exposure (Figure 5.4) and the concentration of Fe(III)-NTA (Figure 5.5). When cells were exposed to 50 μmol/L Fe(III)-NTA, the maximal level of cellular ferritin was present at 24 h. Cellular ferritin content increased in a curvilinear manner after exposure of cells to 0-50 μmol/L Fe(III)-NTA for 20 h. Smaller, although significant ($p<0.05$), increases in ferritin content were also evident in monolayers exposed to increasing concentration of Fe_2-Tf (Figure 5.6).

To examine whether a reduction in extracellular Fe was associated with a decrease in cellular ferritin, cultures were first incubated overnight with 20 μmol/L Fe(III)-NTA. After removal of high Fe medium, monolayers were washed to eliminate residual extracellular Fe. Cultures were incubated with fresh medium without Fe(III)-NTA for an additional 0-36

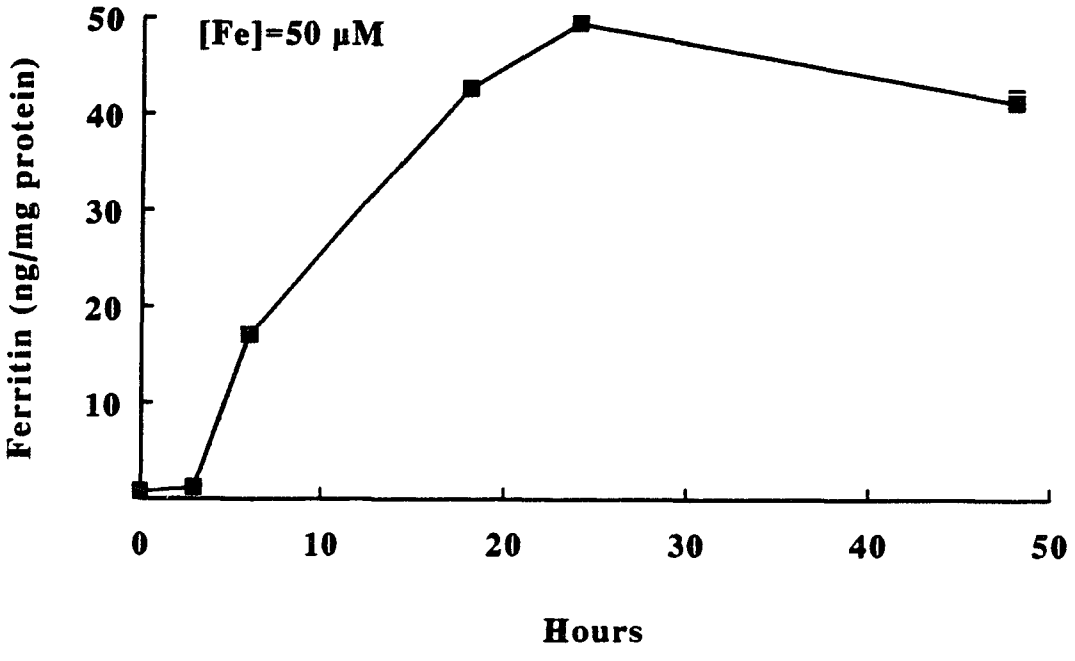


Figure 5.4. Effect of length of exposure to Fe(III)-NTA on ferritin content of Caco-2.

Values are means ± SE from two separate experiments each using three replicate wells. After 6 h or longer incubation of cultures in presence of 50 µmol/L Fe(III)-NTA in apical chamber, cellular ferritin content significantly ($p < 0.05$) increased compared to that at 0 h.

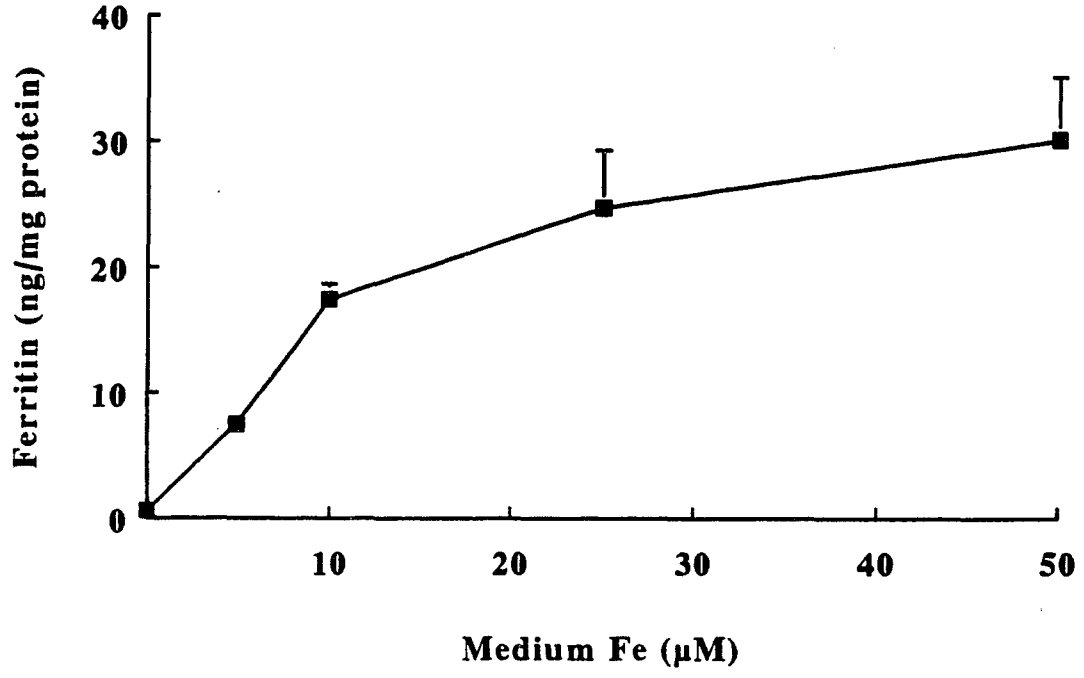


Figure 5.5. Effect of medium concentration of Fe(III)-NTA on ferritin content of Caco-2 at 20 h.

Values are means ± SE from a representative experiment with 3 wells for each concentration of Fe(III)-NTA. The levels of cellular ferritin are significantly ($p < 0.05$) different from control (without Fe(III)-NTA).

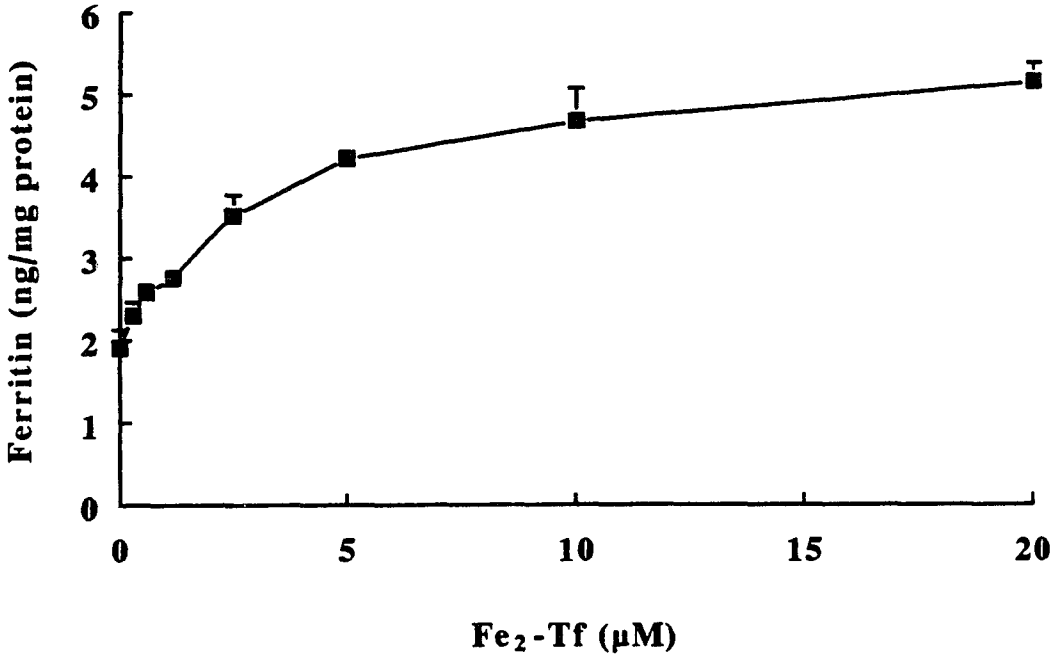


Figure 5.6. Effect of concentration of Fe_2-Tf on cellular level of ferritin in Caco-2 cultures.

Differentiated cultures of Caco-2 cells were incubated for 20 h at 37°C with complete DMEM and 2% FCS containing indicated concentrations of Fe_2-Tf in the BL chamber. Values are means ± SE from three replicate wells. Addition of ≥ 0.6 μmol/L Fe_2-Tf significantly increased cellular ferritin content above control level ($p < 0.05$). The presence of different letters above the error bar indicates that values are significantly different ($p < 0.05$).

h at 37°C. Cellular ferritin content was stable for about 8 h, but thereafter decreased in a linear manner (Figure 5.7). By 36 h after removal of Fe(III)-NTA, the ferritin content of the cell had decreased 60%.

Together, these data clearly demonstrated that Caco-2 cells synthesize ferritin and that the level of the ferritin protein changes in response to the concentration of the trace metal in both the basolateral and the apical compartment.

AP to BL transport of Fe is influenced by cellular ferritin content The rate of Fe transport across the Caco-2 monolayer was 28.4 ± 2.7 pmol/(h·mg protein) when cultures were incubated with 30 nmole Fe(III)-NTA for 5 h (Figure 5.8). Pretreatment of cells with 20 μmol/L Fe(III)-NTA slightly, but significantly ($p < 0.05$), decreased Fe transport from the AP to the BL (Figure 5.8). Next, cultures were pre-incubated in medium containing 10 μmol/L Fe_2-Tf for 36 h. The treatment elevated cellular ferritin content 3.4 fold (2.9 ± 0.4 vs. 12.7 ± 1.3 ng/mg protein in the control and treated cultures, respectively). The rate of Fe transport from the AP (10 μmol/L Fe(III)-NTA added) to the BL compartment by these cultures was only 55% that ofcultures that had not been exposed to additional Fe_2-Tf (9.1 ± 0.5 vs 16.4 ± 1.6 Fe pmol/h·mg protein, respectively;

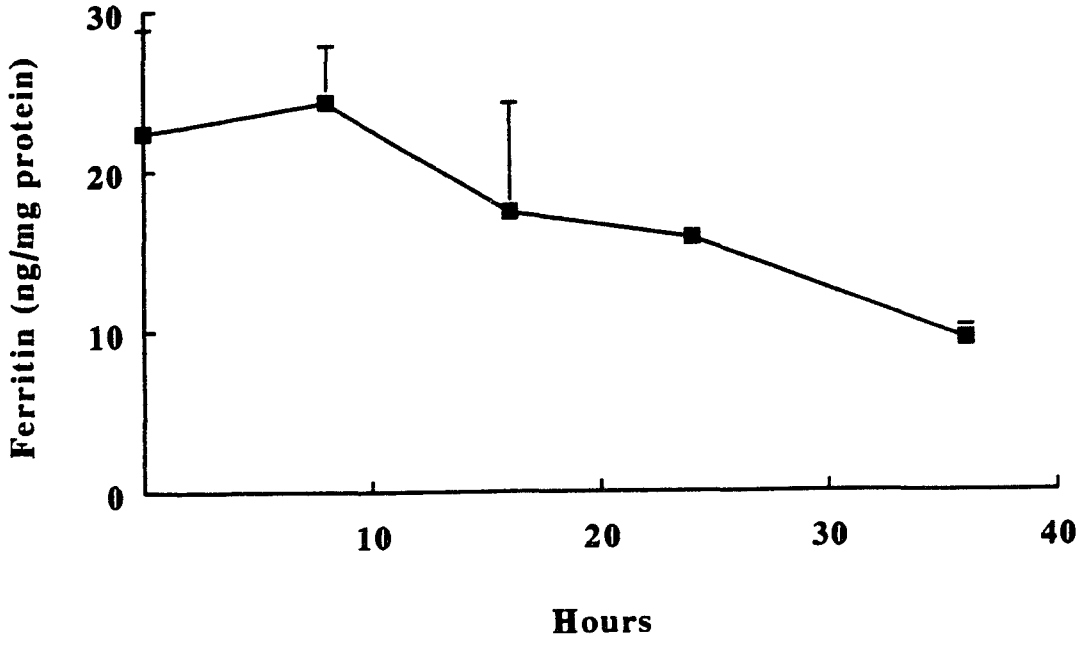


Figure 5.7. Effect of length of incubation in low Fe medium on ferritin degradation by Caco-2 cells.

Differentiated Caco-2 cells grown on microporous membranes were incubated with 20 μmol/L Fe(III)-NTA added to the apical chamber overnight to increase cellular ferritin content. After removal of medium containing Fe(III)-NTA, monolayers were washed 3x with buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Hepes, pH 7.0) and then incubated for 5 min. with removal buffer (140 mmol/L NaCl, 10 mmol/L PIPES, 5 mmol/L BPS, 5 mmol/L $Na_2S_2O_4$, pH 7.0) to eliminate residual Fe on the cell surface. Washing was performed with the buffer at 37°C. After additional incubation for 0-36h with fresh medium without Fe(III)-NTA, cellular ferritin was measured. Values are means ± SE for at least 2 wells from a representative experiment (n=2). The level of cellular ferritin was decreased significantly ($p < 0.05$) after incubation without Fe(III)-NTA ≥ 24 h.

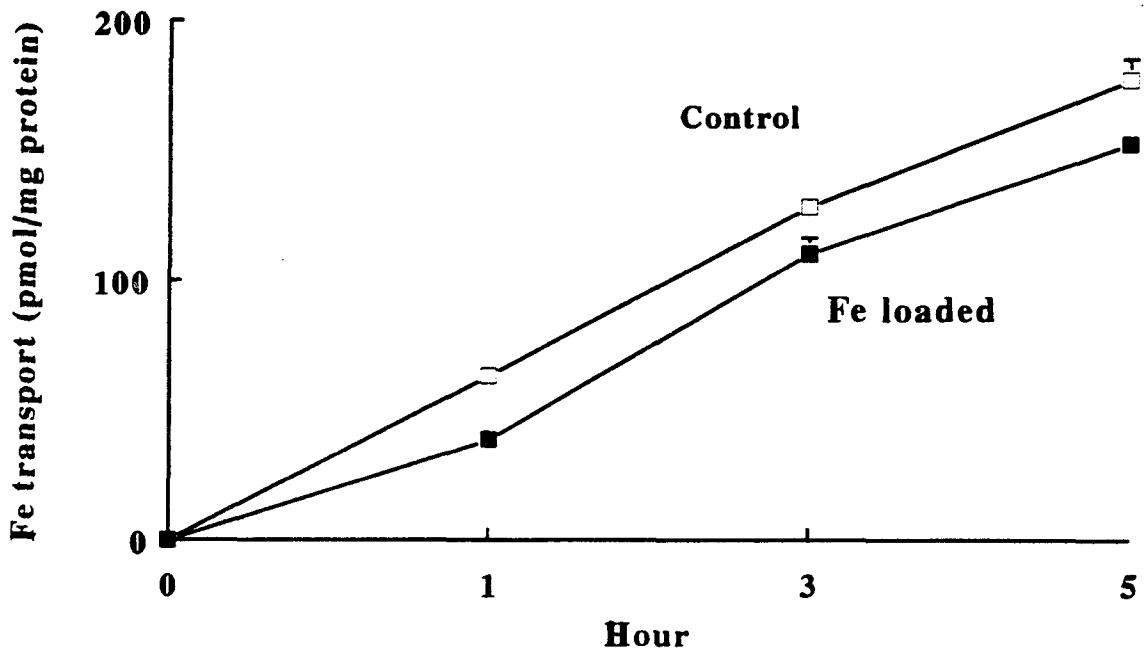


Figure 5.8. Decreased Fe transport from the apical to the basolateral chamber in Fe loaded Caco-2 cells.

After pretreatment of 12d post-confluent cultures of Caco-2 cells with 20 μmol/L Fe(III)-NTA overnight, residual Fe(III)-NTA was removed as described in the legend of Figure 5.7. Uptake buffer (1.5 mL) containing 20 μmol/L ^{59}Fe and 40 μmol/L NTA was added to the apical compartment and the rate of ^{59}Fe transport from the AP to the BL compartment was determined as detailed in Chapter 2. Data are means ± SE for a representative experiment with at least 3 wells. The transport rate of ^{59}Fe was significantly ($p < 0.05$) decreased in Fe loaded cultures of Caco-2 cells.

$p < 0.05$).

Therefore, these observations demonstrate that increases in the cellular content of ferritin are associated with an impaired capacity to transport Fe in the AP chamber across the Caco-2 monolayer.

Monokines increase cellular accumulation of Fe from Fe_2-Tf

Initial studies aimed to define the concentrations of monokines required to eventually test their potential impact on Fe transport across the Caco-2 monolayer. Differentiated cultures were treated with individual monokines for 24 h. Control and monokine-treated cultures were washed before addition of $^{59}Fe_2$-Tf (0.45 μmol/L). Increased basolateral uptake of Fe was demonstrated for cells treated with 10 and 30 ng/mL IL-1 (Figure 5.9). Similarly, treatment with >1 ng/mL IL-6 increased Fe uptake from Fe_2-Tf, although the difference was not significant unless 30 ng/mL of the monokine was present (Figure 5.10). Combined addition of IL-1 and IL-6 (10 ng/mL each) increased cellular uptake of Fe by 30% (Figure 5.11; $p < 0.05$). In contrast, Fe uptake from Fe_2-Tf by cells exposed to TNF (25 ng/mL) did not differ from the control (6.43 ± 0.07 vs 6.35 ± 0.18 Fe pmol/mg protein, respectively; $p > 0.05$). Assuming that TNF may affect cellular Fe transport and metabolism when combined with other monokines, cultures were next exposed to medium

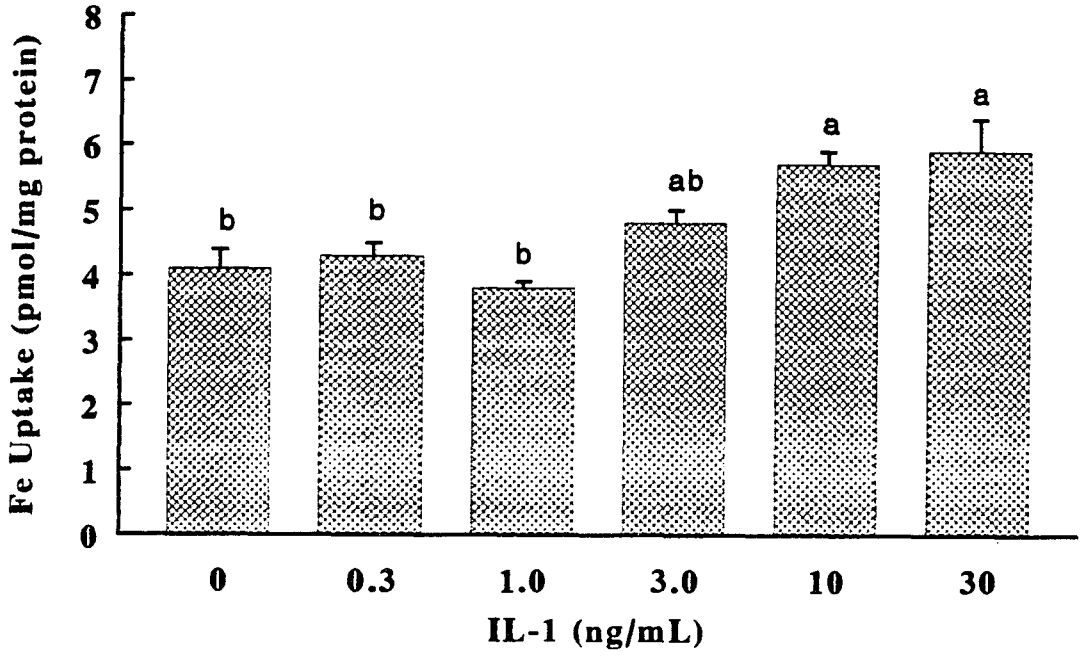


Figure 5.9. IL-1 increases Fe uptake from Fe_2-Tf.

After 24 h treatment with various concentrations of IL-1, cultures of Caco-2 cells grown on microporous membrane were incubated with DMEM and 0.2% BSA (1.5 mL) containing 18 kBq ^{59} Fe(added as 0.45 μmol/L Fe_2-Tf) in the BL compartment for 2 h at 37°C. After removal of $^{59}Fe_2$-Tf containing medium, monolayers of Caco-2 cells were washed 3x with ice cold buffered saline containing 1 mmol/L EDTA to remove non-specifically bound $^{59}Fe_2$-Tf. Values are means ± SE for representative experiments with 3 wells for each concentration of IL-1. The presence of different letters above the error bar indicates that values are significantly different ($p < 0.05$).

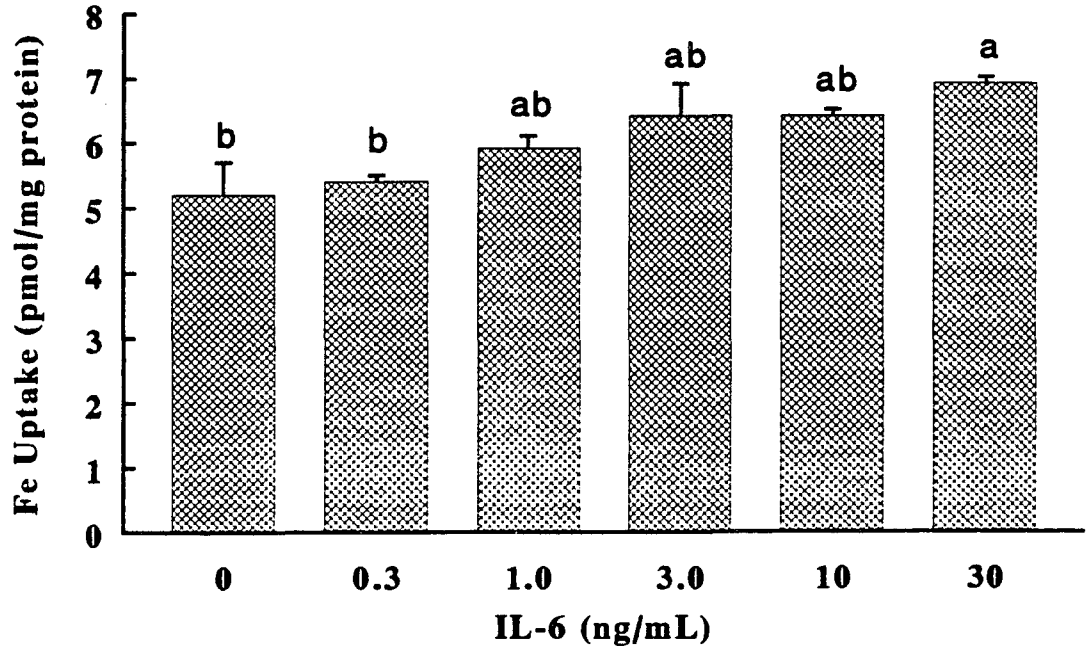


Figure 5.10. IL-6 increases Fe uptake from Fe_2-Tf.

Details are identical to those in the legend of Figure 5.9, except that cells were treated with IL-6 instead of IL-1. The presence of different letters above the error bar indicates that values differ significantly ($P < 0.05$).

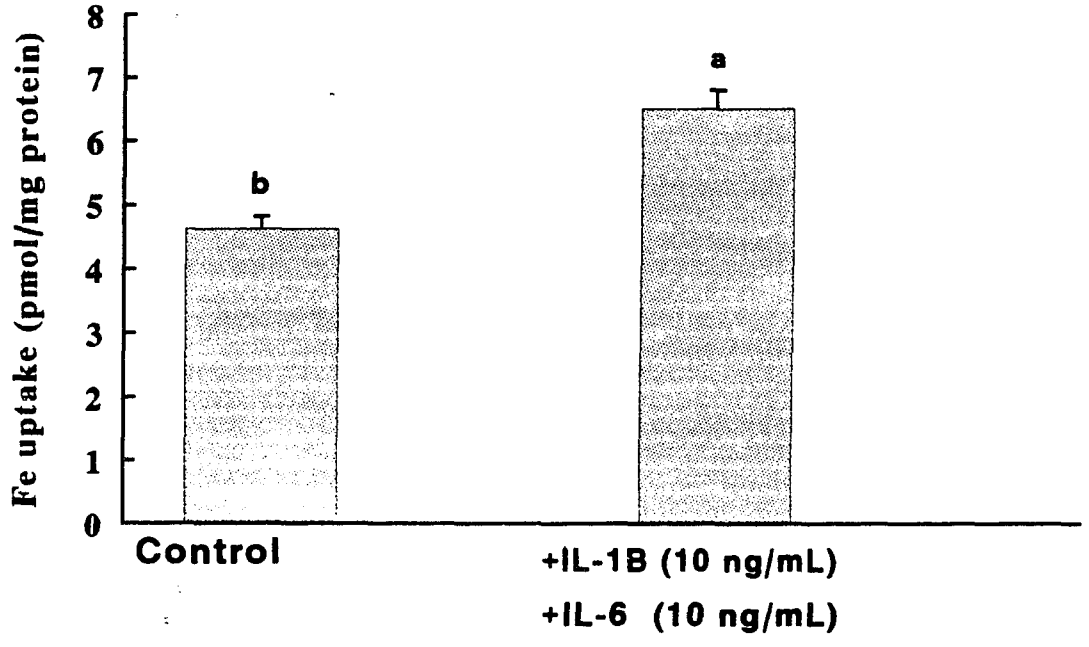


Figure 5.11. Combination of IL-1 and IL-6 increases Fe uptake from Fe_2-Tf.

Details are identical to those in the legend of Figure 5.9., except that cultures of Caco-2 cells were exposed to both IL-1 (10 ng/mL) and IL-6 (10 ng/mL). The combined presence of IL-1 and IL-6 significantly ($p<0.05$) increased ^{59}Fe uptake from $^{59}Fe_2$-Tf compared to that in control cultures.

containing IL-1, IL-6 and TNF (25 ng/mL each). This treatment resulted in a 70% increase in the uptake of Fe from solution containing 1.3 nmoles Fe_2-Tf (Figure 5.12). This mixture of monokines (25 ng/mL each IL-1, IL-6 and TNF) was used in all subsequent studies.

Effects of monokines on cellular ferritin Initially, cultures of Caco-2 were treated with DMEM containing 2% FCS and with or without (control) monokines for 24 h. The only Fe present in the cultures was that associated with Tf in FCS. Cellular ferritin content was significantly ($p < 0.05$) higher in monokine-treated cells than in controls (2.1 ± 0.1 vs. 1.6 ± 0.2 ng/mg protein, respectively). Next, the influence of monokines on the ferritin content of Fe loaded cells was examined after removal of extracellular Fe. To increase cellular Fe and ferritin content, cultures of Caco-2 initially were incubated with 20 μmol/L Fe(III)-NTA in AP compartment for 20 h. This treatment elevated cellular ferritin to 33 ng/mg protein (0 h; Figure 5.13). Medium containing Fe(III)-NTA was removed and monolayers were washed three times with buffer containing 150 mmol/L NaCl, 1mmol/L EDTA, 10 mmol/L HEPES, pH 7.0. Pilot studies showed that this procedure did not adversely affect the cultures. Cells then were incubated either with or without monokines (25 ng/mL each of IL-1, IL-6 and TNF) for 24 h. Cellular

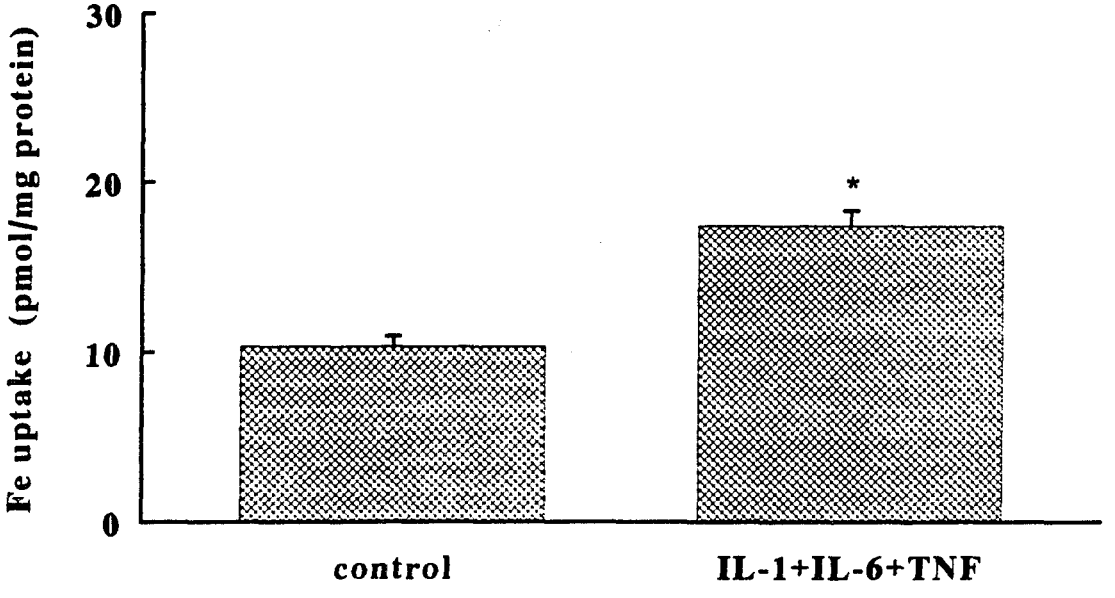


Figure 5.12. Monokines enhance Fe uptake from Fe_2-Tf.

Details are identical to those in the legend of Figure 5.9, except that differentiated cultures of Caco-2 cells were supplemented with monokines (25 ng/mL each of IL-1, IL-6 and TNF) and incubated with 0.87 μmol/L $^{59}Fe_2$-Tf. Uptake of ^{59}Fe from $^{59}Fe_2$-Tf was significantly ($p < 0.05$) higher in monokine-treated Caco-2 cells than that in control cultures.

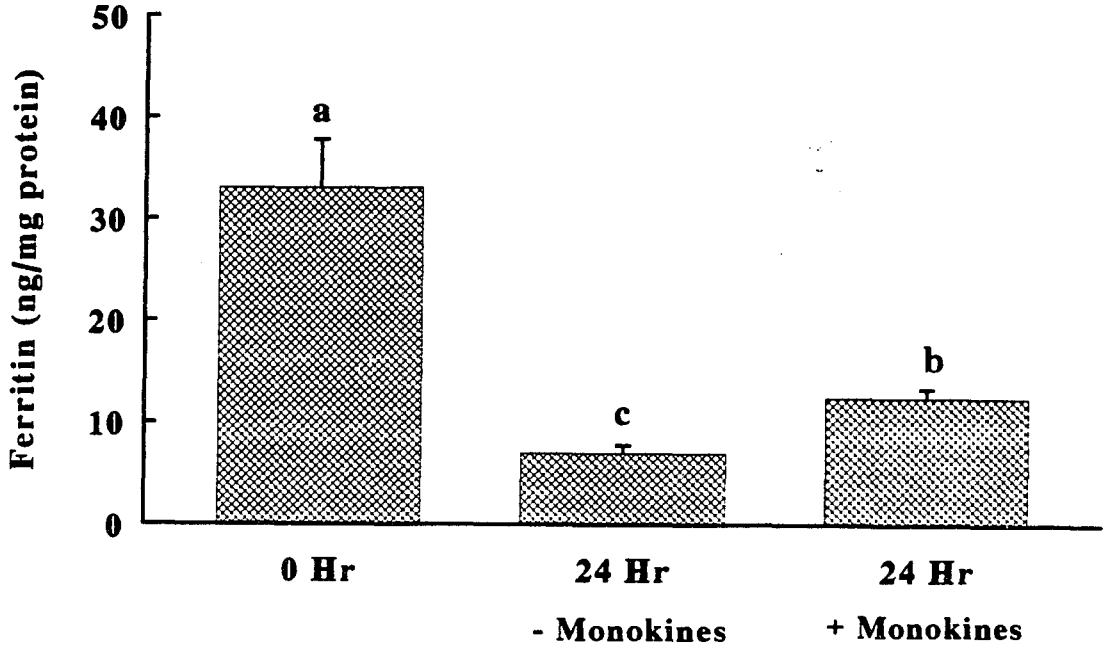


Figure 5.13. Increased retention of ferritin by monokine-treated cells.

Differentiated cultures of Caco-2 cells grown on microporous membrane inserts were exposed to 20 μmol/L Fe(III)-NTA in the AP compartment for 20 h at 37°C. After removal of Fe(III)-NTA and elimination of residual surface Fe (see the legend of Figure 5.7), cells were incubated for 0 or 24 h with or without monokines (25 ng/mL each of IL-1, IL-6 and TNF). Cellular ferritin was assessed as described in the Materials and Methods. Values are means ± SE from three replicate wells. The presence of different letters above the error bar indicates that values are significantly different ($p < 0.05$).

ferritin content was reduced by 79% in control cultures (7.0 ± 0.8 ng/mg protein), whereas monokine-treated cells contained a significantly ($p<0.05$) higher quantity of ferritin (12.4 ± 0.9 ng/mg protein). This observation suggests that monokines may decrease the rate of ferritin protein degradation, perhaps by attenuating efflux of intracellular Fe.

Monokines decrease Fe transport from the AP to the BL compartment The above studies provided information required to test the possibility that monokines inhibited transport of Fe across the Caco-2 monolayer. The mixture of monokines was added to the BL chamber of differentiated cultures. After 16 h, 10 μmol/L Fe_2-Tf was added to the BL chamber and incubation was continued for an additional 24 h to allow Fe uptake and ferritin synthesis. Next, cellular Fe accumulation and transport were examined by adding 10 μmol/L ^{59}Fe(III)-NTA to the AP compartment. The rate of ^{59}Fe transport from the AP to the BL compartment in monokine-treated cultures was 70% that in control cultures (Table 5.1; $p < 0.05$). This difference in the rate of Fe transport was associated with a significantly higher level of Fe and ferritin protein in monokine-treated cells (Table 5.1). In contrast, the rate of ^{14}C-mannitol transport from the AP to the BL (a paracellular process) was not significantly

TABLE 5.1. Monokines decrease the rate of Fe transport from the apical (AP) to the basolateral (BL) compartment by altering cellular ferritin by Caco-2 cells.

	Control	+ Monokines
Rate of AP → BL transport of Fe (pmol/h·mg protein)	17.9 ± 1.0	12.6 ± 0.7*
^{59}Fe accumulation (pmol/mg protein)	272 ± 14	322 ± 15*
Ferritin (ng/mg protein)	10.5 ± 0.2	12.7 ± 0.2*

Differentiated cultures of Caco-2 cells were incubated with complete DMEM plus 2% FCS containing with or without monokines (25 ng/mL each of IL-1, IL-6 and TNF) at 37°C. After 16h, 10 μmol/L Fe_2-Tf was added to the BL chamber and incubation was continued for an additional 24h. Thus, the duration of monokine treatment was 40h. Cellular accumulation and transport of Fe were examined by adding uptake buffer with 10 μmol/L ^{59}Fe(III)-NTA complex to the AP compartment. Cellular accumulation of ^{59}Fe and the rate of ^{59}Fe transport from the AP to the BL compartment were determined as detailed in chapter 2. Values are mean ± SE from triplicate wells. Presence of asterisk as a superscript indicates that values differ significantly ($p < 0.05$) from control.

($p > 0.05$) altered by the monokine treatment. These data suggest that monokines decrease the efficiency of apical Fe transport by enhancing uptake of Fe_2-Tf which increases the synthesis of ferritin, the high affinity intracellular storage protein.

DISCUSSION

It is well established that the efficiency of dietary Fe absorption is decreased when endogenous stores of this essential, but potentially toxic, trace metal are elevated (Linder, 1991). During this period of "mucosal block", dietary Fe continues to be transported across the brush border surface of the enterocyte. However, this is largely retained within the cell instead of being transferred across the BL membrane. The high affinity Fe storage protein ferritin plays a central role in cellular retention of newly acquired Fe from the lumen. Binding within the ferritin core reduces the probability that Fe will interact with its transporter on the BL surface. Because enterocytes migrate from the crypt region to the villus tip from which they are "shed" every 3-4 days, the organism is able to effectively alter the efficiency of Fe absorption in response to the needs of tissues. Despite this general comprehension of the process of dietary Fe absorption, the specific signals that control intestinal Fe transport and cellular metabolism remain unknown.

In order for the Caco-2 cell model to be useful for elucidating the regulation of Fe metabolism and transport in enterocytes, it is important to initially determine that the efficiency of Fe transport from the AP to the BL compartment

is influenced by cellular ferritin content. This necessitated that the development of an assay to quantify ferritin. The ELISA procedure described above provided a sensitive and relatively rapid means of monitoring the level of ferritin in small aliquots of sonicated cells grown in multiwell dishes. My results clearly demonstrated that cellular ferritin content was modulated by the level of the trace metal present in the AP compartment. Moreover, cells containing a higher level of ferritin exhibited decreased ability to transport Fe across the monolayer. These results confirm the earlier report by Alvarez-Hernandez et al. (1991) and support the appropriateness of the Caco-2 cell line as a model to study the regulation of Fe absorption.

Although nutritional scientists generally consider the key role of the enterocyte in nutrient absorption, it is likely that these polarized cells also acquire nutrient and metabolites from the serosal environment. Usually extracellular Fe is bound to its high affinity carrier protein referred to as transferrin (Tf). The transfer of Tf-bound Fe from plasma to cells requires the presence of TfR on the cell surface (Klausner et al. 1983; Sipe and Murphy 1991). Such receptors have been identified on the BL membrane of intestinal epithelial cells in humans (Banerjee et al. 1986; Pietrangelo 1992) and rats (Anderson et al. 1990, 1991, 1994) and the Caco-2 cell line (Halleux and

Schneider 1991; Shah and Shen 1994). Moreover, the levels of TfR on enterocyte BL membrane were inversely related to the whole body stores in normal humans (Banerjee et al. 1986). Minimal attention has been given to the actual role of the TfR in Fe uptake by the enterocyte. Therefore, I first examined whether differentiated cultures of Caco-2 are capable of accumulating Fe from Fe_2-Tf in the BL chamber. The results suggest that Caco-2 cells utilize the TfR pathway since the uptake of Fe from Fe_2-Tf was dependent on temperature and duration of incubation, as well as being saturable. I also found that the cellular level of ferritin increased in response to elevation of the concentration of Fe_2-Tf in the BL chamber. Particularly noteworthy was the observation that exposure of cultures to higher, but physiological, levels of Fe_2-Tf markedly decreased the quantity of Fe transport from the AP to the BL compartment. This discovery suggests that elevations in body Fe status can directly regulate the absorption of dietary Fe.

Attention was next directed to the possible influence of monokines on Fe transport and metabolism in Caco-2 cells. A variety of observations in the literature provided the rational for selecting monokines as potential regulators of intestinal Fe absorption. First, increased efficiency of dietary Fe absorption is associated with physiological changes that do not cause marked attenuation in Fe status.

Thus, it seems likely that factors other than the level of Fe_2-Tf are responsible for enhancing Fe absorption during these periods. Second, infection and inflammation are associated with marked changes in Fe metabolism in several tissues, including liver and the immune system (Beisel 1991; Hirayama et al. 1993; Rogers et al. 1990). Several recent studies have shown that monokines directly alter Fe metabolism in the HepG2 human liver cells (Hirayama et al. 1993; Rogers et al. 1990). Third, it is now recognized that the GI tract is one of the major "immune organs" in the body. At least 40% of all immune cells and more than 80% of antibody secreting cells reside in the gut (Castro and Arntzen 1993; Cook 1994; Kagnoff 1987; Langkamp-Henken et al. 1992; Perdue and McKay 1994). Thus, the basolateral surface of enterocyte is likely to be exposed to relatively high levels of secretory products of activated immune cells. The identification of cytokine receptors on the basolateral surface of enterocytes (Ciacci et al. 1993; Guastafson-Svard et al. 1993; McGee et al. 1991; Varilek et al. 1993) indicates that the activities of the enterocyte are influenced by immune activity. Finally, chronic infection of the GI tract is prevalent in areas where there is a high incidence of Fe deficiency. This suggests that the infectious agents themselves or their activation of the GI immune system may cause decreased absorption of dietary Fe.

If chronic, this situation may lead to Fe deficiency, especially in those groups requiring greater levels of the micronutrient, viz., infants and growing children, and women during their child-bearing years. It is also interesting to note that several non-infectious diseases in which the GI tract is chronically "inflamed", e.g., Crohn's disease and Celiac disease impair the absorption of nutrients (Kagnoff et al. 1987).

My preliminary data clearly show that treatment of Caco-2 cells with monokines increased both uptake of Fe from Fe_2-Tf and cellular ferritin content. Also, the degree to which ferritin decreased after removal of extracellular Fe was attenuated by presence of monokines in medium. These changes were associated with a significant decrease in the rate of Fe transport across the cell monolayer. Together, these results provide strong support for the hypothesis that monokines regulate Fe metabolism by enterocytes.

Therefore, it is possible that prevalent Fe deficiency with endemic infection of the gut in developing countries may be due to impaired dietary Fe absorption by stimulation of immune cells in the GI tract. Although, this study was limited to an examination of the influence of monokines on Fe transport and metabolism, the effects of various possible physiological factors (as discussed above) on Fe and other

nutrient metabolism and transport should be conducted in the future. These results demonstrate that the Caco-2 cell line is an appropriate model for studying the influence of physiological factors on Fe transport and metabolism by enterocytes.

EPILOGUE

The results of this study strongly support the Caco-2 cell line is an appropriate model for investigating the effects of various dietary and physiological factors on Fe transport and metabolism in the human intestine. Although many conditions are known to influence the absorption of dietary Fe, the specific factors that regulate this absorption process have not been identified. The latter studies I conducted indicate that monokines are likely suppress of intestinal absorption of dietary Fe during inflammatory episodes. If time had permitted I would have also liked to have considered whether estrogens are directly responsible for the well recognized increase in the efficiency of Fe absorption during pregnancy and lactation.

The absorption of many nutrients, including Fe, increases during pregnancy and lactation. Several clinical observations and animal studies have demonstrated that the increase in Fe absorption parallels the elevation of the levels of plasma estrogen. However, it is unknown if estrogen directly modulates transport and metabolic events required for Fe absorption by enterocytes. The recent identification of estrogen receptors in intestinal epithelial cells and the demonstrated direct influence of

estrogen on Ca absorption by isolated rat intestinal cells support the possibility that increased plasma estrogen during pregnancy and lactation are directly responsible for stimulating the absorption of dietary Fe.

To examine this hypothesis Caco-2 cultures on microporous membranes would be used. ß-estradiol, an active form of estrogen, would be added to complete DMEM containing 2% FCS that had been previously treated with charcoal to remove endogenous steroids. Initially, the effect of estradiol treatment on Fe uptake from Fe_2-Tf and cellular ferritin content in response to estradiol exposure in the basolateral chamber would be examined to establish the optimal conditions related to dose(s) of estradiol and duration of treatment. Assuming estradiol treatment decreased Fe uptake from Fe_2-Tf and/or decreased cellular ferritin content, additional studies aimed at elucidating the mechanism(S) responsible for these studies would be warranted. Moreover, the impact of estradiol-treatment on apical to basolateral transport of Fe would be investigated in the same manner as completed with monokine-treated cells (Chapter V). The predicted results are that estradiol treatment will enhance Fe transport from the apical to the basolateral compartment by decreasing Fe uptake from Fe_2-Tf and the cellular content of ferritin.

It is likely that new information would be gained from the above study. The elucidation of positive and negative regulators of Fe absorption would allow investigators to begin to design more complex studies that better address the human situation. For example, it would be interesting to determine if monokines might offset the potential stimulatory influence of estradiol on Fe absorption. Insights gained from such in vitro studies would facilitate the design of appropriate experiments using human subjects. Through combined use of in vitro cellular experiments and in vivo studies, effective strategies to combat the devastating consequences of Fe deficiency throughout the world will evolve.

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