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NEW INSIGHTS INTO INTESTINAL HEME AND NON-HEME IRON ABSORPTION: REGULATION BY DIETARY FACTORS

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ABSTRACT

Iron is an essential mineral in all mammals required for many physiological processes, including oxygen transport and storage, electron transfer and DNA synthesis. Iron absorption is crucial in maintaining systemic iron homeostasis because there is no physiological mechanism for iron excretion, which distinguishes iron from other minerals. Dietary factors, such as phytate, ascorbic acid and animal tissues, have been proven consistently to influence intestinal iron absorption. The unique components of animal tissues that enhance intestinal iron absorption are coined as "meat factor". However, the influence of polyphenols on iron absorption and the nature of the "meat factor" remain unresolved. As the major transporters for intestinal iron absorption have been identified in the past decade, the focus has shifted to the molecular mechanism of intestinal heme iron absorption. To address these identified gaps of knowledge, our research focused on defining the effect of polyphenols on iron absorption and elucidating the interactions between heme and non-heme iron during iron absorptive processes using the human intestine-like Caco-2 cell model.

In the first study, our objective was to investigate the effect of several bioactive polyphenolic compounds, including (-) -epigallocatechin-3-gallate (EGCG), grape seed extract (GSE) and green tea extract (GT) on intestinal heme iron absorption. Dietary polyphenolic compounds has attracted increasing attentions recently because they have a wide range of health benefits. Previous studies have revealed the inhibiting effect of polyphenols from black tea on non-heme iron absorption. Our initial study showed the inhibitory effect of EGCG and GSE on non-heme iron absorption. We explored whether polyphenols from green tea and grape seed extract could inhibit intestinal absorption of heme iron in Caco-2 cells. Both EGCG and GSE decreased ($p < 0.05$) transepithelial transport of heme-derived ^{55}Fe. Although apical heme-^{55}Fe uptake was increased ($p < 0.05$) by GSE, the amount of total ^{55}Fe released to basolateral chamber

was negligible. In contrast, EGCG moderately decreased the apical uptake of heme-^{55}Fe, whereas the basolateral iron transfer was extremely low. All three types of polyphenolic compounds tested, including EGCG, GSE and GT, significantly inhibited heme-^{55}Fe absorption in a dose-dependent manner. The addition of ascorbic acid did not modulate the inhibitory effect of these polyphenols on heme iron absorption when the cells were treated with polyphenols at a concentration of 46 mg/L. However, ascorbic acid was able to offset or reverse the inhibitory effect of polyphenolic compounds when lower concentrations of polyphenols were added ($\leq$ 4.6 mg/L). In summary, findings from the first study suggest that bioactive dietary polyphenols inhibit heme iron absorption mainly by reducing basolateral iron exit rather than decreasing apical heme iron uptake in intestinal cells. In addition, the results imply that regular consumption of dietary ascorbic acid can easily counteract the inhibitory effect of very low concentrations of dietary polyphenols on heme iron absorption but cannot counteract the inhibitory actions of higher concentrations of polyphenols.

In the second study, our objective was to explore the mechanism of increased iron absorption promoted by meat factor of animal tissues and whether and how the absorption of heme and non-heme iron interfere with each other. We examined cellular uptake and transepithelial absorption of non-heme ^{55}Fe in human intestine-like Caco-2 cells in response to treatment of different doses of heme or the heme precursor, protoporphyrin IX, for different time periods. We found that non-heme ^{55}Fe absorption across Caco-2 cell monolayer was enhanced by heme via redistributing iron importer divalent metal-transporter-1 (DMT1) from cytosol to apical membrane and iron exporter ferroportin1 (FPN1) from cytosol to basolateral membrane. Interestingly, heme-^{55}Fe absorption was also increased by treatment of non-heme iron. Although non-heme iron alone did not affect FPN1 localization, heme and non-heme iron together synergistically redistributed FPN1 to basolateral membrane. In addition, the heme precursor protoporphyrin IX itself was sufficient to increase non-heme iron absorption, while the

competition between the iron released from the heme moiety and non-heme iron was negligible compared to compensatory increase of FPN1 on basolateral membrane. Finally, non-heme ^{55}Fe absorption was also enhanced adaptively by pretreatment of heme for 2 – 3 days, possible as a consequence of increased FPN1 transcription induced by heme.

In conclusion, our research supports that polyphenols from green tea and grape seed extracts drastically block intestinal absorption of both heme and non-heme iron and that intestinal absorption of heme and non-heme iron are reciprocal of each other via regulation of DMT1 and FPN1 using the Caco-2 cells model.

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LIST OF ABBREVIATIONS

ActD	Actinomycin D
AI	Adequate Intake
ANOVA	Analysis of Variance
AP	Apical
ARE	Antioxidant Response Element
Bach1	BTB and CNC Homology 1
BL	Basolateral
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
Caco-2	Human Colorectal Adenocarcinoma Cells
CO	Carbon Monoxide
Cp	Ceruloplasmin
Dcytb	Duodenal Cytochrome b
DFO	Desferrioxamine
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl Sulfoxide
DMT1	Divalent Metal Transporter 1
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGCG	(-)-Epigallocatechin-3-Gallate
EPO	Erythropoietin
FBS	Fetal Bovine Serum
Fe	Iron
FLVCR	Feline Leukemia Virus Subgroup C Receptor
FPN1	Ferrportin-1
Ft	Ferritin
Fz	Ferrozine
GDF	Growth Differentiation Factor
GPI	Glycosylphosphatidylinositol
GSE	Grape Seed Extract
GT	Green Tea Extract
H_2O_2	Hydrogen Peroxide
Hb	Hemoglobin
HBSS	Ca^{2+}- And Mg^{2+}-Free Hank's Balanced Saline Solution
Heph	Hephaestin
HIF-2α	Hypoxia-Inducible Factor-2α
HJV	Hemojuvelin
HO	Heme Oxygenase

IC50	Half Maximal Inhibitory Concentration
IDA	Iron Deficiency Anemia
IRE	Iron-Response Element
IRP	Iron-Regulatory Protein
KEAP	Kelch-like ECH-associated Protein 1
LPS	Lipopolysaccharide
Maf	Musculoaponeurotic Fibrosarcoma
MARE	Maf Recognition Element
MEL	Murine Erythroleukemia Cells
Nrf2	Nuclear Factor Erythroid 2-Related Factor 2
NTA	Nitrilotriacetic Acid
PBS	Phosphate-Buffered Saline
PCFT/HCP1	Proton-Coupled Folate Transporter/Heme Carrier Protein 1
PCR	Polymerase Chain Reaction
PHD	Prolyl Hydroxylases
PPIX	Protoporphyrin IX
RDA	Recommended Dietary Allowance
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline With Tween 20
TEER	Transepithelial Electrical Resistance
Tf	Transferrin
TfR1	Transferrin Receptor-1
TMPRSS6	Transmembrane Protease Serine 6
TS	Transferrin Saturation
TWSG1	Twisted Gastrulation Protein 1
UL	Upper Limit
UTR	Un-translated Region
VC	Vitamin C
VHL	Von Hippel-Lindau
WGA	Wheat Germ Agglutinin
WHO	World Health Organization

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Chapter 1

LITERATURE REVIEW

ABSTRACT

Regulated iron absorption in the proximal small intestine is essential in maintaining systemic iron homeostasis. This review will describe new insights into intestinal iron absorption and its regulation. The major transporters for intestinal iron absorption have been identified in the past decade and focuses have shifted to the mechanism of intestinal heme iron absorption and more importantly, regulation of intestinal iron absorption. The discovery of the apical heme transporter, proton-coupled folate transporter/heme carrier protein-1 (PCFT/HCP1), has prompted major advances in understanding heme iron absorption. The identification of liver-derived hepcidin as a master regulator of systemic iron homeostasis and a key controller of intestinal iron absorption was a major breakthrough in regulation of intestinal iron absorption. Hepcidin functions to repress intestinal iron absorption in response to a variety of systemic cues, including systemic iron status, erythropoiesis, hypoxia and inflammation, which alter transcription of the hepcidin gene via signals involving HFE, hemojuvelin (HJV) and transferrin receptor 2 (TfR2) on surface of hepatocytes. In addition, the most recent recognition of the hypoxia-inducible factor-2α (HIF-2α) as the inducer of expression of duodenal iron transporters, especially divalent metal transporter 1 (DMT1) and duodenal cytochrome b (Dcytb) and slightly ferroportin-1 (FPN-1), has highlighted new perspectives in regulation of iron absorption and forged the idea that the duodenum is both a crucial sensor and a regulator of iron absorption. Apart from those pathophysiological factors, some dietary factors directly modulate intestinal iron absorption and affect iron bioavailability. In summary, disruptions in intestinal iron absorption or its regulatory

pathways pose a dual challenge to iron homeostasis – iron deficiency anemia and hemochromatosis; understanding the mechanism of these pivotal advances in intestinal iron absorption and its regulation will be beneficial for translational research that improves therapeutic efficacies for iron dysregulation in the near future.

Keywords

Intestinal iron absorption, hepcidin, HIF-2α, IRE/IRP, dietary factors

INTRODUCTION

A. Iron and Its Systemic Homeostasis

Iron is the fourth most abundant element in the Earth's crust and is an essential micronutrient required for survival and proliferation in eukaryotic and most prokaryotic cells and organisms. It is a transition metal readily shifting between its ferric and ferrous states to allow one-electron redox reactions, which on one hand, largely explains its biological function as a component of a diverse group of hemoproteins, including those involved in oxygen transport and storage (hemoglobin and myoglobin), electron transfer (cytochromes) and DNA synthesis (ribonucleotide reductase). In humans, the predominant iron demand is hemoglobin synthesis, which is required for daily production of 200 billion new erythrocytes into blood. Currently, iron deficiency is one of the most common diet-related health problems worldwide, which, in a severe stage, leads to microcytic and hypochromic iron-deficiency anemia, a major public health concern affecting up to 1 billion people. Preventing iron deficiency has thus been a main topic of public concern of World Health Organization (WHO) since 1992.

On the other hand, free iron in excess can be toxic to cells due to its reactivity and capability of producing via Fenton reactions the free radicals that are noxious to lipid membranes, proteins and nucleic acids. When iron overload occurs due to dysregulation of iron metabolism (as in hemochromatosis, which affects more than 1 million people in United States), excessive iron accumulates in parenchymal cells of liver, heart and other tissues, causing damage to vital organs and serious, even life-threatening complications over years.

Since both iron deficiency and iron overload are fatal to cells, iron levels must be tightly regulated to ensure systemic iron homeostasis. As blood is the site of iron entry into and recycling back to the system and of supplying proper amount of iron to tissues, the indicator of systemic

iron homeostasis consists in plasma iron levels. Iron circulates in plasma bound to transferrin (Tf), which has two binding sites for ferric iron with extraordinarily high affinity, serving as the major vehicle for iron delivery into tissues. In humans, transferrin saturation is normally around 30%. Transferrin saturation of <16% signifies iron deficiency, whilst that of >45% implies iron overload. The input of plasma iron consists of both intestinal absorption of 1 – 2 mg iron per day and internal macrophage recycling of 20 – 25 mg iron per day from senescent erythrocytes, while the output of plasma iron includes erythropoiesis utilization of 20 mg iron per day for heme synthesis and irreversible iron losses. These values are subject to person-to-person variability.

B. Importance of Intestinal Iron Absorption

The small intestine plays a key role in maintaining systemic iron homeostasis. The control of systemic iron homeostasis is crucial, since iron deficiency leads to iron deficient anemia, while iron overload results in hemochromatosis. Systemic iron homeostasis requires mechanisms for regulating iron entry into body and mobilization from stores in order to compensate for daily production of 200 billion new erythrocytes and irreversible iron loss resulting from sloughing of skin and mucosal cells as well as blood loss (Figure 1-1). However, there is no regulated pathway for irreversible iron loss from the system. Thus, the net input of iron into the system, intestinal iron absorption, must be tightly modulated to provide adequate iron to keep body iron reservoir replete but not overabundant.

In addition, the duodenum itself is both an important sensor and an important regulator of iron absorption. Historically, the duodenal mucosa was believed to be a key sensor for iron uptake, which was known as the crypt cell sensing hypothesis. Within crypts of Lieberkühn that extend deep into the duodenal lamina propria lie multi-potent stem cells, some of which migrate up the villi and differentiate into mature enterocytes, which are specialized for iron uptake with

high expression of proteins related to iron absorption. Under the crypt sensing hypothesis, crypt stem cells will respond to changes in systemic iron needs by changing its iron contents and subsequently altering expression of proteins related to iron absorption before fully-differentiating into mature enterocytes and migrating to villus tips. The delayed interval between changes in systemic iron needs and that of iron absorption, about 2 days, corresponds well to the period for a crypt stem cell to migrate to villus tip and mature into a fully-differentiated enterocyte. However, there is no ultimate proof for this hypothesis and the underlying molecular mechanism is not completely elucidated yet. Recently, two papers have highlighted mucosal control of iron absorption again by providing compelling data that duodenal mucosa regulates expression of proteins related to iron absorption, especially duodenal cytochrome b (DcytB) and divalent metal transporter 1 (DMT1) via the transcription factor hypoxia-inducible factor-2α (HIF-2α), with prolyl hydroxylases (PHDs) emerging as potential mucosal sensors for mucosal iron or oxygen levels (Mastrogiannaki *et al.* 2009; Shah *et al.* 2009). The HIF/PHD regulatory axis provides a major molecular mechanism to explain the local control of iron absorption in response to iron or oxygen conditions at the intestinal level.

C. Sources of Dietary Iron

There are two dietary sources of iron, including heme iron and non-heme iron. Heme iron is derived from animal products, whereas non-heme iron is mainly found in plant sources. For an average non-vegetarian person in western civilization, heme iron constitutes one-third of total dietary iron but makes up two-thirds of the total absorbed iron, suggesting that heme iron is a more important and more efficiently-absorbed form of dietary iron than non-heme iron. The relative efficiency of dietary heme iron uptake can be attributable to its higher bioavailability. Dietary heme iron is ready to be absorbed and its solubility is enhanced by the presence of

proteins and alkaline conditions, while it is relatively unaffected by other dietary factors. In contrast, dietary non-heme iron, which is usually present in the ferric states in diet, necessities its reduction to the ferrous form by Dcytb or by dietary reducing agents in acidic conditions before it can be absorbed. In addition, non-heme iron tends to be chelated by a variety of dietary factors, such as tannins and phytates, which appear together with non-heme iron in plant products.

Despite the relative efficacy in heme iron absorption from diets, non-heme iron is primarily used in food fortification or supplements in order to combat the prevailing iron deficiency anemia worldwide. In order to test whether heme iron is a better source for supplementing people with iron deficiency, a three-way, randomized, crossover, double-blind postprandial interventional study has been done in seventeen young iron-deficient women with low iron stores (ferritin < 30 μg/L) to analyze the effectiveness of utilization of meat pate as an iron-fortification vehicle and to compare iron fortification in forms of ferrous sulfate, ferric pyrophosphate encapsulated in liposomes or ferric pyrophosphate encapsulated in liposomes plus a hemoglobin-based meat pigment (Navas-Carretero *et al.* 2009). However, the authors concluded that there were no significant differences among these fortifications. The addition of a larger quantity of the meat pigment rich in heme iron should be studied to test its effect as a fortification. Iron forms other than ferrous sulfate and ferric pyrophosphate that were popular in iron fortification, such as ferrous bis-glycinate, ferrous fumarate and ferrous lactate, may well be included in the test as a comparison. In addition, long-term effects of these iron fortifications in addition to the between-day responses of serum iron should be tested further.

D. Iron Requirements

Iron requirements for different life stages are summarized in Table 1-1. As iron overload causes toxicity, there is an upper limit for daily iron intake.

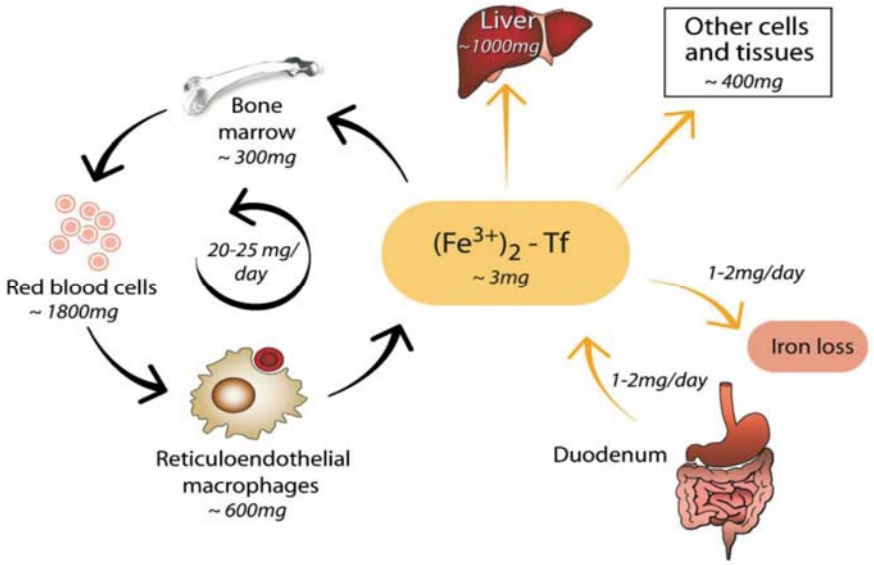

Figure 1-1. Systemic Iron Homeostasis

This figure is cited from Hentze's paper by permission of Cell (Hentze *et al.* 2004). Major pathways of iron traffic between cells and tissues are depicted. Normal (human) values for the iron content of different organs and tissues are stated, and the approximate daily fluxes of iron are also indicated. Iron losses result from sloughing of skin and mucosal cells as well as blood loss. Importantly, no regulated excretion pathway exists to control systemic iron homeostasis. Therefore, the control of systemic iron homeostasis lies in intestinal iron absorption.

Table 1-1. Dietary Iron Requirements (mg/d) by Life Stages[1]

Life Stage Group	Age	RDA/AI*	UL
Infants	0 – 6 mo	0.27*	40
	7 – 12 mo	11	
Children & Adolescents	1 – 3 y	7	
	4 – 8 y	10	
	9 – 13 y	8	
Males	14 – 18 y	11	45
	> 19 y	8	
Females	14 – 18 y	15	
	19 – 50 y	18	
	> 50 y	8	
Pregnancy	14 – 50 y	27	
Lactation	< 18 y	10	
	> 18 y	9	

[1]It represents Recommended Dietary Allowances (RDAs) of iron in ordinary type only and Adequate Intakes (AIs) in ordinary type followed by an asterisk (*). RDAs are set to meet the needs of almost all (97%) individuals in a group. For healthy breastfed infants, the AI is the mean intake. Upper limit (UL) is the maximum level of daily intake that is likely to pose no risk of adverse effects.

As for iron deficiency anemia, the symptoms are fatigue and weakness, decreased physical work performance, delayed motor and mental development, increased susceptibility to infections and increased risks for small or preterm babies. The causes of iron deficiency fall into 2 main categories, i.e., increased iron needs and decreased iron intake. Increased iron needs are commonly attributable to the following factors, including rapid growth, pregnancy, heavy menstruation, blood loss, frequent blood donation and gastrointestinal infections, while decreased iron intake is often associated with restricted diets lacking heme iron sources (e.g., for vegetarians), taking antacids or medicine to treat peptic acid reflux and drinking too much tea, coffee or milk that contains high levels of polyphenols, phytates or calcium. Therefore, high risk groups for iron deficiency are comprised of preterm infants, children, athletes and pregnant women because of rapid growth and inadequate intake, females at childbearing age due to menstruation, and people with chronic infections or restricted diets. The common tests for iron deficiency include hemoglobin test, hematocrit test, complete blood count, serum ferritin levels and transferrin saturation.

MECHANISM OF INTESTINAL IRON ABSORPTION

Dietary iron absorption occurs mainly in the proximal duodenum, where villi and microvilli protrude into intestinal lumen in order to maximize absorptive area. Intestinal iron absorption comprises three sequential steps: apical iron uptake from intestinal lumen into enterocytes, intracellular iron metabolism and transport, and basolateral iron export into circulation (Figure 1-2). Heme iron and non-heme iron are absorbed differently in terms of the first two steps.

A. Intestinal Absorption of Heme Iron

It has been shown that heme is first taken up from intestinal lumen into enterocytes as an intact metalloporphyrin. Within enterocytes, heme is supposed to be catabolized to biliverdin and free ferrous iron by heme oxygenases (HOs). Any free iron released from heme inside the enterocyte ultimately joins the labile iron pool, which is either incorporated into ferritin for transient storage or exported across basolateral membrane via ferroportin-1 (FPN-1) in the same manner as non-heme iron. Intact heme may be transported across the basolateral membrane by the receptor for feline virus subgroup C (FLVCR), where it binds circulating hemopexin. However, our data showed that intact heme export is less than 0.3% during a 7 h incubation of 1 μM heme in human intestinal-like Caco-2 cells, excluding this alternate pathway (Ma *et al.* 2010).

I. Apical Heme Uptake into Enterocytes

The mechanism of apical heme uptake has long been a mystery except for the following two points: (1) Heme is taken up into the enterocytes as an intact metalloporphyrin; (2) Heme is taken up via receptor-mediated endocytosis.

i. Heme Receptor-Mediated Endocytosis

There is a significant amount of evidence, which suggests that apical heme uptake occurs by receptor-mediated endocytosis, although the identity of this heme receptor in human is unknown. This hypothesis originated in 1979 with the discovery of a heme-binding protein in the duodenum of both pigs and humans (Grasbeck *et al.* 1979). A heme-binding protein with similar properties was also characterized on the membrane of the erythroleukemia cell line K562, which are capable of internalizing intact heme as evidenced by the binding and uptake of heme-embedded latex beads and thus resemble apical heme uptake.

The characteristics of apical heme uptake were analyzed in a recent study investigating heme iron uptake by Caco-2 cells from a hemoglobin digest and its response to different iron concentrations (Arredondo *et al.* 2008). The uptake of heme iron was a saturable and temperature-dependent process. Heme iron uptake was inhibited in cells exposed to K^+ depletion or cytosol acidification, suggesting that it could occur through a mechanism involving both a receptor and an endocytotic pathway.

This hypothesis was strengthened by morphological evidences showing that the absorbed heme appears initially on the microvillus membrane of enterocytes, and then becomes internalized into vesicles in the apical cytoplasm for 2-3 hours before disappearing, presumably degraded inside the vesicles (Wyllie *et al.* 1982). There was no heme observed in the basal

cytoplasm or the extracellular space, consistent with heme uptake at the microvillus membrane by an endocytotic pathway and its catabolism within the apical cytoplasm of the cell.

A recent animal study investigating the subcellular location of HO-1, HO-2 and DMT1 in duodenum of rats after administration of heme or ferrous iron has also provided evidence for the hypothesis that enterocytes take up heme via receptor-mediated endocytosis, which undergoes catabolism by HO within vesicles (West *et al.* 2008). The immunofluorescent results showed that HO-1 was distributed evenly throughout the cytoplasm of enterocytes and did not colocalize with endocytotic markers in any condition, while HO-2 staining remained constant with administration of heme, presenting as a dense band in the apical cytoplasm that colocalized extensively with endosomes. The subcellular translocation of HO-2 is therefore consistent with the receptor-mediated-endocytosis hypothesis and may suggest a possible role for this enzyme in heme degradation after heme uptake via receptor-mediated endocytosis. These combined data provide strong evidence for the ability of cells to actively acquire heme by endocytosis. One remaining question for apical heme uptake is that it assumes iron released from heme is transported out of the internalized vesicles in order to join the labile iron pool.

ii. Proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1)

The fact that mammals are capable of acquiring iron from heme implies the existence of a pathway that allows uptake of heme from the gut lumen into intestinal epithelial cells. In 2005, Shayeghi and his co-workers first described a heme carrier protein (HCP1), which is expressed in the apical region of epithelial cells of mouse duodenum (Shayeghi *et al.* 2005). Their identification of HCP1 provides a major missing piece of information in our understanding of apical heme iron uptake. At this early stage, the physiological relevance of this transporter to intestinal heme iron absorption is unclear, but the information that is available will be considered below.

The HCP1 cDNA was initially isolated by a subtractive hybridization of ileal cDNA from duodenal cDNA of hypotransferrinaemic mice. With homology to bacterial metal-tetracycline transporters, HCP1 was identified to mediate heme uptake by cells in a temperature-dependent and saturable manner. HCP1 mRNA was highly expressed in duodenum and regulated by hypoxia. HCP1 protein was translocated to brush-border membrane of duodenal enterocytes during iron deficiency and regulated by iron stores in regard to its localization. The overall data indicate that HCP1 is the long-sought intestinal heme transporter. Although no non-porphyrin competitor was assessed, uptake of radio-labeled heme by transfected CHO cells was competitive with the uptake of excess heme and zinc protoporphyrin, suggesting that this transporter is selective for the porphyrin ring.

Expression and functional analysis of HCP1 in Caco-2 cells, a commonly used model of human intestinal cells, were further analyzed by a molecular biological study. HCP1 mRNA expression in other cell types including HeLa, HEK293, HepG2, Hudu, K562 and U937 was also studied. The uptake of ^{55}Fe-labeled heme was determined under different conditions and HCP1 expression was measured by RT-PCR and immunohistochemistry. HCP1 mRNA and protein expression increased in Caco-2 cells with HCP1 adenoviral plasmid, and consequently ^{55}Fe-heme uptake was higher in these cells. Heme uptake was also increased in fully-differentiated Caco-2 cells when compared to undifferentiated cells. HCP1 expression and heme absorption were maximal in fully-differentiated intestinal cells. Preincubation of cells with DFO had no effect on HCP1 expression or heme uptake, while treatment with $CdCl_2$ to induce HO-1 enhanced HCP1 expression and increased heme uptake into the cells, indicating that HCP1 expression and function were adaptive to the rate of heme degradation by HO-1. Furthermore, HCP1 expression in different cells implies a functional role in tissues other than the duodenum.

On the other hand, HCP1 has been independently identified as a human proton-coupled, high-affinity folate transporter that recapitulates properties of folate transport and absorption in

intestine and in various cell types at low pH (Shayeghi *et al.* 2005). It was first identified by mining the human peptide database Ensembl at low stringency to search for potential folate transporter candidates. HCP1 appears to play a key role in intestinal folate absorption, as evident by a 55%-80% reduction in folate uptake in Caco-2 cells with small interfering RNA of PCFT/HCP1 (Nakai *et al.* 2007). In addition, a mutation in the PCFT/HCP1 gene has been demonstrated to be the molecular basis for hereditary folate malabsorption in human patients. Thus, a major function of HCP1 has been established as proton-coupled folate transport for humans and thus amended the name to PCFT/HCP1.

Interestingly, the folate transporting capabilities of PCFT/HCP1 are at least an order of magnitude higher than that observed for heme, suggesting that folate may be the more physiologically relevant target of this transporter. It is clear that the generation of a knockout model for PCFT/HCP1 is required to assess the importance of this heme/folate transporter in vivo. To clarify the relative capabilities of PCFT/HCP1 for heme and folate transport, heme and folate transport characteristics of PCFT/HCP1 were investigated both in vivo in mice and in vitro in cultured cells to elucidate further the substrate specificity and selectivity of PCFT/HCP1 and the possible interactions of heme and folate at the level of intestinal absorption (Laftah *et al.* 2009). The in vivo study revealed that the addition of folic acid inhibited ^{59}Fe-heme transport in hypoxic mice, while in vitro results showed the increase in ^{3}H-folate uptake from hypoxic mice was reduced by the addition of heme or PCFT/HCP1 antibodies to the medium. Caco-2 cells transiently transfected with PCFT/HCP1 siRNA have 69% reduction in PCFT/HCP1 mRNA when compared with the control. The magnitude of reduction with folic acid uptake was greater (48%) than that of heme (22.5%) with PCFT/HCP1 siRNA; however, both heme and folate uptake were significantly ($p < 0.05$) reduced.

Increased iron absorption is a primary defect of hereditary hemochromatosis. Homozygosity for the C282Y mutation of the HFE gene on chromosome 6p accounts for most

cases of hereditary hemochromatosis in Caucasians. Since HCP1 has recently been identified as a possible heme carrier by in vitro analysis, it might be a major mediator of heme iron absorption by intestine, and mutations within HCP1 may influence body iron stores either independently or as a modifier of clinical expression in individuals with other genetic configurations like HFE C282Y homozygote. A case-control study was conducted to determine whether mutations in the HCP1 gene influence serum iron measures either in those without HFE mutations or with homozygous HFE C282Y by examining the entire coding region of the HCP1 gene in 788 US and Canadian participants selected from the Hemochromatosis and Iron Overload Screening (HEIRS) Study. (Wang *et al.* 2009) For the case group, 298 non-C282Y homozygotes from four racial/ethnic backgrounds (White, Black, Asian, and Hispanic) with high serum ferritin and transferrin saturations were selected, while for the control group, 300 other random participants of the same racial/ethnic backgrounds from the same geographic locations were tested. The authors identified eight different HCP1 genetic variants; each occurred in a heterozygous state. Except one, each was found in a single HEIRS Study participant. Five HEIRS Study participants had non-synonymous coding-region HCP1 variants. Each of these five had transferrin saturations (TS) above the 84th gender- and ethnic/racial group-specific percentile. Thus, the authors concluded that HCP1 variants are uncommon in US or Canadian Whites, Blacks, Hispanics and Asians and people with non-synonymous coding-region mutations of HCP1 appear to have high TS. Studies with a much larger sample size are needed to clarify the distribution and associations of HCP1 variants with other genetic variants.

As many functional and regulatory facets of the molecular machinery for heme catabolism are shared by enterocytes and macrophages, it is reasonable to speculate that HCP-1 could also play a pivotal role in the turnover of hemoglobin (Hb)-derived heme by macrophages, which, in turn, may enlighten the mechanisms of heme absorption in enterocytes. Macrophages constitute the major cellular compartment for Hb degradation and subsequent recycling of heme

iron to erythropoiesis. Endocytosis by macrophage-specific scavenger receptor CD163 is the only known pathway for uptake of cell-free Hb up to date. However, the effectors involved in heme and heme iron handling after CD163-mediated Hb endocytosis had not been investigated yet. The existence of an intracellular pool of HCP-1, which was discovered in enterocytes, might provide clues regarding its role in the export of Hb-derived heme from endosomes and/or lysosomes. The role of HCP1 in macrophage Hb endocytosis pathway, mediated via CD163, and its response to inflammatory factors were therefore investigated (Schaer *et al.* 2008). The study revealed that HCP-1A and 1B were expressed in human macrophages. Within early endosomes, HCP-1A colocalized with endocytosed Hb-haptoglobin (Hp). Hb-Hp passed DMT1B/HCP-1A-positive endosomal compartment on its route from the cell surface to DMT1A-positive lysosomes. HCP-1 mRNA and protein expression were down-regulated by stimulation of macrophages with various TLR agonists and IFN. The profound suppression of HCP-1 expression by inflammatory macrophage activation paralleled the regulation of ferroportin. In contrast, dexamethasone enhanced HCP-1 expression significantly. Based on the spatial relationship, the authors proposed that the Hb scavenger receptor CD163 and HCP-1 constitute a linked pathway for Hb catabolism and heme iron recycling in human macrophages.

II. Heme Iron Catabolism within Enterocytes

There is strong evidence that heme is catabolized within enterocytes after being taken up. Dogs administered with radio-labelled hemoglobin, 90% of the recoverable radioactivity in portal blood over a period of 3 h was present as non-heme iron. The presence of a heme splitting substance in the mucosa was first demonstrated in 1968 (Weintraub *et al.* 1968). Further research revealed that the heme splitting substance was heme oxygenase, due to that heme oxygenase

almost exclusively generates the expected bilirubin IX-α isomer and that heme oxygenase activity is highest in duodenum where heme iron absorption is the highest.

HOs catalyze the oxidation of heme using cytochrome P450, NADPH and O_2 to produce CO, iron and biliverdin IX-α which is rapidly reduced to bilirubin IX-α. There are two well-characterized isoforms of HOs referred to as HO-1 and HO-2, which are products of different genes. A third isoform has been described as HO-3 but it appears to be a brain-specific pseudogene derived from HO-2.

While HO-2 is not inducible, HO-1 expression can be induced by numerous factors including hemin, oxidative stress, inflammation and metals including Cd, Co, Cr, Cu and Fe, etc. Its nature of inducibility by hyperthermia gives it an alternate name, heat shock protein 32. Considering these combined factors, induction of HO-1 expression appears to be related to preventing cell damage under many circumstances by reducing levels of the prooxidant heme and generating the antioxidant bilirubin. There is an in-vivo study exploring the tissue-specific regulation of expression of HO-1 and HO-2 under hypoxemia (Han *et al.* 2010). Two conditions, normobaric hypoxia exposure and HO-$2^{-/-}$ mice, respectively, were utilized to produce the effect of hypoxemia. There was no significant change in the overall expression levels of HO-1 and HO-2 mRNAs and proteins in the lung during the 28-day adaptation of C57BL/6 mice to normobaric hypoxia. However, immunohistochemical analysis revealed increased expression of HO-1 and HO-2 after 28-day normobaric hypoxia in the pulmonary venous myocardium. Moreover, the expression of HO-2 protein was enhanced primarily in the subendocardial myocardium of ventricles under hypoxia, while HO-1 level was increased in the full-thickness walls. Thus, hypoxemia induces expression of both HO-1 and HO-2 proteins in the myocardium. Using C57BL/6 mice lacking HO-2, which manifest chronic hypoxemia, HO-1 protein level in the lung was similar between HO-$2^{-/-}$ mice and wild-type mice. Unexpectedly, HO-1 level was decreased

by 35% in liver of HO-$2^{-/-}$ mice than that of wild-type mice. These results indicate that the expression of HO-1 protein is regulated in a tissue-specific manner under hypoxemia.

As to dietary heme absorbed into enterocytes, it has long been assumed that HO-1 plays a crucial role to split it into free iron, since both HO-1 and heme iron absorption are up-regulated in iron deficiency and HO-1 activity and heme iron absorption are both highest in duodenum, which is also the site of highest expression of PCFT/HCP1. However, HO-1 is generally considered to be a membrane-bound protein located in endoplasmic reticulum with a cytoplasmic catalytic site and have no topological association with endosomes that are thought to contain internalized heme. Therefore, the possibility that HO-2 is also important in heme iron absorption should be strongly considered. Recently, an animal study investigating the subcellular location of HO-1, HO-2 and DMT1 in duodenum of rats after administration of heme or ferrous iron provided evidence for this. (West *et al.* 2008) The immunofluorescent result showed that with the administration of either heme or non-heme iron, HO-1 was distributed evenly throughout the cytoplasm of enterocytes and did not colocalize with endocytotic markers, while HO-2 staining remained constant, presenting as a dense band in the apical cytoplasm that colocalized extensively with endosomes. DMT1 staining was markedly reduced by ferrous iron, but not by heme and did not exhibit colocalization with endocytotic markers. The subcellular translocation of HO-2 is therefore consistent with the RME hypothesis and may suggest a possible role for this enzyme in heme degradation.

Contribution of HO-1 to iron homeostasis has been postulated, since it facilitates iron recycling by liberating iron mostly from heme catabolism. HO-1 also appears to be responsible for the resolution of inflammatory conditions. In a patient with HO-1 deficiency, inflammation and dysregulation of body iron homeostasis, including anemia and liver and kidney hemosiderosis, are evident. A recent study postulated that HO-1 is critical in the regulation of ferroportin-1 (FPN1), the iron exporter, and hepcidin, the key regulator of iron homeostasis

central in the pathogenesis of anemia of inflammation (Kartikasari *et al.* 2009). In regards to FPN1, the authors found that HO-1 induces iron efflux by heme-driven iron-induced FPN1 expression in human THP-1 monocytes. As to hepcidin, the authors observed low hepcidin levels in the HO-1-deficient patient, reflecting increased need for iron in the bone marrow due to the anemia, while they demonstrated that HO-activity did not have a direct modulating effect on expression of hepcidin gene, HAMP, using human hepatoma cells. Furthermore, they evaluated these results with the measured levels of serum hepcidin, inflammatory markers, hematological measurements and iron parameters in blood or serum samples of the HO-1-deficient patient. These findings suggested that dysregulation of iron homeostasis in HO-1 deficiency is the result of both defective iron recycling and erythroid activity-associated inhibition of hepcidin expression. The authors concluded that serum hepcidin levels were low in the HO-1 deficient patient despite the presence of chronic inflammation and iron accumulation, which provided bioavailable iron for erythropoiesis to compensate for anemia and was not directly regulated by HO-1. In addition, free iron released from heme by HO-1 was capable of inducing FPN expression.

B. Intestinal Absorption of Non-heme Iron

Dietary ferrous iron is taken up at the brush border of duodenal enterocytes via DMT1, while dietary ferric iron must first be reduced to its ferrous state before it can be absorbed into intestinal epithelial cells. Cytosol ferrous iron can then be exported into the circulation by the basolateral iron exporter, ferroportin 1 (FPN1), and subsequently be oxidized by hephaestin, a multicopper oxidase homologous to ceruloplasmin. The resulting ferric iron is then loaded onto Tf in the circulation for delivery into tissues.

I. Apical Non-Heme Iron Uptake

As non-heme iron in foods primarily exists in insoluble ferric valence, apical non-heme iron uptake can be divided into two sequential steps: reduction of ferric iron to ferrous iron and apical uptake of ferrous iron. The overall iron acquisition pathway in the brush border of enterocytes involves both a ferrireductase and a ferrous iron transporter, DMT1.

i. Reduction of dietary ferric iron to ferrous iron

Dietary ferric iron must first be reduced to its ferrous form for uptake into duodenal enterocytes. Since ferric iron precipitates at pH > 3 but physiological pH of duodenum ranges from 4 to 7, ferric iron is insoluble and thus not available for absorption in duodenum, while ferrous iron remains soluble with pH up to 7.5 and is available for absorption in duodenum.

Ascorbic acid efficiently reduces ferric iron at low pH. In stomach, the reduction of ferric iron is greatly promoted by the presence of gastric H^+ and dietary ascorbic acid. Even at higher pH in duodenum, ascorbic acid is capable of forming soluble complexes with iron, which increase iron absorption. Iron deficiency leads to the induction of the gene encoding human vitamin C transporter SVCT1 as well as an increase in ascorbate levels in enterocytes (Collins *et al.* 2005).

The remaining ferric iron reaching the apical side of duodenum can be reduced by ferrireductases. In mammals, duodenal cytochrome b (Dcytb, also known as Crbyd1) and its cytochrome b561 homologues perform the role of intestinal ferrireductase.

Dcytb was first identified by a subtractive cloning strategy using iron-deficient rats (McKie *et al.* 2001). It is a plasma membrane protein with six transmembrane domains that exhibits ferrireductase activity, acting in concert with DMT1 for intestinal apical iron uptake. It is predominantly expressed on brush border of duodenal enterocytes and is rapidly induced in response to iron deficiency and hypoxia, highlighting its key role in iron absorption. Over-

expression of Dcytb in MDCK and Caco-2 cells was found to both increase ferric reductase activity and stimulate uptake of ^{59}Fe, suggesting its importance in iron metabolism (Latunde-Dada *et al.* 2008). The ability of Dcytb in reducing iron in enterocytes is markedly enhanced by increased level of ascorbic acid. Studies in Sf9 insect cells revealed that the two heme residues in Dcytb are reducible by ascorbic acid, suggesting that ascorbic acid could serve as an intracellular electron donor to Dcytb.

However, the requirement of Dcytb for intestinal iron absorption has been questioned since the Dcytb-knockout mice fed with a normal-iron diet did not display a severe defect in iron uptake (Gunshin *et al.* 2005). The lack of phenotype has led to suggestions that Dcytb is only one of several intestinal ferrireductases. In addition, as humans are scorbutic, humans may rely more on Dcytb, while mice may survive with self-produced ascorbic acid and other ferrireductase. Studies in Dcytb-knockout mice fed with an iron-deficient diet revealed that iron uptake into the mucosa from the diet tended to be lower in Dcytb-knockout mice but iron transfer from mucosa to the body was more efficient, suggesting that there may be some compensation in iron export into plasma in response to changes in body iron stores.

ii. Apical uptake of ferrous iron via DMT1

DMT1 is the major route for intestinal ferrous iron absorption following reduction of ferric iron. DMT1, also known as natural resistance-associated macrophage protein 2 (Nramp2), solute carrier 11 family A2 (SLC11A2) or solute cation transporter 1 (DCT-1), is a proton-coupled divalent metal transporter mediating the transport of a broad range of divalent metals, including Fe, Cd, Zn, Mn, Cu, Co, Ni and Pb, despite that its importance has only be set definite for ferrous iron transport in vivo (Fleming *et al.* 1997; Gunshin *et al.* 1997). The Nramp2 gene, originally cloned in 1995, is ubiquitously expressed at low levels, with highest expression in proximal duodenum. In the gut, it is primarily located on apical membrane of mature enterocytes in duodenum where its expression is regulated by iron status.

The human DMT1 gene has 17 exons encoding four splice variants that are functionally equivalent. DMT1 isoform 1A mRNA has an alternative promoter starting from exon 1A, while isoform 1B mRNA starts from exon 1B. In addition, there is alternative splicing at 3'-UTR, resulting in splice variants differed by owning and lacking the 3'-end iron-responsive element (+IRE/-IRE), respectively (Mackenzie *et al.* 2007). Other minor splice variants have also been observed. Expression of DMT1 isoforms are tissue-specific and have distinct subcellular distributions. Isoform 1A is found predominantly in duodenum and kidney, while 1B is ubiquitously expressed. Isoform +IRE is mainly expressed at the plasma membrane of epithelial cells for apical iron uptake and responds to iron status, while isoform -IRE is expressed in many cells and essential for uptake of iron into tissues via Tf cycle.

In small intestine, DMT1 exists as isoform 1A, with isform +IRE primarily located to apical membrane and isform –IRE well represented on membranes distinct from apical surface. DMT1 has been studied in both proximal small intestine and the human intestinal-like Caco-2 cells. Targeted DTM-1 gene knockout in mouse intestine was associated with anemia. Knockdown of DMT1 in Caco-2 cells also led to reduced ferrous iron uptake.

Animal models with mutated DMT1 that have hypochromic anemia have clarified the role of DMT1 in intestinal ferrous iron uptake. Its role was first confirmed by the identification of G185R mutation in DMT1 gene in the microcytic (mk) mice and Belgrade (b) rats (Fleming *et al.* 1998). Both models exhibited severe microcytic, hypochromic anemia and iron deficiency due to a defect in intestinal iron absorption as well as iron uptake in peripheral tissues.

In humans, however, given the nature of autosomal recessive disorder, DMT1 mutations of microcytic anemia are rare cases. Unlike in rodents, the first human DMT1 mutation was described in a Czech patient with a severe congenital hypochromic microcytic anemia and iron overload due to a defect of iron uptake into erythrocytes, which was attributable to a homozygous DMT1 point mutation, which exaggerated the skipping of exon 12 (Mims *et al.* 2005). Two other

mutations have been reported in patients leading to iron overload, even at a young age (Beaumont *et al.* 2006). This can be explained by the dual role of DMT1 in duodenum and other major tissues utilizing iron. Although DMT1 in duodenum is necessary for uptake of ferrous iron, these mutants did not completely abolish the role of DMT1 in duodenum; increased heme uptake may also compensate for reduced non-heme iron uptake, resulting in increased total iron absorption due to the anemia signals, while erythrocytes in need of large quantities of iron cannot efficiently utilize iron due to impaired Tf cycle. The increased absorption and decreased utilization, as a consequence, results in iron overload. However, not all DMT1 mutations in human leads to iron overload. A fourth human case with microcytic anemia but no iron overload was found recently due to a G75R point mutation in DMT1, whilst further studies are needed to confirm this.

Another study of non-transferrin-bound iron uptake by cultured cells indicated the possibility of another direct iron uptake system with properties distinct from DMT1 (Barisani *et al.* 1995), but this part has remained elusive. Calcium channels and neutrophil gelatinase-associated lipocalin (NGAL/24-3) appear to mediate the uptake of iron, but their physiological role has not been resolved (Oudit *et al.* 2003; Devireddy *et al.* 2005).

II. Intracellular Iron Metabolism & Transport

Once iron enters the enterocyte, it has 3 possible fates. Some is utilized by intestinal epithelial cell for the synthesis of heme, iron-sulfur clusters and other proteins that are keys for cell functions; some is transiently stored in ferritin (Ft) for future use or export, while others are exported across the basolateral membrane of enterocyte into the circulation. As the part of iron utilized by the cell or stored in Ft would be both lost when the enterocyte is sloughed to the intestinal lumen after 2 – 3 days on the villi, only iron exported into the circulation is considered absorbed.

The portion of iron that is not needed for immediate use or for export is stored in Ft, a ubiquitous and highly conserved protein capable of harboring up to 4500 iron atoms as the core (Harrison *et al.* 1967). Its surface consists of a protein shell of 24 heavy (H) and light (L) chain subunits. Ft sequesters ferrous iron from intracellular labile iron pool, during which the H-subunit is responsible for the rapid oxidation of iron from ferrous form to the chemically less reactive ferrihydrite to accomplish iron detoxification, whereas the L-subunit may support iron nucleation to complete iron storage. Iron release and degradation of Ft shell help mobilize iron for cellular usage or export via FPN-1. Ft degradation can occur through two different mechanisms. In most cases, expression of FPN-1 mediates iron mobilization from Ft preceding Ft degradation by proteasomes, while in the presence of an iron chelator desferrioxamine or events of autophagy, Ft is degraded in lysosome (De Domenico *et al.* 2006).

Intracellular transfer of ferrous iron might be associated with iron chaperones. For example, frataxin acts as an iron chaperone in mitochondria to modulate aconitase activity (Bulteau *et al.* 2004). In regards to the cytosolic iron chaperone between iron transporters and storage proteins, it was recently found that human poly (rC)-binding protein 1 (PCBP1) delivers iron to ferritin (Shi *et al.* 2008). Both in-vivo and in-vitro studies confirmed that PCBP1 bound to iron facilitated iron loading to ferritin, while depletion of PCBP1 inhibited ferritin formation and increased cytosolic iron pools, suggesting its key role in the trafficking of iron into ferritin. However, no other chaperones have been identified or clarified yet.

III. Basolateral Iron Export

Basolateral iron export into circulation from the enterocyte is not only mediated by the iron transporter protein but also requires a ferroxidase. The only cellular iron exporter in mammals identified to date is FPN-1 (Donovan *et al.* 2000). FPN-1 is highly expressed in the

iron-deficient intestine. It resides on the basolateral membrane of enterocytes, where it mediates iron export into circulation in concert with hephaestin (Heph), a ferroxidase homologous (50% identity, 68% similarity) to the abundant plasma ferroxidase, ceruloplasmin (Cp).

The sole basolateral iron transporter, discovered independently by three different labs, is FPN-1 (also called IREG-1, MTP1 and Slc40A1) (Abboud *et al.* 2000; McKie *et al.* 2000). Metal transporter protein 1 (MTP1) was cloned by construction of a library of mRNA sequence enriched for IRP-1 binding, FPN-1 was discovered by positional cloning of the gene responsible for hypochromic anemia in zebrafish mutant *weissherbst*, and iron-regulated transporter-1 (IREG-1) was identified in hypotransferrinemic mice. FPN-1 is a highly conserved protein with 571 amino acids. Expression of FPN1 mRNA and protein are abundant in all major tissues that metabolize iron. The 5'-UTR of Fpn1 mRNA has a functional IRE, subjecting it to induction by high iron status. Mutations in the gene encoding FPN-1 can result in a distinct iron overload disease with autosomal dominant inheritance, i.e., one type of hemochromatosis (Montosi *et al.* 2001). Individuals with heterozygous FPN-1 mutations have iron accumulation in macrophages, but relatively normal serum iron levels. Since there is no robust assay for FPN-1 activity, the mechanism by which these mutations affect protein function is not known yet. The pattern of iron accumulation suggests a defect in the recycling of iron, rather than a primary increase in intestinal absorption. As FPN-1 is highly expressed by the reticuloendothelial macrophages that recycle iron from the hemoglobin scavenged from senescent red blood cells, the recycling of iron through macrophages, around 200 mg per day, is much greater than the influx through intestinal epithelial cells, which is around 2 mg per day. This indicates that the loss of one FPN-1 allele reduces efficiency of recycling, leading to a misinterpretation of body iron stores, which may subsequently result in a signal to increase intestinal absorption to meet iron demands.

Iron export across the basolateral membrane of enterocyte into circulation is facilitated by the membrane-bound multi-copper ferroxidase, Heph. Heph was first identified in sex-linked

anemia (*sla*) mice that have blocked mucosal iron transfer (Vulpe *et al.* 1999). The absence of functional Heph in *sla* mice produced a phenotype of moderate hypochromic anemia by diminishing basolateral iron exit into circulation. Its plasma homologue Cp is known to mediate iron release from liver and tissues other than intestinal mucosa. Both Heph and Cp function to oxidize ferrous iron to ferric iron, allowing its loading onto the high-affinity serum Tf. The difference between Heph and Cp is that Heph contains an extra transmembrane domain. Studies have shown that Heph and FPN-1 colocalize on the basolateral membrane of intestinal epithelial cells and cooperate together for iron export (Han *et al.* 2007). Apart from the iron oxidation capability, other roles for Heph in iron metabolism have not been identified yet, but Cp is found to be required to maintain the localization of FPN-1 on cell surface (De Domenico *et al.* 2007). This situation mimics the FTR1/FET3 system for iron transport in yeast, in which the ferrous iron transporter FTR1 requires the multi-copper ferroxidase FET3 for synergic iron transport as well as correct localization onto the cell surface (Singh *et al.* 2006). As Heph requires copper for its ferroxidase activity, copper deficiency leads to microcytic hypochromic anemia that cannot be resolved by the administration of iron. Apo-Heph without copper is rapidly ubiquitinated and targeted for degradation in proteasomes. Early studies have reported decreased Heph and iron absorption in intestinal enterocytes from copper-deficient animals. In addition, copper-deficient swine is associated with decreased iron assimilation and diminished liver iron.

As to intestinal heme iron absorption, the overwhelming majority of heme is presumably catabolized by heme oxygenase to release free iron, which is then exported via FPN-1. Intact heme may be transported across the basolateral membrane by FLVCR to the bloodstream where it binds circulating hemopexin, but it was found that under normal conditions, intact heme export was not detectable. Previous studies showed that cats viremic with FeLV-C developed profound anemia with a paucity of colony-forming units (CFU-E) and erythroid precursors, suggesting that erythropoiesis is arrested at the CFU-E/proerythroblast stage (Quigley *et al.* 2004). It has been

demonstrated that FLVCR exports cytoplasmic heme and is crucial for erythropoiesis. So far no study has examined function of FLVCR in intestinal heme absorption in vivo, although studies of FLVCR expression in different cell lines suggest this exporter is also expressed in intestine and liver. Since FLVCR acts on membranes of erythroid precursors to regulate heme content, when heme synthesis rates are at their peak just prior to differentiation, it is unlikely that FLVCR is involved with heme uptake at the apical membrane. FPN-1 is therefore crucial in both heme and non-heme iron absorption by enterocytes and the mechanism of its regulation under various conditions is of critical importance for intestinal iron absorption.

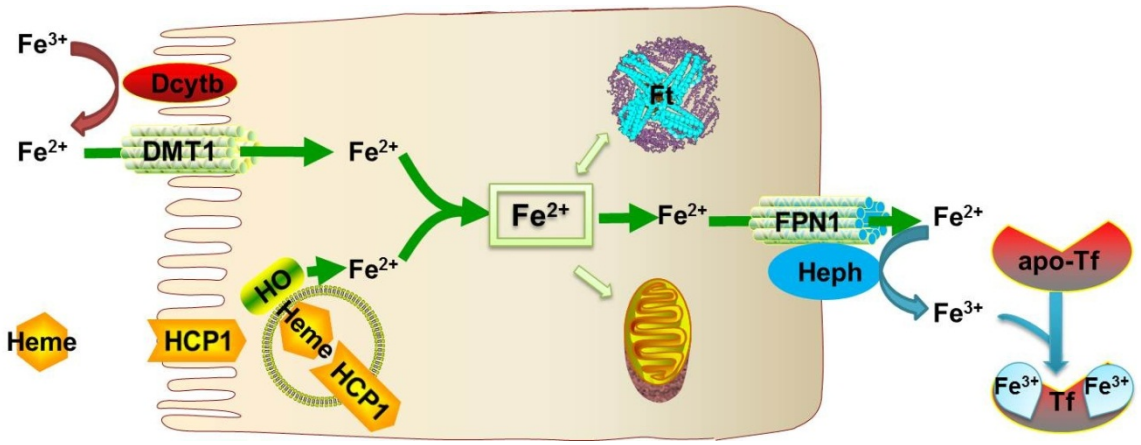

Figure 1-2. Molecular Mechanism of Intestinal Iron Absorption

Dietary non-heme ferric iron is firstly reduced to ferrous iron by Dcytb before it is absorbed via DMT1. Heme is taken up into enterocyte as an intact metalloporphyrin ring possibly via HCP1. Within enterocyte, heme is supposed to be split by HOs to release free ferrous iron. All free ferrous iron ultimately joins the labile iron pool and then either be utilized by the cell, or stored transiently in ferritin, or exported across basolateral membrane via FPN and subsequently be oxidized by Heph. The resulting ferric iron can then be loaded on to Tf for blood circulation.

REGULATION OF INTESTINAL IRON ABSORPTION

Systemic iron imbalance from both iron deficiency and iron overload leads to some of the most common diseases in humans including iron-deficiency anemia, anemia of chronic disease and hemochromatosis. As there is no regulated pathway for iron exit from body, the controlled absorption of iron in duodenum is a sine qua non for maintaining systemic iron homeostasis. On one hand, intestinal iron absorption is balanced by at least three regulatory factors – hepcidin, hypoxia-inducible factor-2α (HIF-2α) and IRE/IRP systems, which act at the post-translational, transcriptional and post-transcriptional levels, respectively, in response to different pathophysiological conditions, such as systemic iron levels and hypoxia. On the other hand, intestinal iron absorption is regulated by some dietary factors at the absorption level. This section discusses how these distinct factors function and coordinate with each other.

A. Regulation of Intestinal Iron Absorption by Hepcidin

The identification of the liver-derived peptide hormone hepcidin as the master regulator of systemic iron homeostasis has driven a major breakthrough in understanding the regulation of intestinal iron absorption and the nature of hemochromatosis. Hepcidin is a tightly-folded cysteine-rich 25-residue peptide matured from an 84 amino-acid prohormone by proteolysis through a consensus furin cleavage (Valore *et al.* 2008). The structure of hepcidin consists of a stable β-sheet element and a β-hairpin loop containing four disulfide bonds (Jordan *et al.* 2009).

Although hepcidin has been shown to possess the antimicrobial activity in vitro, its principal function in the body appears to be an orchestration of systemic iron fluxes. It is secreted from hepatocytes in response to a variety of signals affecting iron homeostasis and, in turn, acts on its downstream targets including but not limited to small intestine, macrophages and liver. The

mechanism of its action is via post-translational modification of FPN-1 to down-regulate iron release into the plasma (Figure 1-3). In specific, hepcidin secreted from liver circulates in plasma bound by α2-macroglobulin (Peslova *et al.* 2009) and binds to FPN-1 on cell surfaces of target tissues, triggering tyrosine phosphorylation, internalization and subsequent ubiquitination and lysosomal degradation of FPN-1 (Nemeth *et al.* 2004) (De Domenico *et al.* 2007). The five N-terminal amino acids of hepcidin is essential for its binding to FPN1 (Nemeth *et al.* 2006), which leads to the subsequent binding and activation of Janus kinase 2 (Jak2) which is required for phosphorylation of FPN1 before internalization (De Domenico *et al.* 2009). With hepcidin, cellular iron export is therefore inhibited due to the absence of cell-surface FPN1. In small intestine, this leads to reduced iron transfer to plasma Tf and iron retention in enterocytes, which will eventually be sloughed into intestinal lumen rather than absorbed.

The clearance of plasma hepcidin is through filtration in kidney or via internalization with FPN1 into cells of target tissues, which are relatively unregulated processes. In contrast, as an effecter hormone, hepcidin production in liver is controlled tightly at the transcriptional level in response to multiple signals affecting iron homeostasis. Both inherited and acquired disorders that perturb hepcidin production in liver result in the disruption of iron homeostasis and cause either iron deficiency or iron overload. Mutations in the hepcidin gene, *HAMP*, lead to juvenile hereditary hemochromatosis with virtually undetectable hepcidin levels, which are extremely rare though. There are four major conditions affecting hepcidin production in liver, including systemic iron status, erythropoietic activity, hypoxia, inflammation and stress, as discussed below (Figure 1-4).

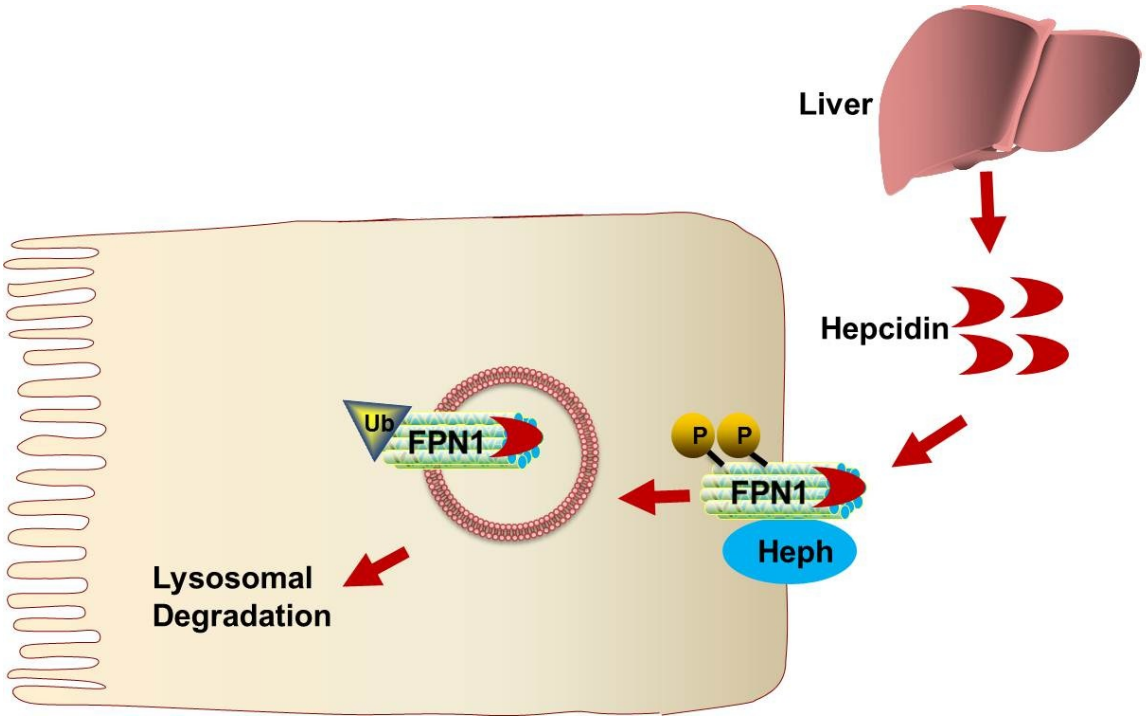

Figure 1-3. Posttranslational Regulation of FPN1 by Hepcidin

Liver-derived hepcidin regulates intestinal iron absorption by binding to FPN. Hepcidin binds to FPN on the basolateral membrane of the enterocyte and induces tyrosine phosphorylation, leading to its subsequent internalization into the cells. FPN is then ubiquitinated and targeted for degradation.

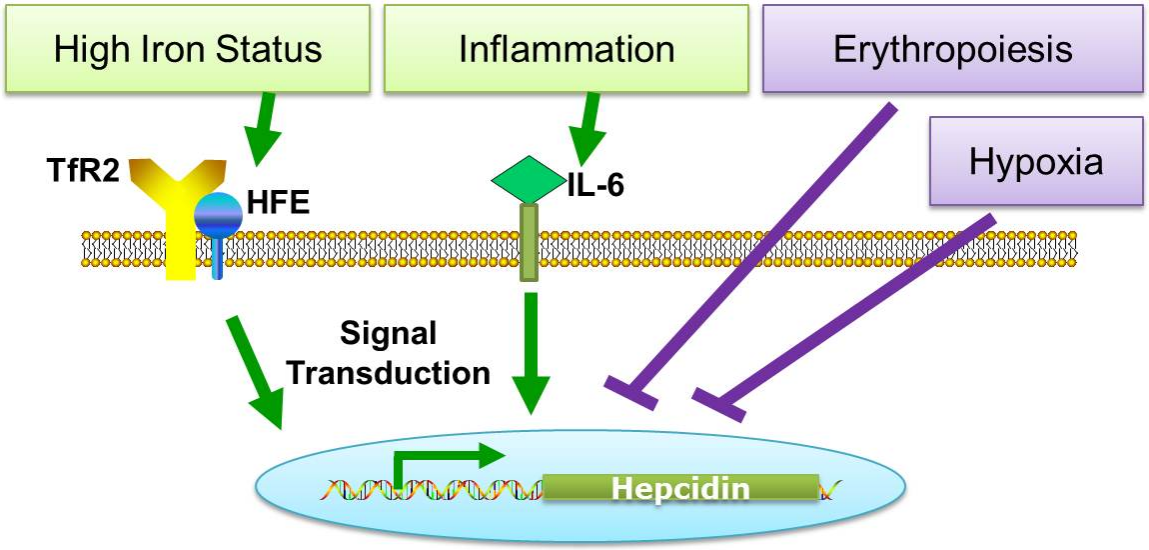

Figure 1-4. Four Conditions Affecting Hepcidin Expression in Liver

There are four conditions affecting hepcidin levels. Firstly, expression of hepcidin is induced by high iron status. HFE serves as a switch between TfR-1 & -2 on hepatocytes by sensing the plasma iron levels. When plasma Tf-bound iron level is high, more Tf-bound iron comes to bind TfR1, promoting the binding of HFE to TfR2, which triggers a signal transduction to induce hepcidin expression. HJV is also capable of inducing hepcidin expression through a BMP/Smad4 signaling pathway. Secondly, expression of hepcidin can be induced by inflammation. This is a host-defense response to withdraw iron from pathogens and malignancies. The pro-inflammatory cytokine, IL-6, activates hepcidin transcription via the Janus kinase/signal transducer and activator of transcription (Jak/STAT) signaling pathway. In addition, hepcidin expression is inhibited by erythropoietic signals and hypoxia. But the molecular mechanism mediating hepcidin inhibition under these two conditions is still uncertain.

I. Regulation of Hepcidin by Systemic Iron Status

Regulation of hepcidin production in response to systemic iron levels has advanced at the molecular level by the identification of proteins involved in hereditary hemochromatosis, including HFE, TfR2 and HJV. Consequently, mutations of these proteins lead to increased levels of iron absorption and recycling attributable to decreased transcription of hepcidin. There are two well-characterized mechanisms of liver hepcidin regulation, TfR1-HFE-TfR2 signaling that senses the amount of Tf-Fe_2 taken up into liver from plasma and BMP-HJV signaling that responds to iron status by transcriptional regulation of bone morphogenetic protein-6 (BMP6). However, the way in which these two pathways integrate or cooperate needs further exploration.

i. TfR1-HFE-TfR2 Signaling

Recent data have shed light on the potential for the HFE-TfR2 signaling pathway in regulating expression of hepcidin in the liver. Mutations in the hemochromatosis gene *HFE* leads to the most common form of hereditary hemochromatosis, which is an adult-onset and autosomal recessive disorder. HFE is expressed in both liver and duodenum, but tissue-specific HFE knockout in mice demonstrated that only HFE in liver is functional in regulating iron homeostasis (Vujic Spasic *et al.* 2007) and liver-specific knockout of HFE captured all phenotypes of total HFE knockout (Vujic Spasic *et al.* 2008). Mutations in TfR2 gene cause another form of hereditary hemochromatosis which may present early, but less severe phenotype than the juvenile form. TfR2 acts together with HFE to regulate hepcidin expression in liver.

As to the mechanism of HFE-TfR2 signaling in regulating iron homeostasis, HFE appears to serve as a switch between TfR1 and TfR2 on hepatocytes by sensing the plasma Tf-Fe_2 levels. TfR1 is ubiquitously expressed, while TfR2 is highly expressed in liver and has lower binding affinity to HFE than TfR1 does (Goswami *et al.* 2006). HFE and plasma Tf-Fe_2 compete

for binding sites on TfR1. Both mice with targeted disruption of TfR2 gene (Wallace *et al.* 2007) and mice bearing an engineered TfR1 mutation with enhanced HFE binding affinity revealed low basal hepcidin expression levels and systemic iron overload similar to HFE-knockout mice. Similar observations have been reported in humans with TfR2 mutations as in hemochromatosis. In contrast, mice with failure in HFE-TfR1 interactions resulted in high hepcidin expression and iron deficiency, which suggests that TfR1 sequesters HFE to prevent its binding to TfR2 to elicit subsequent induction of hepcidin transcription (Schmidt *et al.* 2008). A recent model in summary of these observations proposed that when plasma Tf-Fe_2 level is high, more Tf-Fe_2 comes to bind TfR1 and displace HFE from TfR1 to promote its binding to TfR2, which in turn triggers a signal transduction possibly via the MAPK/ERK pathway (Poli *et al.* 2010), inducing transcription of hepcidin and subsequently lowering iron release into plasma from tissues and plasma Tf-Fe_2 levels (Gao *et al.* 2009). Future research is needed to characterize the interactions among the proteins involved in this TfR1-HFE-TfR2 signaling.

ii. BMP-HJV-SMAD Signaling

The BMP-HJV-SMAD signaling pathway is critical in regulating liver production of hepdicin and consequently, iron homeostasis. Mutations in the gene encoding hemojuvenile (HJV, also known as HFE2) lead to juvenile hemochromatosis, which is the most severe form of hereditary hemochromatosis, apart from that of direct mutations in hepcidin gene *HAMP* (Papanikolaou *et al.* 2004); therefore, HJV is pivotal for regulating hepcidin expression. In addition, mice with either BMP6 or SMAD4 knockout possess severe iron overload to the same extent as mice with HJV knockout do.

Expression of BMP6 is positively regulated by iron status at transcriptional levels in vivo, which is contrary to other BMPs whose roles in iron homeostasis are still undefined (Meynard *et al.* 2009). The roles of other BMPs and the mechanism of BMP6 mRNA activation in response to

high iron levels deserve further investigation. HJV is a BMP co-receptor anchored to cell surface by glycosylphosphatidylinisotol (GPI) and HJV mutants contribute to impaired BMP signaling capacity (Babitt *et al.* 2006). The BMP/HJV complex in conjuction with BMP receptors induces downstream phosphorylation of receptor-regulated SMAD (R-SMAD) and subsequent activation of the sole mammalian co-SMAD factor, SMAD4, liver-specific disruption of which leads to severe iron overload (Wang *et al.* 2005). BMP antagonist dorsomorphin that competes for BMP receptors causes diminished hepcidin transcription and elevated plasma iron levels (Yu *et al.* 2008). SMAD4 forms a complex with SMAD1 or SMAD2 which binds to DNA and induces transcription of hepcidin. Recently, two BMP response elements have respectively been identified at proximal -84/-79 (BMP-RE1) and distal -2255/-2250 (BMP-RE2) of hepcidin promoter, which are critical for both basal hepcidin transcription as well as the hepcidin response to BMP-2 and BMP-6 (Casanovas *et al.* 2009).

As HJV on surface membrane of hepatocytes is pivotal in transmitting regulatory inputs for hepcidin expression, impairment of functional HJV would result in iron overload. On one hand, some stabilizing factor like neogenin may be necessary for maintaining membrane-bound HJV, as mice with neogenin mutations exhibit hepatic iron overload, reduced BMP signaling and low hepcidin levels (Lee *et al.* 2010). On the other hand, in response to low iron status, HJV can be cleaved off of the cell surface and then released as soluble HJV (sHJV) (Lin *et al.* 2005). Contrary to membrane-bound HJV, sHJV lessens the hepcidin expression and enhances plasma iron levels, possibly by competing with membrane-bound HJV for binding to the signal molecules BMPs. It was recently found that furin is capable of cleaving HJV off cell membrane (Silvestri *et al.* 2008). Expression of furin is upregulated in iron deficiency, leading to increased sHJV, reduced hepcidin production and consequently elevated plasma iron levels. The controversial question remains whether the cleavage of preprohormone form of hepcidin to matured hepcidin by furin is also enhanced by iron deficiency or not; if the answer is yes, whether

transcriptional regulation of hepcidin by furin is of overwhelming importance than its post-translational modification by furin needs to be addressed.

Apart from furin, the transmembrane protease serine 6 (TMPRSS6) has also been identified to target HJV for cleavage in vitro (Silvestri *et al.* 2008). This needs to be confirmed by in vivo studies. TMPRSS6 is primarily expressed in the liver. Mice with mutations in TMPRSS6 gene developed microcytic anemia with high hepcidin levels, indicating that TMPRSS6 is essential in detecting iron deficiency and blocking Hamp transcription (Du *et al.* 2008). In humans, mutations in TMPRSS6 gene causes iron-refractory iron deficiency anemia (IRIDA), which is characterized by congenital hypochromic and microcytic anemia unresponsive to oral iron therapy but partially responsive to parenteral iron administration, demonstrating that TMPRSS6 is essential for maintaining iron homeostasis (Finberg *et al.* 2008). As mutation in TMPRSS6 alone causes iron deficiency both in mice and humans, cleavage of HJV by other proteases appears less prominent. The mechanism of the response of TMPRSS6 to iron status remains to be explored. The relative significance of TMPRSS6 and furin in cleaving HJV and regulating iron homeostasis also needs to be determined.

II. Regulation of Hepcidin by Erythropoiesis

Expression of hepcidin is repressed by erythropoietic signals, especially in cases of iron deficiency anemia, hemolytic anemia and anemia with ineffective erythropoiesis. Since erythropoiesis demands 200 mg iron per day, suppression of hepcidin expression by erythropoietic signals is crucial in meeting iron needs. However, the molecular mechanism mediating hepcidin inhibition under this condition is still uncertain.

A study using mouse models with hemolytic anemia or with erythropoietin administration revealed that hepcidin production was repressed in both cases, which however,

was reversed by inhibitors of erythropoiesis, demonstrating that hepcidin suppression in response to anemia requires intact erythropoietic signals (Pak *et al.* 2006). Two erythropoietic signals during anemia that repress hepcidin production have been identified recently, namely, growth differentiation factor (GDF15) and twisted gastrulation protein (TWSG1), which are both erythrokines produced by erythroblast precursors (Tanno *et al.* 2007; Tanno *et al.* 2009). In patients with thalassemia that has deficient globin-chain production during erythropoiesis, GDF15 was overexpressed in accompany with inhibited hepcidin production, while depletion of GDF15 reversed hepcidin suppression, suggesting that GDF15 overexpression arising from erythropoiesis needs contributes to iron overload in thalassemia by suppressing hepcidin expression. Discovered in mice with thalassemia, TWSG1 also has increased expression during early stages of erythropoiesis. As tested in vitro with cultured human cells, TWSG1 inhibits hepcidin production indirectly by suppressing BMP2- and BMP4-dependent signal transduction which results in hepcidin induction. The expression of TWSG1 and its capability of suppressing hepcidin production need to be confirmed in human trials.

III. Regulation of Hepcidin by Hypoxia

Hepcidin production is reduced by hypoxia. It is possible that the iron-dependent prolyl hydroxylases (PHD) that degrades HIFs serve a link between iron sensing and hepcidin regulation by hypoxia, although the physiological significance of this regulation remains unsolved.

After a 20-day feeding period of HIF-1α knockout mice with an iron-deficient diet, hepcidin expression was 10-fold higher than WT littermates, indicating that deletion of HIF-1α affects suppression of hepcidin during iron deficiency (Peyssonnaux *et al.* 2007). In consistence with this, in mice with HIF-1α and HIF-2α stabilization in liver achieved by deletion of von Hippel-Lindau (VHL), hepcidin expression was decreased. The authors also found that HIF-1α is

able to bind the hepcidin promoter in the liver of both mice and humans, supporting the assumption of direct regulation of hepcidin by HIF-1.

Hypoxia may also regulate liver synthesis of hepcidin indirectly by modulating the HJV-BMP signaling pathway or by affecting erythropoietic signals. On one hand, studies have reported that expression of both TMPRSS6 and furin that are involved in cleavage of membrane-bound HJV are induced by hypoxia and by other activators of HIFs. This HIF-dependent upregulation of TMPRSS6 and furin increases sHJV, which in turn decreases hepcidin production in response to BMP signaling in hepatocytes, suggesting a potential role for TMPRSS6 and furin in hepcidin regulation by hypoxia (Silvestri *et al.* 2008; Lakhal *et al.* 2010). On the other hand, hypoxia potently induces expression of erythropoietin (EPO) that stimulates erythropoiesis and consequently suppresses hepcidin production. In mice with HIF-1α and HIF-2α stabilization in liver, EPO mRNA levels were strongly upregulated, which was consistent with increased erythropoiesis. EPO injections promoted decreased hepcidin levels secondary to enhanced erythropoiesis (Robach *et al.* 2009). At present, the exact mechanism for this remains unknown. Overall, whether HIF mainly regulates hepcidin transcription directly through binding or indirectly via affecting other signaling pathways needs further investigation.

IV. Regulation of Hepcidin by Inflammation and Stress

In view of its nature as an antimicrobial peptide, hepcidin is promptly induced by inflammation, which plays a critical role under the circumstances of anemia of chronic diseases. On one hand, mice injected with lipopolysaccharide (LPS) had enhanced hepcidin expression even in the context of iron overload; on the other hand, pretreatment of hepcidin protected mice from a lethal dose of LPS. In addition, hepcidin-knockout mice could be rescued from LPS toxicity by injection of hepcidin, suggesting the crucial role of hepcidin in modulating

inflammatory responses (De Domenico *et al.* 2010). As to the specific mechanism of increasing hepcidin production by inflammation, IL-6 and IL-1 were both shown to be potent inducers of hepcidin (Lee *et al.* 2005). IL-6 activates hepcidin transcription via the Janus kinase/signal transducer and activator of transcription (Jak/STAT) signaling pathway (Wrighting *et al.* 2006). Apart from this, hepcidin is also upregulated via the BMP/SMAD signaling pathway in the context of inflammation. However, IL-6 knockout mice with chronic inflammation mimicked their WT littermates in terms of increased hepcidin production, which indicated that other cytokines may be involved as well. IL-1 showed the capability of increasing hepcidin transcription in mice independently of IL-6.

Apart from inflammation and infection, endoplasmic reticulum (ER) stress also induces hepcidin expression, resulting in hypoferremia and spleen iron sequestration in mice (Vecchi *et al.* 2009). The response is mediated by the cyclic AMP response element-binding protein H (CREBH), an ER stress-activated transcription factor, which trans-activates the hepcidin promoter. In CREBH knockout mice, hepcidin induction by toxins or accumulation of unfolded protein in the ER is defective, indicating a role for CREBH in ER stress-regulated hepcidin expression.

B. Regulation of Intestinal Iron Absorption by HIF-2α

Hypoxia is important for the regulation of iron homeostasis. HIF was initially discovered as the major oxygen-regulated transcription factor that controls the expression of EPO and therefore relates tissue oxygen supply to red cell production. It has long been known that hypoxia induces an early increase in intestinal iron absorption after 6 hrs of the onset of hypoxia, preceding any alteration in plasma iron, erythropoiesis or hepcidin, suggesting hypoxia itself directly regulates iron absorption. Recently, it has been revealed that HIF-2α induces expression

of Dcytb and DMT1 drastically, and to some extent FPN1, at the transcriptional level, resolving the long-sought missing piece in regulation of Dcytb for intestinal iron absorption (Figure 1-5).

On one hand, hypoxia is a suppressor for a growing list of proteins involved in iron metabolism including hepcidin (the master regulator of iron homeostasis), TfR1, ceruloplasmin and HOs, which are known as HIF-responsive targets (Peyssonnaux *et al.* 2008). HIF binding to the promoter of hepcidin leads to the suppression of hepcidin expression in hepatocytes and increases in iron uptake to meet the erythropoietic demand. In addition, HIF-2α regulates expression of the mitochondrial aconitase chaperone protein frataxin, thereby controlling iron utilization in mitochondria (Oktay *et al.* 2007). HIF protein levels are negatively regulated by iron and oxygen by an oxygen-sensing mechanism. In the presence of oxygen, a regulatory subunit of HIF is modified by the action of prolyl hydroxylases (PHD) and is then able to interact with the E3 ubiquitin ligase von Hippel-Lindau tumor suppressor protein (pVHL) and subsequently targeted for degradation via the ubiquitin/proteasome pathway (Epstein *et al.* 2001). The HIF system has tissue specificity through variable expression of the several HIF α subunit isoforms (HIF-1α, HIF-2α, HIF-3α) and PHD isoforms (PHD1, PHD2, PHD3). Since not only oxygen, but also iron and ascorbate, are required for PHDs in order to modify the subunit of HIF, PHDs can also potentially act as iron sensors and HIF as an iron-sensitive transcription factor. Under hypoxia or following iron chelation, the activity of PHDs is inhibited, leading to the accumulation and translocation of HIF into nucleus. The extra requirement of PHDs for ascorbate adds a degree of complexity to the regulation of HIFs. Oxygen, iron and ascorbate are closely linked and duodenal ascorbate levels tend to change inversely with iron status (Atanasova *et al.* 2005). Further studies are required to figure out how these factors coordinately regulate iron absorption via regulation of PHDs.

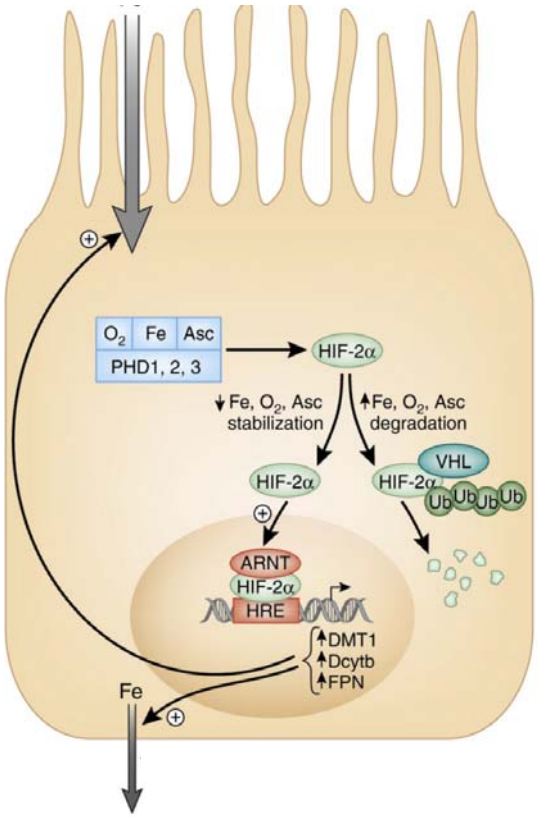

Figure 1-5. Transcriptional Control of Intestinal Iron Transport Genes by HIF-2α

This figure is cited from Simpson's paper by permission of Cell Metabolism (Simpson *et al.* 2009). HIF-2α directly binds to the HRE on the promoter region of Dcytb and DMT1 and induces their transcriptions. In the presence of iron and oxygen, HIF is modified by prolyl hydroxylases (PHD) and is subsequently targeted for degradation via the ubiquitin/proteasome pathway. Under iron-replete conditions, the expression of Dcytb and DMT1 are not induced and iron uptake is low due to minumal expression of Dcytb and DMT1. Under hypoxia or iron-deficient conditions, the activity of PHDs is inhibited, leading to the accumulation and translocation of HIF-2 alpha into nucleus, which subsequently induces expression of Dcytb and DMT1 and slightly FPN-1, leading to increased iron uptake into intestine.

On the other hand, two recent papers have provided compelling evidence that regulation of iron transport proteins by the transcription factor HIF-2α enables the duodenum to serve as both an important sensor and an essential regulator of iron absorption. DMT1 and particularly Dcytb are among the genes most highly upregulated by both iron deficiency and hypoxia in the duodenum. In the first study, the authors showed that HIF-2α but not HIF-1a mRNA was induced in the duodenum by iron deficiency (Shah *et al.* 2009). In the intestinal-specific VHL knockout (VHL KO, blocking HIF degradation) mice, there was a large induction of HIF-2α but not HIF-1α expression and both Dcytb and DMT1 were among the genes most highly activated in duodenum of these mice, suggesting that both Dcytb and DMT1 were transcriptionally regulated by HIF-2α. In contrast, in double VHL/Arnt knockout mice, the effects on iron metabolism were all reversed (Arnt is required for the formation of functional HIF-1α and HIF-2α transcription complexes). The Arnt KO mice actually became anemic with lower serum iron when fed an iron-deficient diet due to the lack of HIF-2α -mediated transcriptional response of Dcytb and DMT1 genes, thus providing strong physiological evidence for the role of HIF-2α.

In the second paper, the authors directly and selectively deleted HIF-1α and HIF-2α in the intestine (Mastrogiannaki *et al.* 2009). While intestinal deletion of HIF-1α had no effect on iron metabolism, intestinal HIF-2α KO had a similar phenotype to intestinal Arnt KO mice with markedly reduced mRNA levels for DMT1, Dcytb, Fpn, and hepcidin as well as decreased iron stores, serum iron and hemoglobin even on normal diet. The data suggested that the hepcidin and liver iron sensing mechanism were functioning correctly but were unable to counteract the altered expression of intestinal iron transporters. Both studies showed that the modulation of HIF-2α in the intestine altered serum iron and iron stores, with HIF-2α intestinal KO and Arnt intestinal KO, causing anemia and VHL intestinal KO which resulted in iron overload.

The recent advance has not only shed a light on how Dcytb, DMT1 and ferroportin are transcriptionally regulated by hypoxia, but has also provided a major molecular mechanism to

explain how local changes in enterocyte iron or oxygen alter duodenal transporter expression and dietary iron absorption. Therefore, these may suggest that alterations in HIFs at the intestinal level can override systemic regulation via hepcidin and provide alternative therapeutic strategies for interventions in control of iron absorption in iron-overload conditions that focus on local control of intestinal iron absorption. Hypoxia is known to increase intestinal heme iron absorption. As to proteins involved in intestinal heme iron absorption, PCFT/HCP1 mRNA was highly regulated by hypoxia. There are suggestions that HIF-2α may exert more control on the apical iron uptake pathway (Dcytb and DMT1) than the basolateral iron transport (FPN and Hephaestin). It would be interesting to examine whether hypoxia regulates PCFT/HCP1 functions in the apical uptake of heme iron to the same extent as it regulates DMT1 and Dcytb that function in the apical uptake of non-heme iron. In addition, the expression of HO-1 and HO-2 is regulated by hypoxia on a tissue-dependent manner (Han *et al.* 2010). Immunohistochemical analysis revealed that the increased expression of HO-1 and HO-2 after 28-day normobaric hypoxia in the pulmonary venous myocardium. Moreover, the expression of HO-2 protein was enhanced primarily in the subendocardial myocardium of ventricles under hypoxia, while HO-1 level was increased in the full-thickness walls. Thus, hypoxemia induces expression of both HO-1 and HO-2 proteins in the myocardium. However, it has not been tested whether HOs are regulated by hypoxia in small intestine.

C. Regulation of Intestinal Iron Absorption by IRE/IRP System

While key events of intestinal iron absorption are regulated post-translationally by hepcidin and transcriptionally by HIFs, another system that predominantly controls intestinal iron absorption is the IRE/IRP system, which is essential to maintain both structural and functional integrity of intestine (Muckenthaler *et al.* 2008). Both IRPs, including IRP1 and IRP2, are

expressed in the villi and crypts of Lieberkühn. In villus where enterocytes takes up dietary iron, IRP activity is subject to fluctuations in luminal iron levels (Figure 1-6). Consequently, with low dietary iron in small intestine, iron storage in Ft and iron utilizations in enterocytes are decreased, while apical iron uptake via DMT1 is enhanced, thereby increasing total iron absorption into the circulation. On the contrary, in crypts of Lieberkühn where the precursor cells lie, IRP activity is responsive to changes in plasma iron levels as the precursor cells acquire iron from the blood via TfR1. Thereby, when plasma Tf-bound Fe level changes, intestinal precursor cells respond by regulating IRP activity to subsequently modulating iron absorptive proteins during its maturation into enterocytes, which is known as crypt sensing hypothesis.

When cells are iron deficient, IRPs are active and bind to cis-regulatory hairpin structures known as IREs in untranslated regions (UTRs) of mRNAs of target genes to block translation or stabilize mRNA. Binding of IRPs to IRE at the 5'-UTR of target mRNAs, including mRNAs of Ft, FPN1, mitochondrial aconitase and HIF-2α, blocks their translation, while binding to IRE in 3'-UTR of mRNAs for TfR1 and DMT1 maintains their stability. In contrast, in iron-replete enterocytes, IRPs are inactivated; specifically, IRP1 is converted into a cytosolic aconitase by its 4Fe-4S cluster insertion, while IRP2 is targeted for degradation via the newly discovered E3 ubiquitin ligase complex consisting of F-box/LRR-repeat protein 5 (FBXL5), S-phase kinase-associated protein 1 (SKP1), cullin1 and RING-box protein 1 (RBX1) (Salahudeen *et al.* 2009; Vashisht *et al.* 2009). FBXL5 is degraded by the proteasome under low cellular iron levels or under hypoxia, which is attributable to the N-terminal amino acids of FBXL5, which fold appropriately into a hemerythrin-like domain with a di-iron-oxygen center only in presence of iron and oxygen. With high cellular iron levels, stabilized FBXL5 binds to IRP2 and recruit E3 ligase complex, promoting ubiquitination and subsequent degradation of IRP2 by proteasomes.

I. Down-Regulation by IRE/IRP (Ft, FPN1)

IREs located on the 5'-UTR of mRNAs, including but not limited to the iron storage protein Ft and iron exporter FPN1, are subject to IRP binding in order to inhibit their translation when iron status is low (Figure 1-7). Firstly, both ferritin H- and L- subunits are post-transcriptionally downregulated by IRPs in iron deficiency and vice versa, although whether decreased Ft production in enterocytes contributes to increased intestinal iron absorption remains uncertain.

Secondly and most notably, FPN1 mRNA possesses a functional IRE in its 5'-UTR, which is responsive to IRP binding during iron deficiency, indicating that low iron status would result in a block in FPN-1 mRNA translation in enterocytes, which is contradicted by the observation that intestinal iron absorption is enhanced in iron deficiency. A recent study has provided a mechanism that resolves this apparent paradox by showing that FPN-1 mRNA undergoes an alternate splicing leading to a fraction of the mRNA that lacks the IRE (Fpn1B) and thereby cannot be regulated via IRP/IRE mechanism, demonstrating that FPN-1 translation is only partially controlled by IRE/IPR system in small intestine (Zhang *et al.* 2009). Nonetheless, simultaneous ablation of both IRPs in the mouse intestine elicited a strong elevation in FPN1 expression despite increased hepcidin levels, indicating that the IRE/IRP system is as critical as hepcidin in securing physiological FPN1 expression in enterocytes.

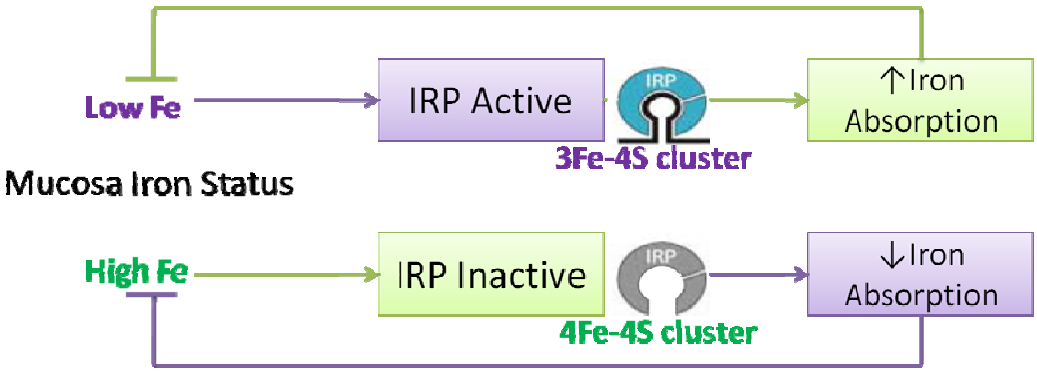

Figure 1-6. Feedback Loops of Intestinal Iron Absorption via IRE/IRP System

Free iron acts on its own level through elaborate feedback loops. IRPs actively bind to iron response element on mRNAs under low iron conditions. Ft and FPN1 have IRE at 5'-UTR of mRNA, while TfR1 and DMT1 have IREs at 3' of mRNA. When iron status is low, IRP binds to 5'-IRE of Ft and FPN1 mRNA, which blocks translation and downregulates levels of Ft and FPN1, while it also binds to 3'-IRE of TfR and DMT1 mRNAs, which stabilizes mRNA and increases their expression levels to intestinal iron absorption. When iron status is high, IRPs become inactive and cannot bind to IREs. The translation of Ft and FPN1 precedes and the mRNA of TfR1 and DMT1 are degraded to reduced intestinal iron absorption. Thus, free iron acts on its own level through these elaborate feedback loops.

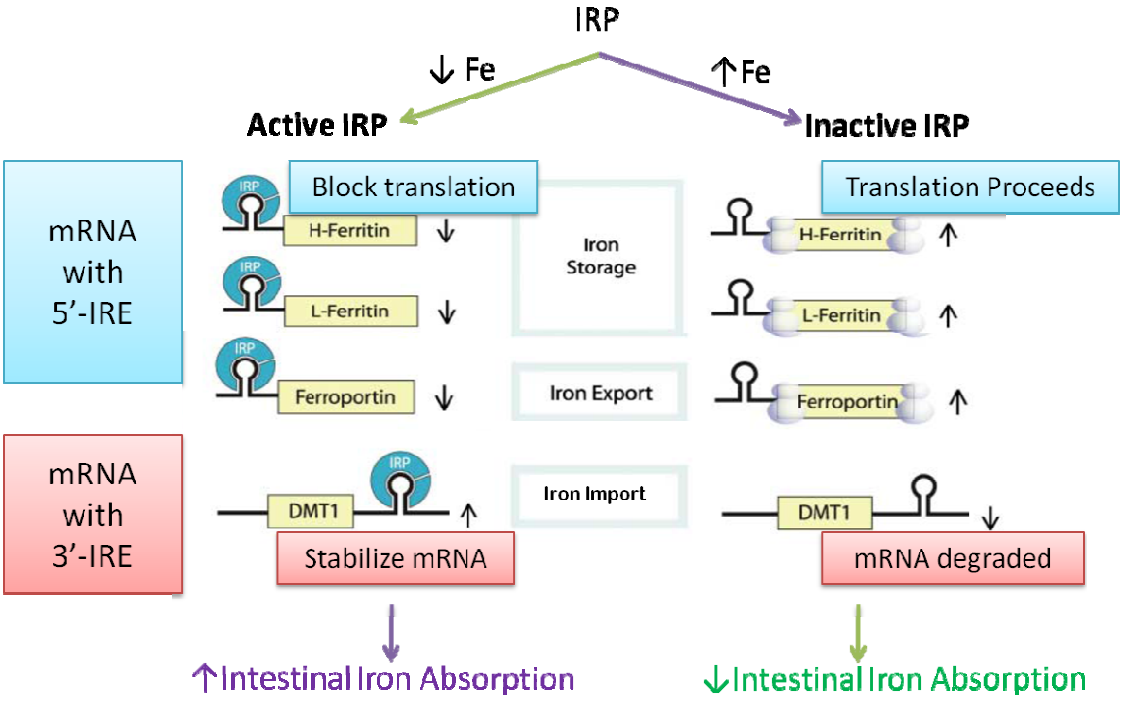

Figure 1-7. Regulation of Intestinal Iron Absorption by IRE/IRP System

This figure is cited and slightly modified from Hentze's paper by permission of Cell (Hentze *et al.* 2004). Proteins involved in iron storage, iron export, and iron uptake are coordinately regulated by the interaction of the iron regulatory proteins (IRPs) with conserved RNA secondary structures, the iron-responsive elements (IREs). The binding of IRPs to single IREs in the 5'-UTRs of mRNAs blocks their translation, while IRP binding to multiple IREs in the 3'-UTR stabilizes the TfR-1 mRNA.

II. Up-Regulation by IRE/IRP (DMT1)

The IRPs positively regulate DMT1 mRNA expression via a single 3'-UTR IRE (Figure 1-7). DMT1 mRNA has at least four different isoforms containing or lacking IRE in the 3'-UTR by alternative promoter and alternative splicing. In small intestine, DMT1 exists primarily as isoform 1A with IRE, which dominates the apical membrane. Despite the fact that DMT1 expression is not altered in mice lacking either IRP1 or IRP2, possibly by compensation for each other, simultaneous ablation of both IRPs in the mouse intestine caused a marked decrease in the IRE-containing isoforms of DMT1 mRNA, suggesting that the IRPs positively regulate DMT1 expression (Galy *et al.* 2008). The positive effect of the IRPs on DMT1 expression can therefore coordinate the IRE/IRP-mediated apical iron uptake with the hepcidin-mediated regulation of basolateral iron export. As the down-regulation of FPN1 by hepcidin blocks iron export, intracellular iron level is increased, reducing IRP activity and consequently decreasing DMT1 expression, which in turn lessening apical iron uptake and diminishing total iron absorption. However, the exact molecular mechanism by which the IRPs regulate DMT1 expression is unknown, possibly by stabilization of DMT1 mRNA similar to that of TfR1 mRNA.

D. Regulation of Intestinal Iron Absorption by Dietary Factors

Intestinal iron absorption is affected not only by the above pathophysiological conditions, such as body iron status and inflammation, but also by some dietary factors. Regarding the availability of non-heme iron absorption, it is conditioned by several dietary factors, such as meat, ascorbic acid, fiber and phytate. Dietary factors such as fiber and phytate mainly decrease non-heme iron absorption by chelating iron and forming insoluble complex in the intestinal lumen. Intestinal non-heme iron absorption in humans is promoted by animal tissues including pork, beef,

poultry and fish (Cook *et al.* 1976). Single-meal radioisotope absorption studies consistently revealed that beef, fish, chicken and calf thymus all increased non-heme iron absorption 2 – 3-fold, while egg albumin and the water extracts of beef had no effect on iron absorption (Bjorn-Rasmussen *et al.* 1979). Animal studies also demonstrated the enhancing effect of meat on iron absorption (Gordon *et al.* 1989). Substituting washed beef for lactalbumin increased the changes in hemoglobin concentration of rats fed with bran-derived iron significantly to 4 folds. More recently, a randomized cross-over trial in 21 young women with low iron stores (ferritin < 30 μg/l) showed that the addition of fish to the bean meal significantly increased iron absorption (Navas-Carretero *et al.* 2008). A randomized cross-over study in 19 healthy female subjects showed that non-heme iron absorption in the group with the addition of pork meat was significantly higher compared to those with vegetarian diet (Bach Kristensen *et al.* 2005).

Regarding the effect of dietary factors on intestinal heme iron absorption, not many studies have conducted to investigate into it. It is only established that heme iron absorption is enhanced by the presence of animal tissues or soy proteins, while it is inhibited by calcium, although the underlying mechanisms have not been completely elucidated. Animal tissues promote not only non-heme iron absorption, but also intestinal heme iron absorption (Hallberg *et al.* 1979). Soy proteins reduced non-heme iron absorption significantly, although it improved heme iron absorption (Lynch *et al.* 1985). Phytate is a major inhibitory factors in soy-protein isolates, which decreases non-heme iron absorption but does not affect heme iron absorption (Hurrell *et al.* 1992).

The factors in animal products that promote iron absorption are coined as “meat factor”, the nature of which remains unresolved. Although some evidence indicates that some proteins or peptide in meat may fulfill this function, it is still possible that some non-protein substances are involved (Hurrell *et al.* 2006). It seems that some cysteine-containing peptides enhance iron absorption due to their reducing and solubilizing capability (Taylor *et al.* 1986). Studies in Caco-2

cells indicated that carbohydrate fractions from cooked fish, especially glycosaminoglycans, contribute to the enhancing effect of meat on iron uptake by the enterocyte (Huh *et al.* 2004). In addition, L-alpha-glycerophosphocholine might also play a role in the enhancement of non-heme iron absorption by meat (Armah *et al.* 2008). However, there are also human studies with controversial results showing that neither cysteine nor glycosaminoglycans increases iron absorption (Bjorn-Rasmussen *et al.* 1979; Storcksdieck genannt Bonsmann *et al.* 2007). It has recently been found in macrophages that the iron export protein FPN1 is transcriptionally co-regulated with HO-1 by heme and that the protoporphyrin ring of heme is sufficient to increase FPN1 transcription (Marro *et al.* 2007). The murine macrophages treated with either 2 μM hemin (a chemical compound analogous to physiological heme) or 2 μM PPIX for 8 h showed a marked increase in FPN1 mRNA levels by approximately 10 fold. In specific, heme binds to cysteine residue of the transcription repressor Bach1, replacing it on the antioxidant response element (ARE) of FPN1 gene with the transcription activator Nrf2 and inducing transcription of FPN1 gene in macrophages (Figure 1-8). Although FPN1 is transcriptionally upregulated by heme in macrophages, because of the splice variants of FPN1 as well as different regulatory systems in macrophages and intestine, whether this happens in small intestine and whether the protein levels of FPN1 are increased by heme treatment have not been corroborated. Although heme is a component in animal products, no studies has investigated into the effect of heme on intestinal non-heme iron absorption yet.

Apart from the above factors, studies have revealed the possibility that some bioactive polyphenols might inhibit intestinal iron absorption. A study of iron absorption in humans found lower than expected absorption figures when tea was drunk with the meal. Since tea is a popular drink in a number of countries where iron deficiency is a major nutritional problem, this study undertook a formal investigation about the effect of tea on iron absorption (Disler *et al.* 1975). The authors concluded that tea inhibits the absorption of non-heme iron and uncooked

hemeoglobin iron to a significant extent. As polyphenols, especially epigallocatechin gallate (EGCG), is the major component of green tea, it is well possible that some polyphenols like EGCG could inhibit intestinal iron absorption. A recent study investigating whether beverages including wine, beer and tea, as well as some of their specific constituents, especially ethanol and polyphenolic compounds, affect the intestinal folate uptake revealed that all tested beverages and polyphenols significantly inhibited the uptake of folate by Caco-2 cells (Lemos *et al.* 2007). Some of the tested phenolic compounds, namely myricetin, EGCG and isoxanthohumol, markedly inhibited folate uptake. Resveratrol, quercetin and kaempferol were able to inhibit the transport of folate, but only in the concentration of 100 μM. Since dietary folate and heme share the transporter PCFT/HCP1 for absorption, the absorption of heme may also be modulated by dietary bioactive polyphenols.

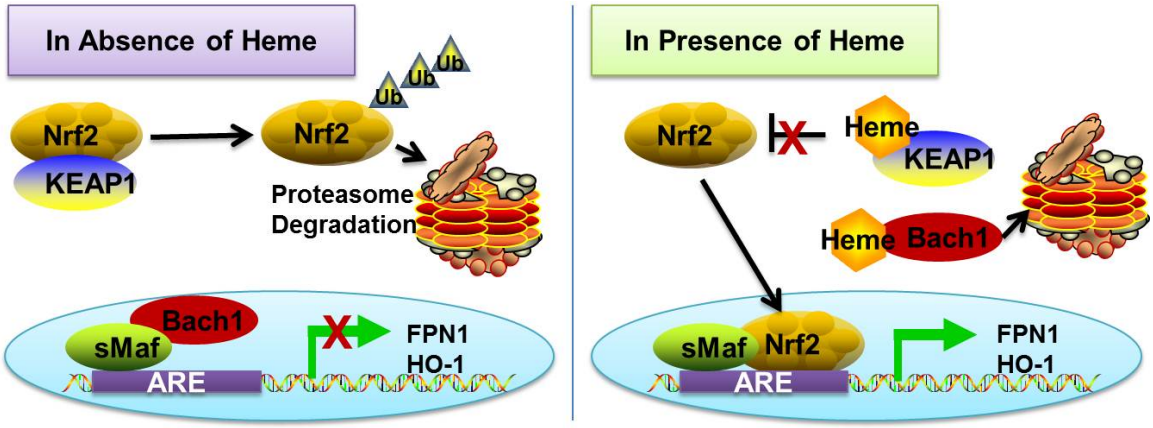

Figure 1-8. Regulation of FPN Expression by Heme in Macrophages

Heme accumulation leads to dissociation of transcriptional repressor Bach1 from the antioxidant responsive elements (ARE)/Maf recognition elements (MARE) enhancer, degradation of Kelch-like ECH-associated prFotein 1 (KEAP1), and accumulation of transcriptional activator Nrf2 in the nucleus. Nrf2 binds to small MAFs leading to enhanced transcription of FPN and HO-1.

CONCLUSIONS

Disruptions in intestinal iron absorption or its regulatory pathways pose a dual challenge to iron homeostasis – iron-deficiency anemia and iron overload diseases. Regulated intestinal iron absorption delicately equilibrates the demand for maximal iron absorption to prevent iron deficiency with the need to minimize iron intake to preclude iron overload. The three factors that modulate intestinal iron absorption in response to pathophysiological conditions reflect local and systemic signals of iron needs and represent three levels of control. The IRE/IRP system in representative of cellular iron status leads to the post-transcriptional up-regulation of DMT1 with the shortest response time. The HIF-2α indicating low local oxygen levels transcriptionally induces expression of DMT1, Dcytb and slightly FPN-1. The peptide hormone, hepcidin, signifying continuous high systemic iron challenges, causes FPN-1 internalization and degradation at the post-translational level. The presence of these three levels of regulation allows for precise control of intestinal iron absorption to maintain systemic iron homeostasis. Apart from the pathophysiological factors, however, intestinal iron absorption is modulated by some dietary factors, which affect intestinal iron absorption disregarding local and systemic signals of iron need and impress the overall iron bioavailability from meals.

Impressive progress has been made during the last decade in understanding intestinal iron absorption and its regulation. Future research is needed to resolve the following aspects. An urgent issue is to address the crosstalk among these three regulatory mechanisms of intestinal iron absorption and unravel how they integrate with each other. The regulatory roles of HFE and TfR2 may be further explored. The regulation of IRP1 and IRP2 itself in intestinal iron absorption is worth probing into. In addition, the fundamental molecular mechanisms for intestinal heme iron absorption and cellular iron biology are waiting for answers. How proteins involved in iron absorption coordinate under different conditions that affect intestinal iron absorption? What are

the structures of these transporters? How does intracellular iron traffic? Moreover, we also need to determine interactions of dietary iron with other metals and dietary factors. Finally, research efforts need to be devoted to regulations of iron homeostasis under different disease status so as to provide understandings or solutions for some common disorders such as inflammatory bowel diseases and cancers. For example, during inflammation, how alteration in iron metabolism bridges inflamed tissues and the immune system. The increasing new knowledge will form a complete picture of intestinal iron absorption and systemic iron homeostasis. Advances in mechanism of intestinal iron absorption and its regulation will be beneficial for translational research that improves therapeutic efficacies for iron dysregulation in the near future. The current research may well be translated into new drugs to treat iron-deficiency anemia and iron overload diseases.

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Chapter 2

STATEMENT OF HYPOTHESIS

The hypothesis of this dissertation was first developed during our studies regarding regulation of intestinal iron absorption. As reviewed in Chapter 1, intestinal non-heme iron absorption is regulated by several dietary factors, while studies of intestinal heme iron absorption are limited. In this dissertation, we focus on elucidating the role of two novel dietary factors for intestinal heme and non-heme iron absorption and determining the respective mechanism behind their effects on intestinal iron absorption.

In the first part of our study, we aim to determine the role of polyphenols on intestinal heme iron absorption. Polyphenols are a class of natural compounds widely distributed in fruits and vegetables. For example, green tea is a rich source of polyphenols. Tea is the second most popular drink worldwide, just next to water. Green tea constitutes approximately 20% of total tea consumption. Apart from the popularity as a beverage, polyphenols are also widely consumed as dietary supplements or in food fortification. For example, EGCG, the major polyphenolic ingredient of green tea, is widely used as a dietary supplement. GSE, containing a variety of polyphenols, is added to bread or meat products to boost the antioxidant activity of the specific food. Polyphenols have been attracting more and more attentions recently due to their reported health benefits, such as enhancing immune response and reducing risks of cancer. However, we are concerned whether polyphenols may incur an adverse effect on intestinal iron absorption because of their chelating capabilities. Previous studies in our lab using Caco-2 cells revealed that polyphenols such as EGCG and GSE drastically reduced intestinal non-heme iron absorption. Dietary factors affecting intestinal non-heme iron absorption usually target for apical uptake of

non-heme iron. The inhibitory effect of these polyphenols, however, differs from other dietary factors in that they mainly blocked basolateral iron release rather than affecting apical non-heme iron uptake. Because heme and non-heme iron share the same pathway for basolateral export into circulation (Figure 1-2), we postulated that these polyphenols would also inhibit heme iron absorption by blocking its basolateral release. Therefore, we hypothesize that polyphenols are a class of dietary inhibitor for intestinal heme iron absorption. In addition, because the amount of GT ingested varies greatly depending on the origination of green tea, brewing time and temperature, we are concerned if a tiny amount of GT would be capable of blocking intestinal iron absorption. Thus, we also aim to examine the dose-dependent effect of these polyphenols.

In the second part of our study, we aim to determine the role of heme on intestinal non-heme iron absorption. Animal tissues such as meat, poultry and fish are known to promote intestinal non-heme iron absorption in humans but the mechanism has never been adequately explained. The unique components of animal tissues that enhance non-heme iron absorption are termed as meat factor. While it was suggested to be some cysteine-rich proteins in animal products, previous studies indicated that certain non-protein substances in animal products could also be responsible for the enhanced non-heme iron absorption. Studies of those proposed meat factors, however, have been controversial and unsatisfactory. So we are wondering what else might be responsible for enhanced intestinal non-heme iron absorption as a meat factor. There is emerging evidence suggesting that some nutrients directly modulate the expression of enterocyte iron transporters. Interestingly, it has recently been reported that the expression of ferroportin-1 (FPN1) is up-regulated by heme treatment in macrophages. Thus, it is possible that heme may induce intestinal iron absorption by modulating the expression of FPN1 and other proteins involved absorption and metabolism. Therefore, we hypothesize that heme could be a meat factor that readily boosts intestinal non-heme iron absorption. In addition, we hypothesize that pretreatment of heme adaptively increases succeeding intestinal non-heme iron absorption by

inducing expression of the iron exporter FPN1 and possibly other genes involved in iron absorption.

Accordingly, four specific aims were designed to test the above hypotheses and addressed respectively in the following four chapters:

SPECIFIC AIM 1 (Preliminary Study)

Aim 1: To characterize intestinal heme iron absorption using intestinal Caco-2 cells.

A. To establish experimental condition and methods;
B. To assess the levels of ^{55}Fe-heme uptake and transport with different heme concentrations;
C. To determine the effect of time and temperature on ^{55}Fe-heme uptake and transport in Caco-2 cells;
D. To examine the proportion of heme released as free iron from Caco-2 cells.

SPECIFIC AIM 2 (Part I – Polyphenols)

Aim 2: To determine the effect of bioactive polyphenols, including EGCG, green tea extract and grape seed extract, on intestinal heme iron absorption using Caco-2 cell model.

A. To examine the effect of EGCG, green tea extract and grape seed extract on ^{55}Fe-heme uptake and transport in Caco-2 cells;
B. To examine the effect of very low amounts of EGCG, green tea extract and grape seed extract on iron uptake and transport in Caco-2 cells;
C. To assess whether the inhibitory effect of EGCG, green tea extract and grape seed extract on intestinal iron absorption could be reversed by other dietary factors;
D. To determine the mechanism by which EGCG, green tea extract and grape seed extract inhibit heme iron absorption.

SPECIFIC AIM 3 (Part II – Heme: Immediate Effect)

Aim 3: To determine the direct interactions between heme and non-heme iron during iron absorption processes using intestinal Caco-2 cells.

A. To examine the immediate effect of heme on intestinal non-heme iron absorption in Caco-2 cells;
B. To determine the mechanism by which heme stimulates non-heme iron absorption;
C. To assess the immediate effect of non-heme iron on heme iron absorption;
D. To determine the mechanism by which non-heme iron enhances heme iron absorption.

SPECIFIC AIM 4 (Part II – Heme: Adaptive Effect)

Aim 4: To explore adaptive effect of heme consumption on intestinal non-heme iron absorption and metabolism using Caco-2 cell model.

A. To determine the adaptive effect of long-term heme treatment on intestinal non-heme iron absorption;
B. To investigate possible mechanisms by analyzing the adaptive effect of heme treatment on the expression of FPN1.

SIGNIFICANCE OF RESULTS

Intestinal iron absorption is tightly regulated to ensure iron homeostasis. As there are limited studies regarding the mechanism and regulation of intestinal heme absorption, the results of the first aim will be the basis for study of subsequent aims and for future studies in this area.

The results of the second aim will highlight a new class of dietary factor, bioactive polyphenols, that impair intestinal absorption of both heme and non-heme iron and be the first report to clarify the mechanism how tea and some bioactive dietary polyphenols inhibit iron transport across the enterocyte. If these are also true for in vivo studies, then polyphenols would

be recommended to be separated from meals for people with iron deficiency and may serve as a treatment for people with iron overload.

The results of the third aim will answer whether and how intestine balances between absorption of the two forms of dietary iron. In addition, they will provide a new perspective to characterize intestinal heme and non-heme iron absorption jointly and serve as a basis for future study in this area. They will also be inspiring if intestinal heme and non-heme iron absorption boost each other, which would be beneficial for people with iron deficiency to enhance their iron absorption. It will not only partially explain how meat enhances non-heme iron absorption, but also illuminate a new way of iron supplementation.

Last but not the least, the results of the final part will establish a new mechanism for regulating intestinal iron absorption through modulating transcriptional levels of FPN1 by heme. In addition, they will substantiate whether and how long-term consumption of animal products increases intestinal non-heme iron absorption, resolving the missing piece in understanding the adaptive effect of meat on iron absorption.

The overall results could be applied practically as a dietary strategy to enhance iron absorption for people with iron deficiency to take into consideration and for people with iron overload to take caution.

Chapter 3

CHARACTERIZATION OF HEME IRON ABSORPTION

ABSTRACT

There are two types of dietary iron, heme iron, derived from hemoglobin and myoglobin in animal products, and non-heme iron, which is mainly from plant sources. The absorption pathways for heme and non-heme iron differ greatly. The mechanism of heme iron absorption remains elusive and studies regarding heme iron absorption are limited. The purpose of this study was to characterize apical uptake and transepithelial transport of heme iron from a hemoglobin digest in human intestinal Caco-2 cells and its response to different heme concentrations. We studied the effect of time, temperature, and excessive heme or non-heme iron on apical uptake and transepithelial transport of heme iron in Caco-2 cells. Our results showed that heme iron uptake and transport were both time- and dose-dependent. Excessive non-heme iron did not compete with heme iron absorption. Heme iron absorption was inhibited in cells incubated at 4°C. The overwhelming majority of iron in heme (> 99.7%) was released into basolateral chamber as heme-free iron. In summary, the absorption of heme iron is a saturable and temperature-dependent process.

Keywords

Intestinal iron absorption, heme iron, Caco-2 cells

INTRODUCTION

The importance of dietary heme iron cannot be underestimated (Carpenter *et al.* 1992). Heme iron only constitutes one-third of total dietary iron for an average non-vegetarian person in western countries, but makes up two-thirdss of the total absorbed iron, suggesting that heme iron is more efficiently absorbed than non-heme iron. The absorptive processes of heme and non-heme iron are different. Although mechanism of intestinal heme iron absorption is still uncertain, the following aspects have been proposed or substantiated. Before heme iron can be absorbed, heme must be released from dietary hemoglobin or myoglobin by proteolytic enzymes in stomach and small intestine (Conrad *et al.* 1967). Heme iron absorption mainly occurs in the duodenum. As heme is soluble in an alkaline environment, no binding proteins are necessary for its duodenal absorption. Apical heme uptake is suggested to occur via receptor-mediated endocytosis, although the characteristic of this receptor in humans is unknown. The heme carrier protein (HCP1) is proposed as a protein involved in apical heme uptake (Shayeghi *et al.* 2005). After the heme group enters the enterocyte as an intact metalloporphyrin, it is delivered to a microsomal compartment in the apical side of enterocyte, where it is split by heme oxygenases (HO) into free ferrous iron, carbon monoxide, and biliverdin, which is then rapidly converted to bilirubin (Weintraub *et al.* 1968). Free iron released from heme inside enterocytes joins the labile iron pool in cytoplasm and is then either utilized by the cell or transiently stored in ferritin, or transferred across the basolateral membrane of the enterocyte via ferroportin-1 (FPN1), the only iron exporter in mammalians (Donovan *et al.* 2000), and then oxidized to ferric ion by the hephaestin (Heph) on the basolateral surface prior to release into the circulation (Vulpe *et al.* 1999).

Cell culture models have been increasingly used for the study of iron absorption, and Caco-2 cell line is without any doubt the most competitive cell model in this regard (Halleux *et al.* 1994). It spontaneously differentiates into polarized columnar cells with tight junctions and

exhibits many morphological and functional properties of mature enterocytes, including polarization, the formation of brush border microvilli and the presence of brush border associated enzymes and transporters (Pinto *et al.* 1983). Analysis of heme iron uptake in Caco-2 is usually preceded by hemoglobin digestion to simulate the conditions in vivo. Culturing the cells on a microporous insert allows for precise measurement of iron transport from apical to basolateral chamber. Fully-differentiated Caco-2 cells express HO-1, whose activity can be induced by heme or heavy metals like cadmium (Cable *et al.* 1993). There is a good correlation between iron uptake in Caco-2 cells and human iron bioavailability for a number of dietary factors known to affect iron absorption (Yun *et al.* 2004). Caco-2 cells mimic in-vivo dietary iron absorption in many ways and have been established as a good model for ranking foods with respect to bioavailability, for mechanistic studies of iron absorption, and for studies of dietary factors influencing absorption.

The aim of this study was to characterize heme iron uptake and transport using human intestine-like Caco-2 cell model and its response to different heme iron concentrations.

MATERIALS AND METHODS

Reagents

The ^{55}Fe (in the form of $^{55}FeCl_3$) was obtained from Perkin–Elmer Life Sciences (Boston, MA). Hanks' balanced salts solution (HBSS), glutamine, nonessential amino acids and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Unless otherwise noted, all other reagents were obtained from Sigma Chemical (St. Louis, MO), VWR (West Chester, PA), or Fisher Scientific (Springfield, NJ).

MEL Cell Culture and Synthesis of ^{55}Fe-Hemoglobin (Hb)

The murine erythroleukemia (MEL) cell line was a generous gift from Dr. Robert Paulson at Pennsylvania State University (University Park, PA). The MEL cells were grown in the same conditions as described above for the Caco-2 cells. As previously reported, Hb was synthesized using MEL cells as previously reported (Ma *et al.* 2010). Briefly, to induce Hb synthesis, erythroid differentiation of cells was induced by adding dimethyl sulfoxide (DMSO). $(^{55}Fe)_2$-Tf was prepared from apo-Tf and ^{55}Fe and purified. The level of Tf saturation was estimated from the A_{465}/A_{280} ratio, which was routinely found to be 0.046, consistent with complete saturation on both sites of Tf for iron binding (Huebers *et al.* 1978). To produce ^{55}Fe-Hb, MEL cells were seeded at 10,000 cells/cm^3 and were treated with 2 μmol/L $(^{55}Fe)_2$-Tf and 2% DMSO. After a 6-d treatment, the cells were harvested and washed three times with phosphate-buffered saline (PBS, pH 7.0) and then collected by centrifugation for 5 min at 800 xg, 4°C.

Spectrophotometric Quantification of Hb and Hb Digestion

Hb concentrations in MEL cell lysates were measured by the benzidine assay as previously described (Ma *et al.* 2011). Briefly, pellets of MEL cells (70 x 10^6 of cells) harvested after 6 d were re-suspended in 0.5 mL distilled water and lysed by 4 cycles of freezing (in dry ice/ethanol for 3 min) and thawing (in a water bath at 37°C for 3 min). The cell lysate were centrifuged at 14,000 xg for 15 min at 40°C using an Eppendorf 5402 centrifuge (Hamburg, Germany). Benzidine assay was performed in duplicate by adding the reagents in the following order: 100 µL supernatant, 900 µL deionized water and 100 µL freshly prepared benzidine-HCl (10 mg/ml in 0.5% acetic acid). The reaction was started by the addition of 40 µL 30% H_2O_2. The contents were mixed well and after exactly 90 sec the absorbance was measured at 604 nm. The Hb concentration was then calculated based on the measured absorbance using a calibration curve obtained with purified Hb standard (Sigma, Saint Louise, MO) measured in the same way as the supernatant. Both Hb-^{55}Fe prepared from MEL cell lysis and commercially bought bovine Hb were digested with 0.1% pepsin at pH 2.0 overnight at 37°C. The solution was then centrifuged for 5 min at 14,000 xg at 4°C. The digestion rate was estimated by measuring the remaining Hb content in the supernatant using benzidine assay as described above. Pellets of ^{55}Fe-heme and heme were dissolved in 10 mmol/L of NaOH and further diluted in iron uptake buffer (final pH 7). The ^{55}Fe-heme specific activity was between 0.40 Ci/mol and 0.45 Ci/mol heme.

Caco-2 Cell Culture

The human Caco-2 cell line HTB37 was purchased from American Type Culture Collection (Rockville, MD). Stock cultures were maintained at 37°C in complete medium in a humidified atmosphere of 95% air and 5% CO_2 and used for experiments within 20 serial passages. The complete culture medium contained Dulbecco's Modified Essential Medium (DMEM) supplemented with 25 mM glucose, 2 mM glutamine, 100 µM non-essential amino acids, 100 U/l penicillin G, 100 mg/l streptomycin and 10% FBS. For experiments, 5.0 × 10^4

cells/cm^2 in 1.5 ml complete DMEM were seeded on 3 μm microporous membrane inserts (4.9 cm^2, BD Biosciences, Bedford, MA) coated with collagen type I (5 μg/cm^2) on 6-well plates. The basolateral chamber was filled with 2.5 ml complete DMEM. The culture medium was changed every 2 days, and cells were used after 17-day post-confluence for experiments. The Caco-2 cell monolayer was routinely checked for tight junctions at 17-day postconfluence with transepithelial electrical resistance (TEER) >250 Ω/cm^2, which indicates full differentiation under normal cell culture conditions.

^{55}Fe Transport Study

After washing the cell monolayer three times with Ca^{2+}- and Mg^{2+}-free Hank's balanced saline solution (HBSS) containing 137 mM NaCl, 5.36 mM KCl, 1.3 mM $CaCl_2$, 410 μM $MgSO_4$, 490 μM $MgCl_2$, 337 μM Na_2HPO_4, 440 μM KH_2PO_4, 4.17 μM $NaHCO_3$ and 5.55 mM dextrose at 37°C, cells were incubated at 37°C with 1.5 ml of ^{55}Fe/Fe(NTA)$_2$ or ^{55}Fe-heme in iron-uptake buffer in the apical compartment and 2.5 ml DMEM in the basolateral compartment. The uptake buffer contained 130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES, pH 7.0. Transepithelial iron transfer from apical to basolateral chamber and cellular iron accumulation were determined. An aliquot of 200 μL was removed from the basolateral chamber at the indicated time points and replaced with an equivalent volume of pre-warmed DMEM; time course data were corrected to account for this sample replacement. To measure the cellular levels of ^{55}Fe, cell monolayers were washed three times with ice-cold wash buffer containing 150 mM NaCl, 10 mM HEPES, pH 7.0, and 1 mM EDTA to remove any nonspecifically bound radioisotope. Cells were homogenized in PBS containing 0.3% Triton X-100, and ^{55}Fe was quantified by liquid scintillation counting in glass vials. Protein levels were assessed using Bio-Rad protein assay kit (BioRad Laboratory Inc., Hercules, CA). All ^{55}Fe transport studies were repeated for at least 3 times.

Statistical Analysis

Values are expressed as non-transformed means ± SEM. The experiments were repeated at least three times with more than three wells for each treatment. Data were analyzed by 1-way ANOVA with the following post-hoc tests for multiple comparisons using Prism 5.0 software (GraphPad). Differences were considered significant when $p < 0.05$.

RESULTS

3.1 Dose-dependent Heme-^{55}Fe Uptake and Transport in Caco-2 Cells

To study the characteristic of uptake and transport of different concentrations of heme in fully-differentiated Caco-2 cells, we first analyzed the amounts of heme taken up into the cells and those exported into basolateral chamber when different concentrations of heme-^{55}Fe were applied to the apical chamber for 1 h.

Our results showed that the apical heme-^{55}Fe uptake in fully-differentiated Caco-2 cells was dose-dependent and saturable (Figure 3-1 C & D). The maximal apical uptake of heme-^{55}Fe was observed when the concentration of heme-^{55}Fe in the uptake buffer was above 50 μmol/l. Analysis of the data using the Michaelis-Menten model indicates that the apparent Km and Vmax for apical heme-^{55}Fe uptake were 4.76 ± 0.26 μmol/l and 1.54 ± 0.06 nmol/mg proteins, respectively. The rate of apical heme-^{55}Fe uptake was reduced with higher concentration of heme-^{55}Fe. As 10 grams of beef produces about only 1 μM heme in digestive juices, we also explored the characteristics of apical heme-^{55}Fe uptake when lower concentrations of heme-^{55}Fe were applied. The dose-response curve of apical heme-^{55}Fe uptake was almost linear when the concentration of heme was between 0.5 and 10 μmol/l (Figure 3-1 E).

More importantly, the transepithelial transport of heme-^{55}Fe across the fully-differentiated Caco-2 cell monolayer was also dose-dependent and saturable, with maximum transport occurring at the concentration of 50 μM (Figure 3-1 A & B). The rate of transepithelial transport of heme-^{55}Fe was decreased with increased concentration of heme-^{55}Fe.

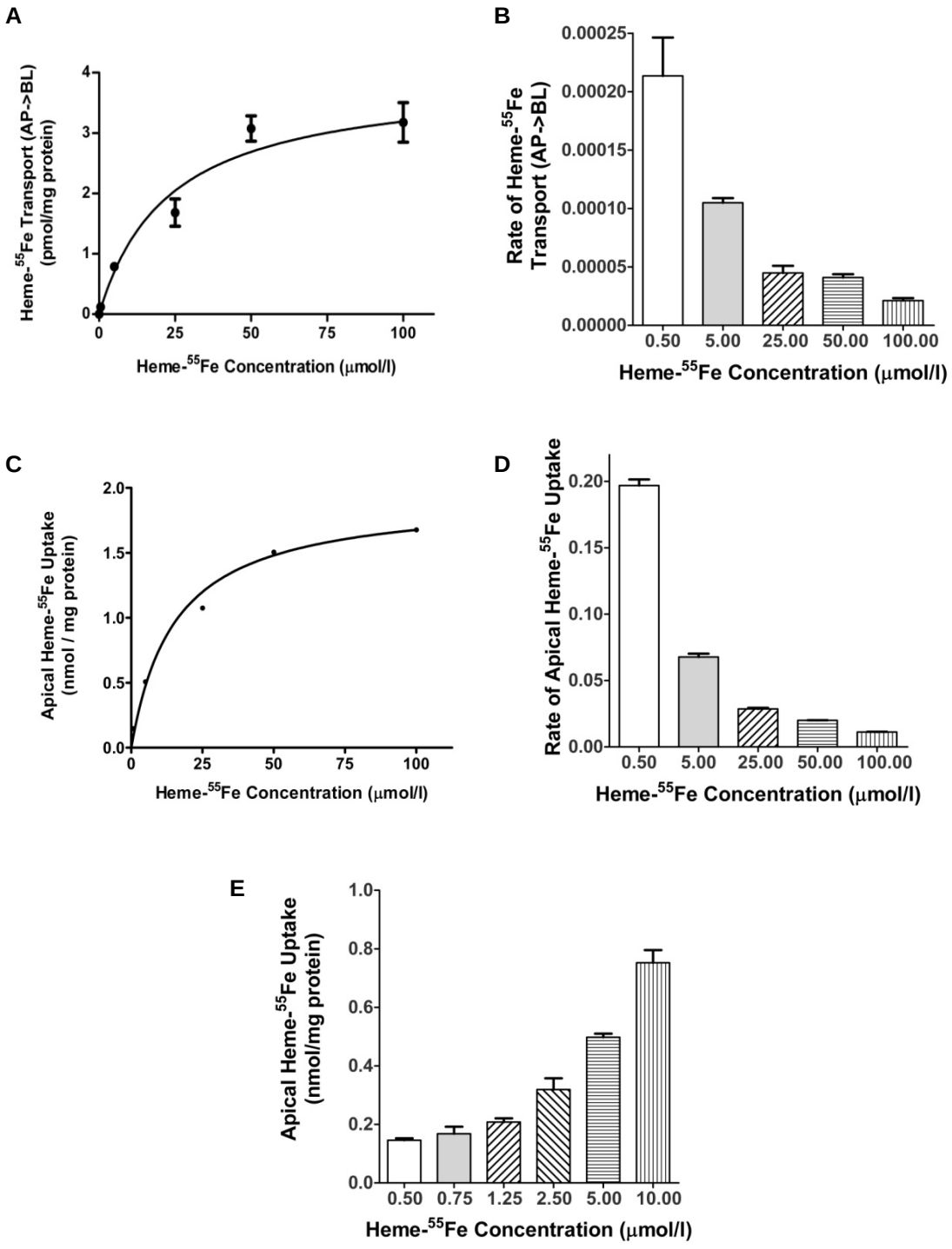

Figure 3-1. Saturable transepithelial transport and apical uptake of heme-^{55}Fe in Caco-2 cells

Dose-dependent transepithelial transport of heme-^{55}Fe in fully-differentiated Caco-2 cells treated with 0.5 μM, 5 μM, 25 μM, 50 μM and 100 μM of heme-^{55}Fe for 1 h (A & B); Saturable apical uptake of heme-^{55}Fe with different doses of heme-^{55}Fe at 1 h (C & D); Dose-dependent apical heme-^{55}Fe uptake with low doses of heme-^{55}Fe for 1 h (E). Values are means ± SEM, n = 6.

3.2 Time-dependent Transepithelial Transport of Heme-^{55}Fe in Caco-2 Cells

Next, to explore the time effect on heme-^{55}Fe transport across the fully-differentiated Caco-2 cell monolayer, we analyzed the amount of ^{55}Fe exported to basolateral chamber for up to 7 h to mimic the time interval that food generally stays in small intestine. The quantity of heme-derived ^{55}Fe transferred from the apical to the basolateral compartment of Caco-2 cell monolayer was linearly increased between 1 and 7 h of incubation (Figure 3-2). We will use this time interval of 7 h throughout the thesis for iron transport study unless otherwise noted.

3.3 Temperature-dependent Heme-^{55}Fe Uptake and Transport in Caco-2 Cells

To determine the effect of temperature on heme-^{55}Fe absorption in Caco-2 cells, heme-^{55}Fe uptake and transport studies were performed at both 4°C and 37°C. The results showed that transepithelial transport of heme-^{55}Fe from apical to basolateral chamber was decreased by 66.7 ± 10.3% at 7-h point at 4°C compared to that at 37°C ($p < 0.05$) (Figure 3-3 B). The apical heme-^{55}Fe uptake was also reduced by 57.2 ± 2.7% at 4°C compared to that at 37°C ($p < 0.05$) (Figure 3-3 A). Thus, both apical uptake and transepithelial transport of heme-^{55}Fe in fully-differentiated Caco-2 cells are temperature-dependent. Low temperature decreases total transepithelial transport of heme-^{55}Fe mainly by lessening its apical uptake.

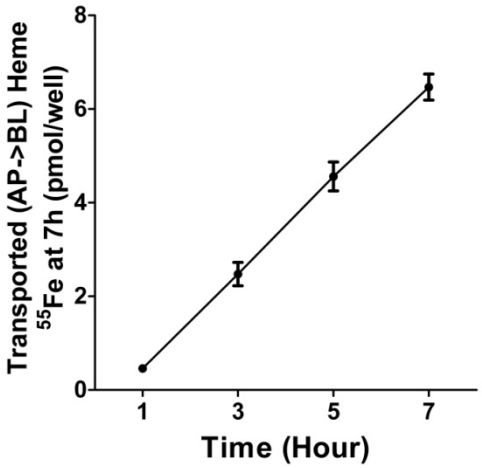

Figure 3-2. Time-dependent transepithelial transport of heme-^{55}Fe in Caco-2 cells

Transepithelial transport of heme-derived ^{55}Fe in fully-differentiated Caco-2 cells treated with 0.5 μM of heme-^{55}Fe for 1, 3, 5 and 7 h. Values are means ± SEM, n = 5.

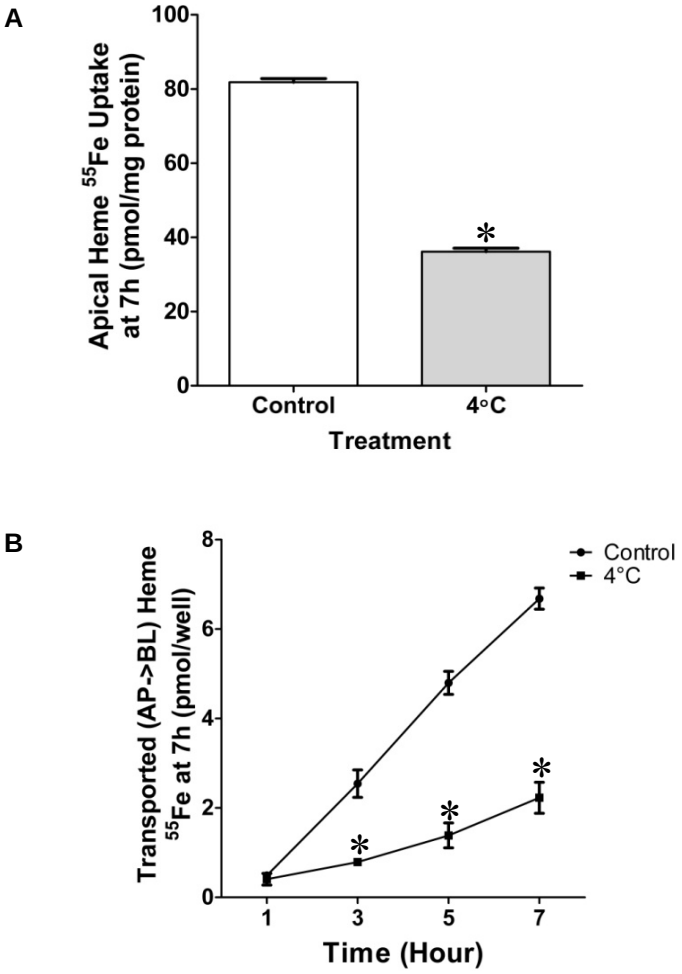

Figure 3-3. Temperature-dependent uptake and transport of heme-^{55}Fe in Caco-2 cells

Apical uptake (A) and transepithelial transport (B) of heme-^{55}Fe in fully-differentiated Caco-2 cells treated with 0.5 μM of heme-^{55}Fe for 7 h at 4°C compared to those at 37°C. Values are means ± SEM, n = 4; * indicates that the treatment has a significant effect, $p < 0.05$.

3.4 Effect of Excessive Iron on Heme-^{55}Fe Absorption in Caco-2 Cells

To study the effect of excessive iron on heme-^{55}Fe uptake and transport in Caco-2 cells, competition studies were executed to determine if extra heme or non-heme iron would compete with absorption of 0.5 μM heme-^{55}Fe. Competition studies showed that non-radiolabeled heme iron in 50X molar excess decreased heme-^{55}Fe uptake and transport at 7h significantly to 84.1 ± 1.3% and 44.9 ± 8.5%, respectively ($p < 0.05$) (Figure 3-4). This is expected because high concentration of heme iron (25 μM) has much lower rates of uptake and transport compared with low concentration of heme iron (0.5 μM) (Figure 3-1 B & D).

However, extra non-heme iron did not compete with absorption of 0.5 μM heme-^{55}Fe (Figure 3-5). It was assumed that intestinal absorption of heme and non-heme compete at the level of basolateral export by sharing the only iron exporter FPN1, but based on our result, they do not compete with each other although they do share FPN1 for basolateral export. Surprisingly, non-heme iron in 50X molar excess even increased heme-^{55}Fe transport significantly, which we will discuss in more details in Chapter 5.

3.5 Basolateral Release of Heme-^{55}Fe as Heme-free Iron in Caco-2 Cells

To test the hypothesis that heme iron is exported into basolateral chamber as heme-free ferrous iron rather than being intact heme, iron transport study with 0.5 μM heme-^{55}Fe was performed and at the end of 7h, basolateral medium was collected for detection of any intact heme. Our results showed that the overwhelming majority of heme iron (> 99.7%) was released into basolateral chamber as heme-free iron.

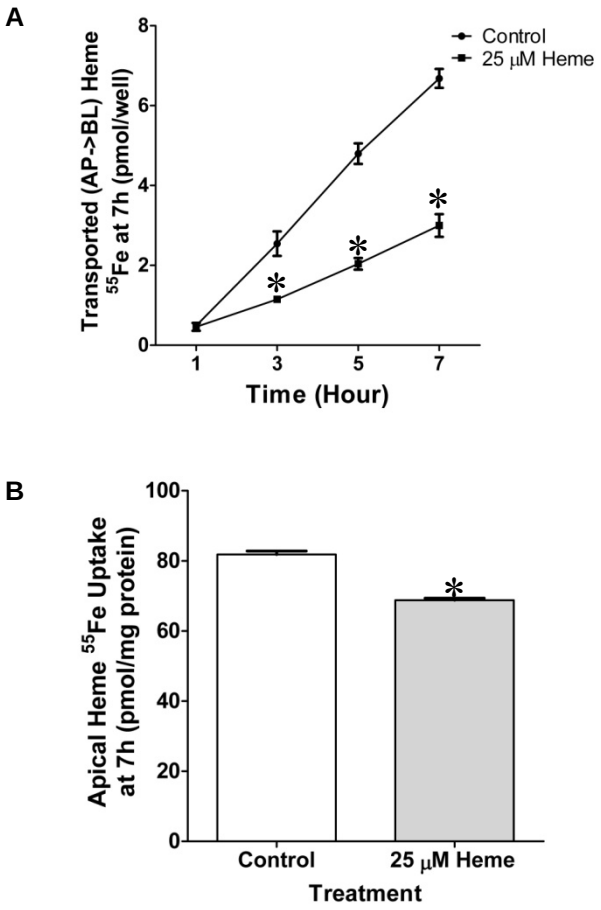

Figure 3-4. Competition studies between heme-^{55}Fe and extra heme in Caco-2 cells

Transepithelial transport (A) and apical uptake (B) of heme-^{55}Fe in fully-differentiated Caco-2 cells treated with 0.5 μM of heme-^{55}Fe in absence and presence of 50X molar excess of non-radiolabeled heme for 7 h. Values are means ± SEM, n = 4; * indicates that the treatment has a significant effect, $p < 0.05$.

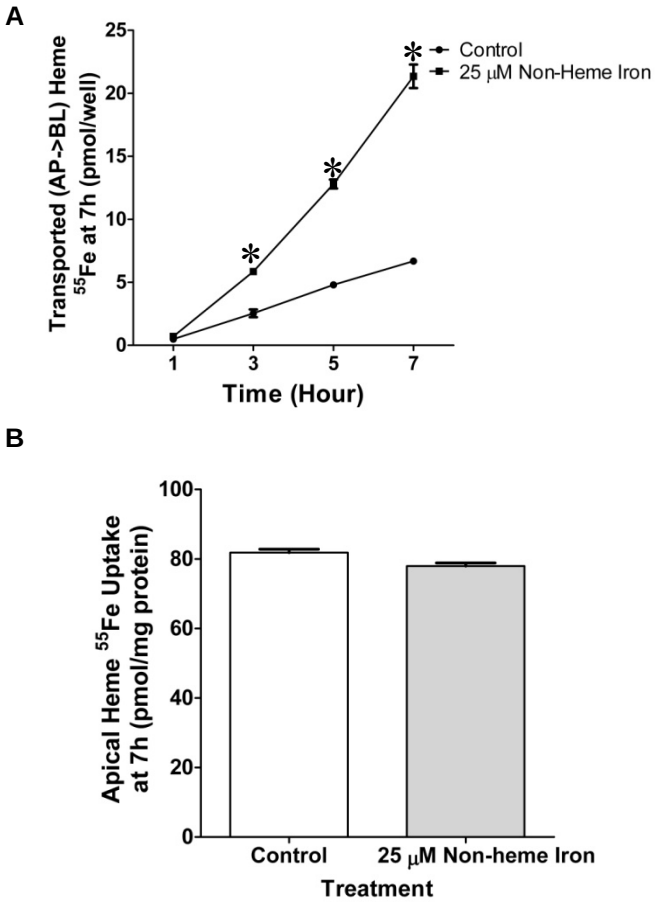

Figure 3-5. Competition studies between heme-^{55}Fe and 50X non-heme iron in Caco-2 cells

Transepithelial transport (A) and apical uptake (B) of heme-^{55}Fe in fully-differentiated Caco-2 cells treated with 0.5 μM of heme-^{55}Fe in absence and presence of 50X molar excess of non-radiolabeled non-heme iron for 7 h. Values are means ± SEM, n = 4; * indicates that the treatment has a significant effect, $p < 0.05$.

DISCUSSION

The present experiments were designed to characterize intestinal heme iron absorption using Caco-2 cells. Caco-2 cell model has proven to be a useful model for studying the characteristics of intestinal iron uptake and metabolism due to its comparable result as in vivo studies (Halleux *et al.* 1994; Yun *et al.* 2004). Overall, we found that the apical uptake and transepithelial transport of heme-^{55}Fe were dose-dependent and saturable in Caco-2 cells, which mimic the cases in animals and humans. The rate of heme-^{55}Fe absorption decreased with increased amount of heme-^{55}Fe in apical chamber when the concentration of heme-^{55}Fe was above 10 μM. In addition, the apical uptake of heme-^{55}Fe was temperature-dependent in Caco-2 cells. The transepithelial transport of heme-^{55}Fe also decreased at 4°C possibly due to reduced apical heme uptake. As apical uptake of heme is both saturable and temperature-dependent, it could occur through a receptor-mediated endocytic pathway as reported previously (Wyllie *et al.* 1982; Lara *et al.* 2005). Finally, the third major finding was that the overwhelming majority of heme iron was exported into basolateral chamber as heme-free iron, suggesting that heme was largely degraded within Caco-2 cells to release free ferrous iron.

Heme in foods was found primarily in Hb or myoglobin. The source of heme iron used in this study was a digest of Hb. Hb digestion is an enzymatic hydrolytic process that occurs in stomach and intestinal lumen; thus, imitating this digestive process will give a better model for heme uptake by enterocyte and absorption into blood. In addition, heme iron from a digest is absorbed better than intact Hb under normal conditions. Increasing Hb hydrolysis enhances heme iron absorption (Vaghefi *et al.* 2002).

Both animal and human studies have shown that the quantity of heme iron absorbed is correlated to that ingested. In rats and guinea pigs tested, although the percentage of absorption decreased with increasing doses of heme, the total amount of heme absorbed increased

(Bannerman 1965; Weintraub *et al.* 1965; Han *et al.* 1999). In humans, the observations were similar; with larger concentration of heme iron, relative percent absorption was less while the absolute total absorption was more (Turnbull *et al.* 1962). Our results in Caco-2 cells were also similar and confirmed that heme absorption is dose-dependent. The rate of both apical uptake and basolateral release of heme-^{55}Fe were reduced with higher concentration of heme-^{55}Fe, despite that the total amounts were enhanced. In addition, the dose-response curve of apical heme-^{55}Fe uptake was almost linear when the concentration of heme was between 0.5 and 10 μmol/l at 1 h of incubation.

Foods generally stay in small intestine for approximately 7 – 8 hours before they reach the colon (Sizer *et al.* 2010). As heme iron absorption mainly occurs in the duodenum, we chose the time interval of 7 h for heme iron absorption in Caco-2 cells to mimic that in small intestine. Our results showed that heme-^{55}Fe absorption across the Caco-2 cell monolayer was linearly increased between 1 and 7 h of incubation. We will use this time interval of 7 h throughout the thesis for iron transport study unless otherwise noted.

We also investigated if heme-^{55}Fe absorption were responsive to uptake of different levels of heme and non-heme iron. The level of iron ingested has been shown to be a factor affecting intestinal iron absorption. Enterocytes respond to luminal dietary factors by increasing or decreasing expression of relevant proteins involved in intestinal iron absorption or altering relevant enzymatic activities. Similar to the dose-dependent study, our results showed an inverse relationship between heme iron concentration and heme iron absorption. Excessive non-radiolabeled heme iron decreased heme-^{55}Fe absorption. However, extra non-heme iron did not compete with absorption of heme-^{55}Fe. It has been proposed that cells exposed to heme iron could not sense the iron uptake (Cermak *et al.* 1993; Tapia *et al.* 1996). Both apical uptake and transepithelial transport rates of heme-^{55}Fe were high in presence of excessive non-heme iron. Although apical uptake of heme iron remained at a similar level in presence of excessive non-

heme iron, the cells exported heme-derived ^{55}Fe at a higher rate regardless of increased total intracellular iron levels.

It has been recently identified that feline leukemia virus subgroup C receptor (FLVCR) is capable of exporting heme out of cells as intact heme instead of free iron (Quigley *et al.* 2004). It has been demonstrated that FLVCR exports cytoplasmic heme and is crucial for erythropoiesis. As to intestinal heme iron absorption, no studies have yet directly examined FLVCR function. Since FLVCR on membranes of erythroid precursors functions to regulate heme content when heme synthesis rates are at their peak just prior to differentiation, it is highly unlikely that FLVCR is involved with intestinal heme absorption. Our study in Caco-2 cells revealed that heme iron absorbed across intestinal absorptive cells is mainly exported as free iron (> 99.7%) instead of intact heme, excluding the possibility that FLVCR plays any important role in intestinal heme iron absorption. There are strong evidence that heme is split into free iron within enterocyte instead of being exported as intact in both animal and human studies. For example, for dogs administered with intragastric radiolabelled hemoglobin, 90% of the recoverable radioactivity in samples of portal blood over a period of 3 h was present as non-heme iron (Brown *et al.* 1968). Given that FLVCR is expressed in intestinal cell lines including Caco-2 cells, it may function to facilitate heme transport for cells before full differentiation rather than exerting a role in intestinal heme iron absorption.

In conclusion, this chapter provides the preliminary data and experimental condition for the following chapters. Our results showed that similar to the cases in animals and humans, heme iron absorption was also enhanced with increasing dose of heme despite decreased relative percentage absorbed in our Caco-2 cell model. Ease of use, homogeneity and resemblance to human studies of Caco-2 cells as a model for intestinal iron absorption make them advantageous to further examine pathophysiological conditions and dietary factors that affect heme and non-heme iron absorption. In addition, we confirmed that apical uptake of heme iron in Caco-2 cells is

a saturable, dose-dependent and temperature-dependent process. Transepithelial transport of heme iron in Caco-2 cells is dose-dependent and linearly increased between 1 – 7 h intervals. Finally, we substantiated that heme iron is mainly absorbed into basolateral side as heme-free iron.

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Chapter 4

BIOACTIVE DIETARY POLYPHENOLS INHIBIT HEME IRON ABSORPTION IN CACO-2 CELLS

ABSTRACT

Because dietary polyphenolic compounds have a wide range of health effects, including chelation of metals such as iron, it is prudent to test whether the regular consumption of dietary bioactive polyphenols impair the utilization of dietary iron. Our previous study showed the inhibitory effect of (-) -epigallocatechin-3-gallate (EGCG) and grape seed extract (GSE) on non-heme iron absorption. The aims of this study were to investigate whether EGCG and GSE affect intestinal heme iron absorption and if so, whether small amounts of EGCG, GSE, and green tea extract (GT) could inhibit heme iron absorption, and to test whether the inhibitory action of these polyphenols could be offset by ascorbic acid. The fully-differentiated intestinal Caco-2 cells grown on microporous membrane inserts were incubated with heme-^{55}Fe in uptake buffer containing various concentrations of EGCG, GSE or GT in the apical compartment for 7 h. Both EGCG and GSE decreased ($p < 0.05$) transepithelial transport of heme-derived ^{55}Fe. Although apical heme-^{55}Fe uptake was increased ($p < 0.05$) by GSE, the amount of ^{55}Fe released to basolateral chamber was negligible. In contrast, EGCG moderately decreased the apical uptake of heme-^{55}Fe, whereas the basolateral iron transfer was extremely low. All three polyphenolic compounds tested, including EGCG, GSE and GT, significantly inhibited heme-^{55}Fe absorption in a dose-dependent manner. The addition of ascorbic acid did not modulate the inhibitory effect of these polyphenols on heme iron absorption when the cells were treated with polyphenols at a concentration of 46 mg/L. However, ascorbic acid was able to offset or reverse the inhibitory

effect of polyphenolic compounds when lower concentrations of polyphenols were added (≤ 4.6 mg/L). Ascorbic acid modulated the heme iron absorption without changing the apical heme uptake, the expression of the proteins involved in heme metabolism and basolateral iron transport, and HO activity, indicating that ascorbic acid may enhance heme iron absorption by modulating the intracellular distribution of ^{55}Fe. Expression of HOs, FPN1 and Heph proteins are not changed by EGCG, GSE and GT during the 7h transport study. Overall, our data suggest that bioactive dietary polyphenols inhibit heme iron absorption mainly by reducing basolateral iron exit rather than decreasing apical heme iron uptake in intestinal cells, possibly by forming insoluble products within the cell. In addition, the results imply that regular consumption of dietary ascorbic acid can easily counteract the inhibitory effect of low concentrations of dietary polyphenols on heme iron absorption but cannot counteract the inhibitory actions of high concentrations of polyphenols.

Keywords

Intestinal iron absorption, heme iron, green tea, EGCG, GSE

Practical Application

Bioactive dietary polyphenols inhibit heme iron absorption in a dose-dependent manner. The small amounts of polyphenolic compounds present in foods are capable of reducing heme iron transport across the intestinal enterocyte. However, the inhibitory effect of dietary polyphenolic compounds on heme iron absorption can be offset by ascorbic acid and can possibly be avoided by decreasing the consumption of polyphenols while simultaneously taking ascorbic acid.

INTRODUCTION

Recent studies suggest that bioactive dietary polyphenols have health-promoting benefits pertaining to a variety of disorders (Renaud *et al.* 1992; Yang *et al.* 1993; Ness *et al.* 1997; Tijburg *et al.* 1997; Basu *et al.* 2010). Bioactive dietary polyphenolic compounds are naturally occurring chemicals found in foods including fruits, some types of grain, wine, and tea. Green tea, made from the leaves of the plant Camellia sinensis, is one of the most popular beverages consumed worldwide. The beneficial effects of green tea are attributable to its polyphenolic compounds, particularly the catechins. (-)-Epigallocatechin-3-gallate (EGCG) is the most abundant catechin of green tea and is regarded as the most bioactive disease-preventing polyphenolic compound in green tea (Bose *et al.* 2008). Grape seed extract (GSE), which contains various polyphenolic compounds including gallic acid, catechin, EGCG, epigallocatechin, epicatechin-3-gallate, epicatechin, and proanthocyanidins, also has been reported to have protective effects on various forms of cardiac disorders (Sato *et al.* 1999; Bagchi *et al.* 2003; Tsang *et al.* 2005; Frederiksen *et al.* 2007). Bioactive polyphenol-rich extracts derived from both green tea and grape seed have the potential to protect foods and beverages from oxidation and are widely used as supplements or food additives. Their antioxidant activities have been shown to be maintained by the ability of their constituent, phenolic compounds, to scavenge free radicals and to chelate metals (Guo *et al.* 1996; Apak *et al.* 2004; Mandel *et al.* 2006).

Iron is an essential trace mineral for human life. In eukaryotic cells and most prokaryotic cells, iron is notably required for survival and proliferation, as a constitute of a diverse group of hemoproteins, including those involved in oxygen transport and storage (hemoglobin and myoglobin), electron transfer (cytochromes) and DNA synthesis (ribonucleotide reductase) (West *et al.* 2008). Although iron is quite abundant in the environment, iron deficiency is still the most

common nutritional deficiency worldwide, which in a severer stage leads to iron deficiency anemia, a major public health concern affecting up to 1 billion people (World Health Organization 2007). This is caused not only by low intake of this essential trace metal but also by its poor bioavailability as well. Systemic iron homeostasis requires mechanisms for regulating iron entry into and mobilization from stores, in order to counterview iron loss (1-2 mg/day) and to compensate for daily production of 200 billion new erythrocytes (Hentze *et al.* 2004). Since there is no efficient way for iron elimination from body, intestinal iron absorption is tightly modulated and even self-modulated to keep iron homeostasis. There are two types of dietary iron, heme iron and non-heme iron. The importance of dietary heme iron cannot be underestimated, as in western civilization, heme iron only constitutes one-third of total dietary iron for an average non-vegetarian person, but makes up two-thirds of the total absorbed iron, suggesting that heme iron is more efficiently absorbed than non-heme iron (Carpenter *et al.* 1992).

Before heme can be absorbed, heme must be released from dietary hemoglobin (Hb) and myoglobin by proteolysis in the lumen of the stomach and small intestine (Conrad *et al.* 1967). Heme is firstly taken up from intestinal lumen into enterocytes as an intact metalloporphyrin. The proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) is proposed as a protein involved in dietary heme uptake in duodenal enterocytes (Shayeghi *et al.* 2005). After heme iron enters the enterocyte, it is then cleaved by the heme oxygenase (HO) to release free ferrous iron, CO, and biliverdin (Raffin *et al.* 1974). Despite different modes of apical uptake for heme and non-heme iron, iron released from heme inside vesicles would be transported to the cytoplasm to join the labile iron pool and then transferred across the basolateral membrane of the enterocyte into the circulation using the same pathway as for non-heme iron. The newly transported iron is then distributed to the basolateral side or to iron-binding proteins (e.g. heme, non-heme iron-binding proteins, and ferritin). Finally, iron from the labile iron pool is either incorporated into ferritin for transient storage or be exported across basolateral membrane via ferroportin-1 (FPN-

1) (Abboud *et al.* 2000; Donovan *et al.* 2000; McKie *et al.* 2000), where it is oxidized to ferric iron by hephaestin prior to release into the circulation (Vulpe *et al.* 1999).

Because the antioxidant properties of the polyphenols include chelating metals like iron and heme is the type of dietary iron with relatively more importance for non-vegetarians, it is prudent to examine the effects of the bioactive polyphenols on intestinal heme iron absorption. The objective of this study were to investigate whether EGCG and GSE affect intestinal heme iron absorption and if so, whether tiny amounts of these polyphenolic compounds are still capable of inhibiting heme iron absorption. Because green tea is a major natural source of EGCG and is one of the most popular beverages in a number of countries where iron deficiency is a major nutritional problem, the effect of green tea extract (GT) on heme iron absorption and its relationship to dietary ascorbic acid were also examined.

MATERIALS AND METHODS

Reagents

EGCG (TEAVIGO^{TM}, purity >95%), GSE and GT were purchased from DSM Nutritional Products (Parsippany, NJ), Partoeno (Bordeaux, France) and Pharmanex Inc. (Provo, UT), respectively. For GSE, its chemical characteristics and degree of polymerization have been documented (Tsang *et al.* 2005). As to GT, it is a mixture of catechins, including EGCG as the major component (43.0% by weight), followed by epicatechin-3-gallate (13.7%), epicatechin (6.0%), gallocatechin gallate (5.6%), epigallocatechin (4.0%), gallocatechin (2.3%), catechin (2.0%) and catechin gallate (1.4%) (Lu *et al.* 2005). In this study, the concentration of EGCG, GSE or GT was expressed as weight per milliliter of buffer bathing the apical side of cells [mg/L]. The ^{55}Fe (as $^{55}FeCl_3$) was obtained from PerkinElmer (Boston, MA). Hanks' balanced salts solution (HBSS), glutamine, non-essential amino acids and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Unless otherwise noted, all other reagents were obtained from Sigma Chemical (St Louis, MO), VWR (West Chester, PA) or Fisher Scientific (Springfield, NJ).

MEL Cell Culture and Synthesis of ^{55}Fe-Hemoglobin (Hb)

The murine erythroleukemia (MEL) cell line was a generous gift from Dr. Robert Paulson at Pennsylvania State University (University Park, PA). The MEL cells were grown in the same conditions as described above for the Caco-2 cells. As previously reported, Hb was synthesized using MEL cells as previously reported (Ma *et al.* 2010). Briefly, to induce Hb synthesis, erythroid differentiation of cells was induced by adding dimethyl sulfoxide (DMSO). $(^{55}Fe)_2$-Tf was prepared from apo-Tf and ^{55}Fe and purified. The level of Tf saturation was estimated from the A_{465}/A_{280} ratio, which was routinely found to be 0.046, consistent with

complete saturation on both sites of Tf for iron binding {Huebers, 1978 #1124}. To produce ^{55}Fe-Hb, MEL cells were seeded at 10,000 cells/cm^3 and were treated with 2 μM (^{55}Fe)$_2$-Tf and 2% DMSO. After a 6-d treatment, the cells were harvested and washed three times with phosphate-buffered saline (PBS, pH 7.0) and then collected by centrifugation for 5 min at 800 xg, 4°C.

Spectrophotometric Quantification of Hb and Hb Digestion

Hb concentrations in MEL cell lysates were measured by the benzidine assay as previously described (Ma *et al.* 2011). Briefly, pellets of MEL cells (70 x 10^6 of cells) harvested after 6 d were re-suspended in 0.5 mL distilled water and lysed by 4 cycles of freezing (in dry ice/ethanol for 3 min) and thawing (in a water bath at 37°C for 3 min). The cell lysate were centrifuged at 14,000 xg for 15 min at 40°C using an Eppendorf 5402 centrifuge (Hamburg, Germany). Benzidine assay was performed in duplicate by adding the reagents in the following order: 100 μL supernatant, 900 μL deionized water and 100 μL freshly prepared benzidine-HCl (10 mg/ml in 0.5% acetic acid). The reaction was started by the addition of 40 μL 30% H_2O_2. The contents were mixed well and after exactly 90 sec the absorbance was measured at 604 nm. The Hb concentration was then calculated based on the measured absorbance using a calibration curve obtained with purified Hb standard (Sigma, Saint Louise, MO) measured in the same way as the supernatant. Both Hb-^{55}Fe prepared from MEL cell lysis and commercially bought bovine Hb were digested with 0.1% pepsin at pH 2.0 overnight at 37°C. The solution was then centrifuged for 5 min at 14,000 xg at 4°C. The digestion rate was estimated by measuring the remaining Hb content in the supernatant using benzidine assay as described above. Pellets of ^{55}Fe-heme and heme were dissolved in 10 mM of NaOH and further diluted in iron uptake buffer (final pH 7). The ^{55}Fe-heme specific activity was between 0.40 Ci/mol and 0.45 Ci/mol heme.

Caco-2 Cell Culture

The human Caco-2 cell line HTB37 was purchased from American Type Culture Collection (Rockville, MD). Stock cultures were maintained at 37°C in complete medium in a humidified atmosphere of 95% air and 5% CO_2 and used for experiments within 20 serial passages. The complete culture medium contained Dulbecco's Modified Essential Medium (DMEM) supplemented with 25 mM glucose, 2 mM glutamine, 100 μM non-essential amino acids, 100 U/l penicillin G, 100 mg/l streptomycin and 10% FBS. For experiments, 5.0×10^4 cells/cm^2 in 1.5 ml complete DMEM were seeded on 3 μm microporous membrane inserts (4.9 cm^2, BD Biosciences, Bedford, MA) coated with collagen type I (5 μg/cm^2) on 6-well plates. The basolateral chamber was filled with 2.5 ml complete DMEM. The culture medium was changed every 2 days, and cells were used after 17-day post-confluence for experiments. The Caco-2 cell monolayer was routinely checked for tight junctions at 17-d postconfluence with transepithelial electrical resistance (TEER) >250 Ω/cm^2, which indicates full differentiation under normal cell culture conditions.

^{55}Fe Transport Study

Transepithelial heme-derived ^{55}Fe transfer from apical to basolateral compartment were determined by scintillation (Han *et al.* 1994). After washing the Caco-2 cell monolayer three times with Ca^{2+}- and Mg^{2+}-free HBSS, cells were incubated at 37°C with 1.5 mL iron uptake buffer containing 1 μM ^{55}Fe-heme and the indicated bioactive compounds in the apical compartment and 2.5 mL DMEM in the basolateral compartment for 7 h. The iron uptake buffer (pH 7.0) consists of 130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose and 50 mM HEPES. During the 7 h incubation, 200 μL media was transferred from the basolateral chamber to glass vials at the indicated time points (1, 3, 5 and 7 h) and replaced by an equivalent volume of pre-warmed DMEM; time course data were corrected to account for this sampling replacement. The rate of ^{55}Fe transport across the cell monolayer was increased during the 7 h incubation, and

transport rates [pmol/well] were calculated by linear regression analysis (Table 4.1 & 4.2). To measure the cellular levels of ^{55}Fe, cell monolayer were washed three times with ice-cold wash buffer to remove any nonspecifically bound radioisotope. The wash buffer (pH 7.0) contains 150 mM NaCl, 10 mM HEPES and 1 mM EDTA. This washing step was effective in removing all surface bound iron since additional wash steps using solution containing 100 mM bathophenanthroline disulfonate (BPS, Fe^{2+} chelator) and desferrioxamine (DFO, Fe^{3+} chelator) did not further reduce cellular ^{55}Fe content after the wash. Cells were homogenized in PBS containing 0.3% Triton X-100, and ^{55}Fe was quantified by liquid scintillation counting in glass vials. Cellular protein levels were consistently 1.2 ± 0.2 mg/well, as assessed by Bio-Rad protein assay kit (BioRad Laboratory Inc., Hercules, CA). The level of heme iron (1 μM) used for iron transport studies is similar to that obtained from 10 g of cooked beef per meal (Hazell *et al.* 1982).

Western Blot

Western blot was performed to determine protein levels of HO-1, HO-2, Ft, FPN-1, Heph, and TfR1 in Caco-2 cells, which were assessed as previously described (Han *et al.* 2007; Ma *et al.* 2010). Protein samples were extracted from Caco-2 cells treated with heme and various polyphenols in the absence or presence of ascorbic acid for 7 h. Total protein concentrations were determined using a Bio-Rad protein assay kit. Protein samples (40 μg) was mixed with Laemmli buffer, boiled for 10 min, resolved by a 12% SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. The membranes were stained with reversible Ponceau dye for 5 min to confirm equal amount of total proteins for each lane and washed for several times by 10 mM Tris-Base and 150 mM NaCl (TBS, pH 7.4) containing 0.05% Tween 20 (TBST). The membranes were first blocked by 5% non-fat powered milk in TBS at room temperature for 1h and then incubated for 2 h at room temperature with an affinity-purified HO-1, HO-2, FPN-1,

Heph, TfR1 or Ft antibody (1:2000) in TBST. The membranes were washed several times with TBST and then incubated for 1 h at room temperature with peroxidase-linked goat anti-rabbit IgG (1:3000) in TBS containing 5% non-fat powered milk. The visualized antigens by ECL were detected via ChemiDoc XRS system (Bio-Rad). The blots were stripped and reprobed with anti-calnexin antibody to confirm equal loading and transfer.

HO Activity Assay

HO activity was assessed on the basis of the rate of bilirubin production (Maines 1996). HO contribution reflects the total activity of both HO-1 and HO-2 present in the sample. Caco-2 cells treated with or without polyphenols and ascorbic acid for 7 h were harvested in ice-cold 0.3 ml PBS with 1% Triton X-100 and homogenized. Hemin was added to cell lysates with a final concentration of 10 μM, which were then incubated at 37°C with shaking for 30 min. The reaction was terminated by placing the samples on ice. The samples were centrifuged 2000 rpm at 4°C for 10 min to remove cell debris. The amount of bilirubin in the supernatant was examined following the QuantiChrom^{TM} bilirubin assay kit (BioAssay Systems, Hayward, CA). All assays above were prepared under dim light. The HO activity is expressed as nmol bilirubin formed per minute per mg protein.

Statistical Analysis

Values were expressed as non-transformed means ± SEM, n = 3 - 6. Data were analyzed by 2-way ANOVA using R software and by 1-way ANOVA with the following post-hoc tests for multiple comparisons using Prism 5.0 software (GraphPad). Differences were considered significant when $p < 0.05$.

RESULTS

4.1 EGCG and GSE Inhibit Transepithelial Heme Iron Transport

The addition of polyphenols EGCG or GSE (46 mg/L) to the uptake buffer decreased ($p < 0.05$) the rate of ^{55}Fe transfer across the cell monolayer when heme concentration was 1 μM (Table 4.1). The rate of iron transfer across the cell monolayer was decreased by 86 ± 4.2% and 90 ± 4.6% by EGCG and GSE, respectively. During 7 h of incubation, both EGCG and GSE decreased the transepithelial iron transfer across the cell monolayer by 74 ± 9.1% and 79 ± 0.8%, respectively (Figure 4-1). EGCG and GSE also inhibited the transepithelial transport of ^{55}Fe when heme ^{55}Fe was applied at a higher concentration (25 μM) in the uptake buffer. The addition of these bioactive dietary polyphenols did not alter transepithelial electrical resistance values, thereby confirming the integrity of the monolayer for EGCG or GSE-added cells. The cellular assimilation of heme ^{55}Fe was decreased by 18 ± 3.0% by EGCG but increased by 77 ± 5.4% by GSE during the 7-h assay. The control cells accumulated 84 ± 2.8 pmol/well of heme ^{55}Fe during 7 h of incubation. The total protein level was almost the same for each well and was not changed by the addition of EGCG or GSE.

4.2 Polyphenols Do Not Alter Expression of Proteins Involved in Intestinal Iron Absorption

Addition of EGCG and GSE did not alter levels of HO proteins and other proteins involved in basolateral iron export in intestinal cells. A major protein band with a molecular mass of approximately 35 and 40 kD was detected by the HO-1 and HO-2 antibodies, respectively. The

expression of HO-1 and -2 was not changed by EGCG and GSE during the 7-h iron transport assay (Figure 4-2A). The addition of EGCG and GSE also did not change levels of the 2 major proteins involved in the basolateral iron export in the enterocyte, FPN1 and Heph. In addition, cellular levels of TfR1 and ferritin protein were not altered by treatments with EGCG and GSE during iron transport study (Figure 4-2B). Our quantitative analysis data showed that the levels of these proteins in cells treated with EGCG or GSE did not differ compared with control.

4.3 No Effect of Ascorbic Acid on the Inhibitory Action of EGCG and GSE

The treatment of 46 mg/L of EGCG or GSE significantly reduced the rate of heme-derived ^{55}Fe transport across Caco-2 cell monolayer by 91.4 ± 3.90% and 91.4 ± 4.41%, respectively. The question remained as to whether this inhibitory effect could be reversed by dietary ascorbic acid. The effects of 100 μM of ascorbic acid on the EGCG- or GSE-mediated inhibition of heme absorption were investigated using Caco-2 cells.

The addition of 100 μM ascorbic acid did not counteract the inhibitory effect of EGCG and GSE on heme-derived ^{55}Fe transport across Caco-2 cell monolayer (Figure 4-3). The rate of heme-derived ^{55}Fe transport was significantly decreased by 91.1 ± 1.91% and 91.8 ± 3.22% for cells treated by 46 mg/L of EGCG and GSE in presence of ascorbic acid, respectively, compared to the control. These inhibited transport rates were similar to those for cells treated with EGCG and GSE alone. Heme-^{55}Fe transport remained unchanged with addition of 100μM ascorbic acid in the presence of the 46 mg/L of EGCG or GSE, suggesting that the inhibitory effect of 46 mg/L of EGCG or GSE on heme absorption is not altered by dietary ascorbic acid.

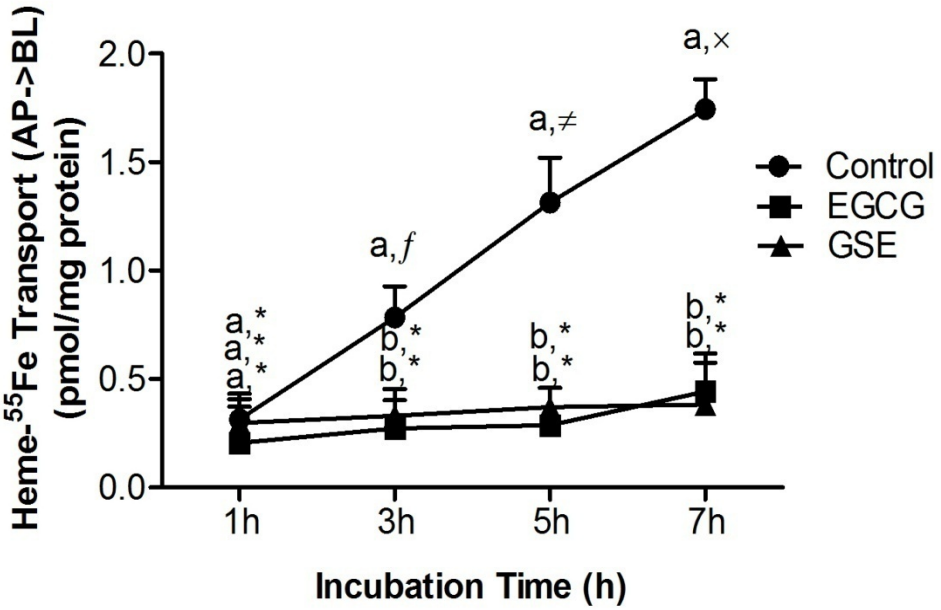

Figure 4-1. EGCG and GSE inhibit heme iron transport across Caco-2 cell monolayer

Transepithelial transport (A & B) and apical uptake (C & D) of heme-^{55}Fe in fully-differentiated Caco-2 cells treated with 0.5 μM of heme-^{55}Fe in absence and presence of 50X molar excess of non-radiolabeled non-heme iron for 7 h. Values are individual replicates (B & D). Values are means ± SEM, n = 4 (A & C); * indicates that the treatment has a significant effect, $p < 0.05$.

Table 4-1. Rate of heme-^{55}Fe Transport[1]

Treatment	Rate of heme ^{55}Fe transfer (AP to BL chamber), pmol/(hr•well)
Control	0.341 ± 0.013
EGCG	0.050 ± 0.015*
GSE	0.037 ± 0.016*

1 Data are means ± SEM, n = 5. Means with * differ, p < 0.05; the rate of transepithelial transport of heme ^{55}Fe across the differentiated Caco-2 cell monolayer was calculated during 7 h incubation by linear regression analysis (control, r = 0.999; EGCG, r = 0.943; GSE, r = 0.984).

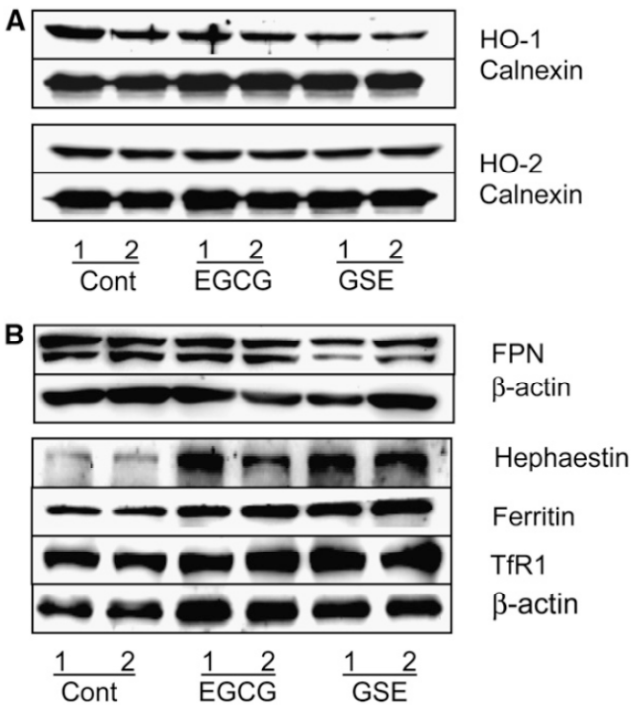

Figure 4-2. Effects of EGCG and GSE on the expression of proteins involved in intestinal heme iron absorption

Representative western blots of HO-1 and HO-2 protein expression from Caco-2 cells treated with 0.5 μM heme in iron uptake buffer containing 46 mg/L EGCG or GSE for 7 h (A). Representative Western blots for FPN, Heph, ferritin, and TfR1 from Caco-2 cells treated with 0.5 μM heme in iron uptake buffer containing 46 mg/L EGCG or GSE for 7 h (B).

Table 4-2. Linear Regression Analysis of the Rate of heme-^{55}Fe Transport[1]

R^2-value	Dose of Polyphenols		
	0.46 mg/L	4.6 mg/L	46 mg/L
Control	0.9900		
EGCG	0.9355	0.9271	0.915
GSE	0.9531	0.9787	0.9413
GT	0.9563	0.9937	0.8162
VC	0.9945		
EGCG+VC	0.9484	0.9334	0.9796
GSE+VC	0.962	0.9619	0.9703
GT+VC	0.9989	0.9802	0.8676

1 The rate of transepithelial transport of heme Fe across Caco-2 cell monolayer was calculated during 7 h incubation by linear regression analysis. Values are means ± SEM, n = 6.

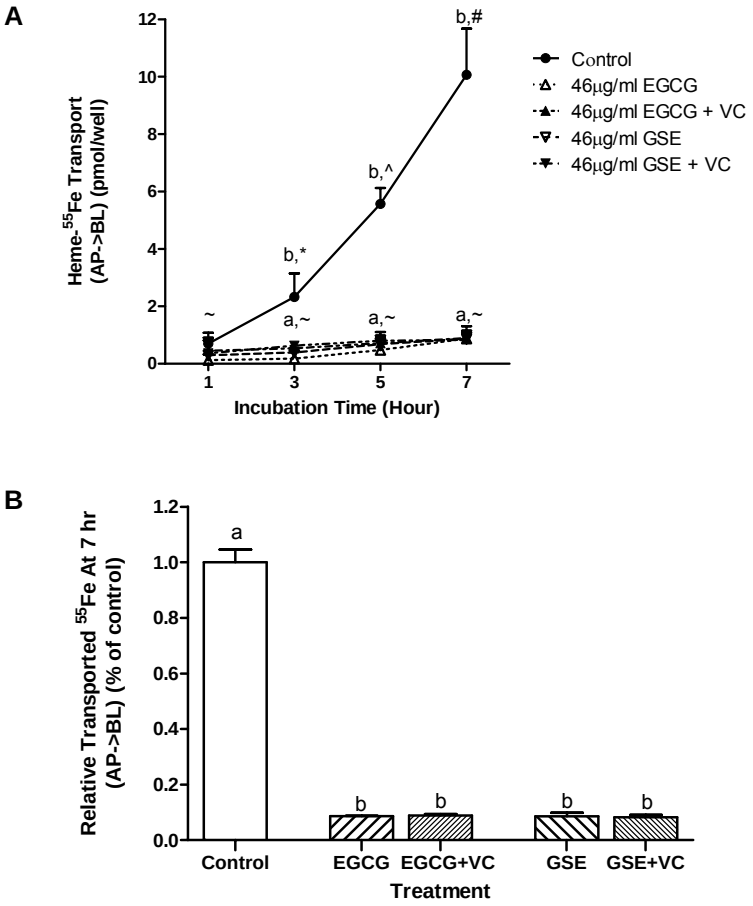

Figure 4-3. Ascorbic acid does not reverse the inhibitory effect of EGCG and GSE on heme iron absorption

(A) Transepithelial transport for heme-derived ^{55}Fe during 7 h incubation. Values are means ± SEM, n = 6. Means at a time without a common letter differ, $p < 0.05$. Within a treatment, means without a common symbol differ, $p < 0.05$. (B) Relative amounts of ^{55}Fe transported from the apical (AP) to the basolateral (BL) chamber for 7 h incubation. Data are means ± SEM, n = 6. Means without a common letter differ, $p < 0.05$. EGCG or GSE was added at the concentration of 46 mg/L. VC (vitamin C): ascorbic acid. Ascorbic acid was added at 100 μM.

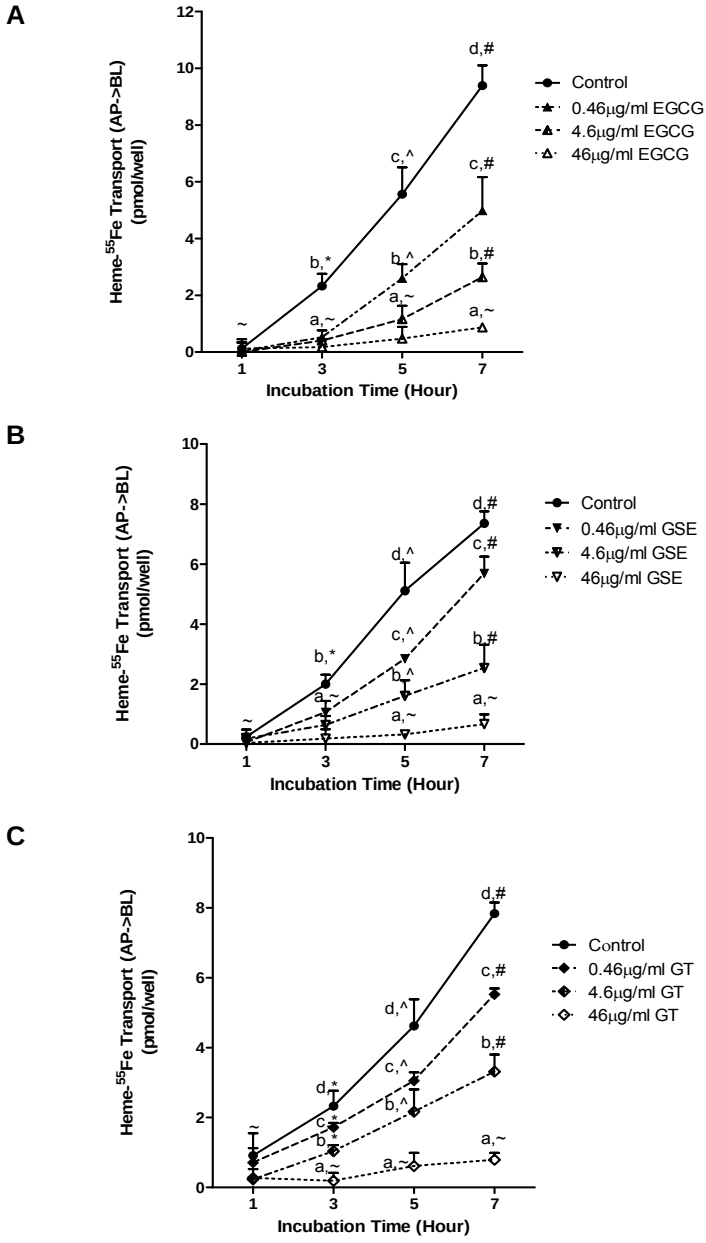

Figure 4-4. Polyphenols inhibit transepithelial transport of heme-derived ^{55}Fe in a dose-dependent manner in Caco-2 cell cells

(A) EGCG, (B) GSE, and (C) GT. Values are means ± SEM, n = 6. Means of different doses at the same time point without a common letter differ, $p < 0.05$. Means of different time points for the same treatment without a common symbol differ, $p < 0.05$.

4.4 Dose-Dependent Inhibitory effect of EGCG, GSE and GT

Because EGCG and GSE almost completely blocked intestinal heme-^{55}Fe absorption at the concentration of 46 mg/L, the effects of lower concentrations of these polyphenols on heme iron absorption were tested. Since green tea is the major natural source of EGCG, the effect of GT on heme-^{55}Fe absorption was also examined. All of the tested polyphenolic compounds, including EGCG, GSE and GT, exerted a dose-dependent inhibitory effect on heme-derived ^{55}Fe transport across the Caco-2 cell monolayer, with half maximal inhibitory concentration (IC50) values (95% confidence interval) of 3.65 (2.01 – 6.65) mg/L, 3.00 (1.93 – 4.67) mg/L and 5.14 (4.02 – 6.56) mg/L, respectively, when 1 μM heme was applied to the apical uptake buffer.

The treatment of EGCG at a lower concentration, 4.6 mg/L, also significantly ($p < 0.05$) decreased the transepithelial heme-derived ^{55}Fe transport to 28.1 ± 5.17% of the control, following a 7-h incubation time (Figure 4-4A). The addition of EGCG to the uptake buffer, even at a very low concentration (0.46 mg/L), significantly ($p < 0.05$) reduced the amount of heme-derived ^{55}Fe transferred from the apical to the basolateral compartment of Caco-2 cell monolayer by 53.1 ± 12.60% of the control. The quantities of heme-derived ^{55}Fe transport across the cell monolayer for treatments with different doses of EGCG were linearly increased over the 7-h time course. Treatment of EGCG at 4.6 and 0.46 mg/L reduced the rate of heme-derived ^{55}Fe transport significantly ($p < 0.05$) by 64.5 ± 4.68 % and 30.9 ± 10.52 %, respectively.

Treatment by GSE at the lower concentrations of 4.6 and 0.46 mg/L significantly ($p < 0.05$) decreased the transepithelial transport of heme-derived heme-derived ^{55}Fe to 34.4 ± 10.56% and 77.3 ± 7.58% of the control, respectively, during a 7-h incubation (Figure 4-4B). The quantities of heme-derived ^{55}Fe transport with different doses of GSE also demonstrated time-dependent growth between 1 and 7 h-incubation. Treatment of GSE at 4.6 and 0.46 mg/L reduced

the rate of heme-derived ^{55}Fe transport significantly ($p < 0.05$) by 63.2 ± 13.2 % and 23.7 ± 11.7 %, respectively.

As indicated by the EGCG and GSE results, the addition of 46 mg/L GT also markedly inhibited the transepithelial transport of heme-derived ^{55}Fe across the cell monolayer to 10.1% ± 2.5% of the control (Figure 4-4C). The amounts of ^{55}Fe transported from the apical into the basolateral chamber were significantly ($p < 0.05$) reduced to 42.3 ± 6.20% and 70.5 ± 2.16% of the control by the addition of 4.6 and 0.46 mg/L of GT, respectively, during the 7-h incubation. The time course of heme-derived ^{55}Fe transport incubated with different doses of GT was also linear between 1 and 7 h. Treatment of GT at 4.6 and 0.46 mg/L reduced the rate of heme-derived ^{55}Fe transport significantly ($p < 0.05$) by 57.5 ± 9.85 % and 35.6 ± 8.47 %, respectively, indicating the retarding effect of GT are slightly weaker than that of purified EGCG.

EGCG, GSE, and GT at all different concentrations tested above also inhibited heme-derived ^{55}Fe transport across the cell monolayer when ^{55}Fe-heme was added at either a lower concentration (0.5 μM) or a higher concentration (5 μM). The transepithelial electrical resistance was not altered by the addition of EGCG, GSE, or GT, confirming the integrity of the Caco-2 cell monolayer under the conditions of the different treatments. The total cellular protein levels were not affected by treatments during the 7-h incubation.

4.5 Counteracting Effects of Ascorbic Acid on Lower Doses of Polyphenols

The next study was conducted to examine whether the regular consumption of ascorbic acid was capable of alleviating the inhibitory effect of lower concentrations of EGCG, GSE, and GT on heme iron absorption. The inhibitory effect of 0.46 mg/L EGCG on the transepithelial transport of heme-^{55}Fe was completely reversed by ascorbic acid, and the amounts of ^{55}Fe transported across the cell monolayer were 28% higher ($p < 0.05$) relative to the control (Figure

4-5A). In contrast, ascorbic acid was not able to counteract the negative impact of a higher concentration of EGCG (4.6 mg/L) on heme iron transport, although the level of inhibition was alleviated. The amount of ^{55}Fe transported across the cell monolayer was decreased by 35.7% ± 2.05% in cells treated with 4.6 mg/L EGCG plus ascorbic acid (Figure 4-5A) but by 71.9% ± 5.17% in cells treated with 4.6 mg/L EGCG alone (Figure 4-5A), compared with control, over a 7-h time course. These suggest that the inhibitory effect of very low dose of EGCG on intestinal heme iron absorption can be easily counteracted by dietary ascorbic acid, while EGCG of higher concentrations are more potent in inhibiting intestinal heme iron absorption

Similarly, the inhibited heme ^{55}Fe transport by 0.46 mg/L GSE was completely reversed by ascorbic acid and was even enhanced above the control level. The amount of transported ^{55}Fe into the basolateral chamber was 41% higher ($p < 0.05$) than that of the control during the 7-h incubation (Figure 4-5B). The transepithelial ^{55}Fe transport was decreased by 59.2% ± 2.45% in the presence of 4.6 mg/L GSE alone (Figure 4-5B), but ascorbic acid significantly alleviated GSE's inhibitory action, and the resulting ^{55}Fe transport was only decreased by 24.5% ± 4.09% compared with the control (Figure 4-5B).

Ascorbic acid also completely reversed the inhibitory effect of 0.46 mg/L GT on heme iron absorption. The transepithelial transport of ^{55}Fe was enhanced by 48% ± 0.4% by ascorbic acid in the presence of 0.46 mg/L GT, compared with the control, during 7-h incubation (Figure 4-5C). The addition of ascorbic acid was not able to reverse the inhibitory effect 4.6 mg/L GT on heme-^{55}Fe transport, although the negative effect was mitigated. The addition of ascorbic acid, however, did not alter the inhibitory effect of 46 mg/L GT, as shown for EGCG and GSE (Figure 4-3B); the rate of ^{55}Fe transport remained significantly reduced by 83.6 ± 5.58%. Surprisingly, ascorbic acid itself significantly enhanced the amounts of ^{55}Fe transported into the basolateral chamber by 97.9% ± 25.3% compared with the control. However, the apical uptake of heme-^{55}Fe was not changed by ascorbic acid. As demonstrated from our ferrozine-based colorimetric assay,

free iron was not detected in the apical uptake solution containing heme-^{55}Fe, indicating that ^{55}Fe was added as heme-^{55}Fe rather than as free ^{55}Fe during the iron transport study. Most of the ^{55}Fe (>97%) transported from the apical to the basolateral compartment across the cell monolayer was heme free iron.

4.6 Effect of Polyphenols and Ascorbic Acid on Expression of Iron Relevant Proteins and HO Activity

To investigate the possible mechanisms by which polyphenols and ascorbic acid modulate heme iron absorption, the expression of proteins involved in intestinal heme iron metabolism and basolateral iron transport and total HO activity were assessed. The expression of HO-1 and -2 proteins was not changed by ascorbic acid, EGCG, GSE, GT, or by the cocktail of ascorbic acid and each of these polyphenols. Similarly, the expression of other proteins involved in iron metabolism and basolateral iron transport was not modulated by ascorbic acid and polyphenolic compounds (Figure 4-6). The total HO activity detected in the control group was 0.77 ± 0.027 nmole bilirubin/(min • mg protein), and was not affected by ascorbic acid and/or by polyphenolic compounds ($F = 1.814$, p-value = 0.1113) (Figure 4-7).

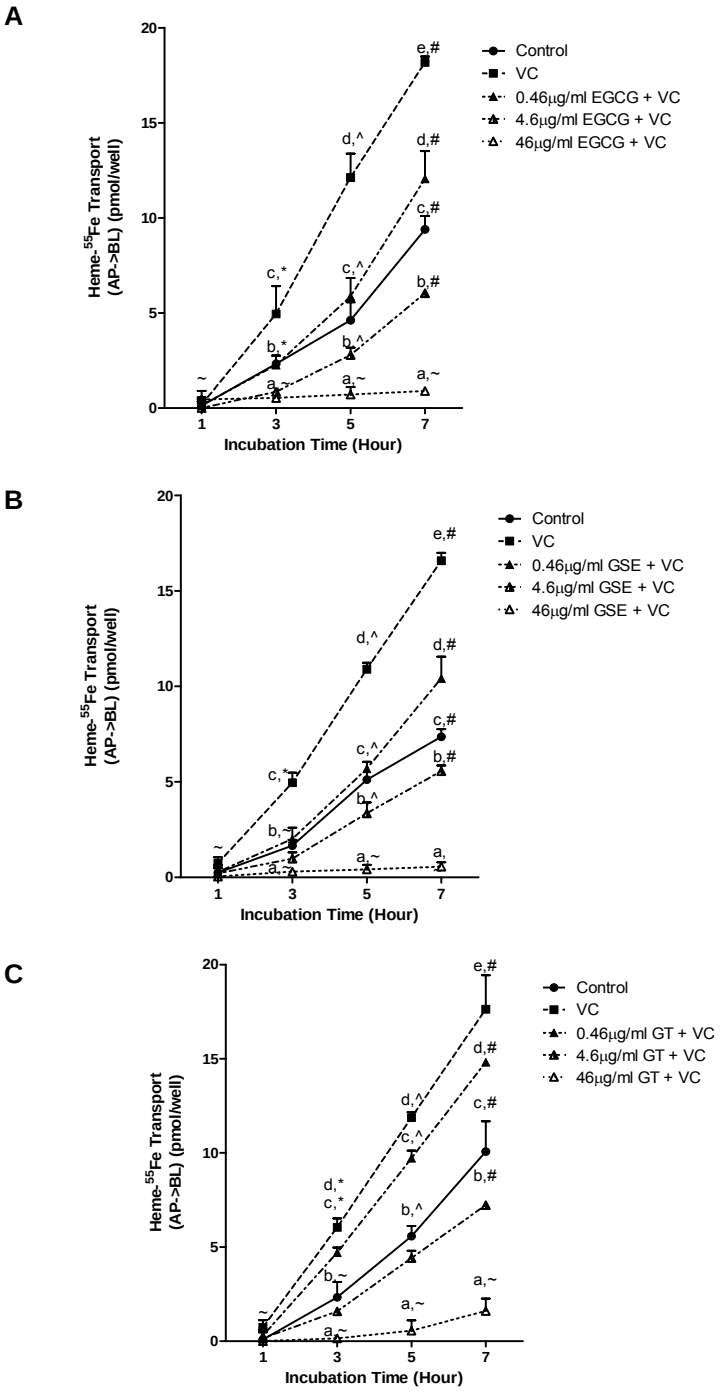

Figure 4-5. Ascorbic acid can reverse or alleviate the inhibitory effect of polyphenols at concentrations of ≤ 4.6 mg/L on heme iron absorption in Caco-2 cells

(A) EGCG, (B) GSE, and (C) GT. Ascorbic acid was added at 100 μM. Values are means ± SEM, n = 6. Means of different doses at the same time point without a common letter differ, $p < 0.05$. Means of different time points for the same treatment without a common symbol differ, $p < 0.05$.

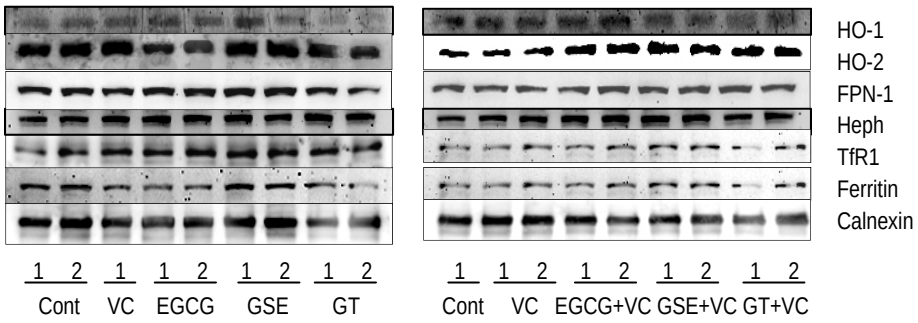

Figure 4-6. Effects of polyphenols and ascorbic acid on HO activity

(A) Quantification of bilirubin production. (B) Effects of polyphenols and ascorbic acid on HO activity. Cells were treated with 46 mg/L EGCG, GSE, GT, and 100 μM ascorbic acid for 7 h. Values are means ± SEM, n = 6. No significant differences were found in HO activity.

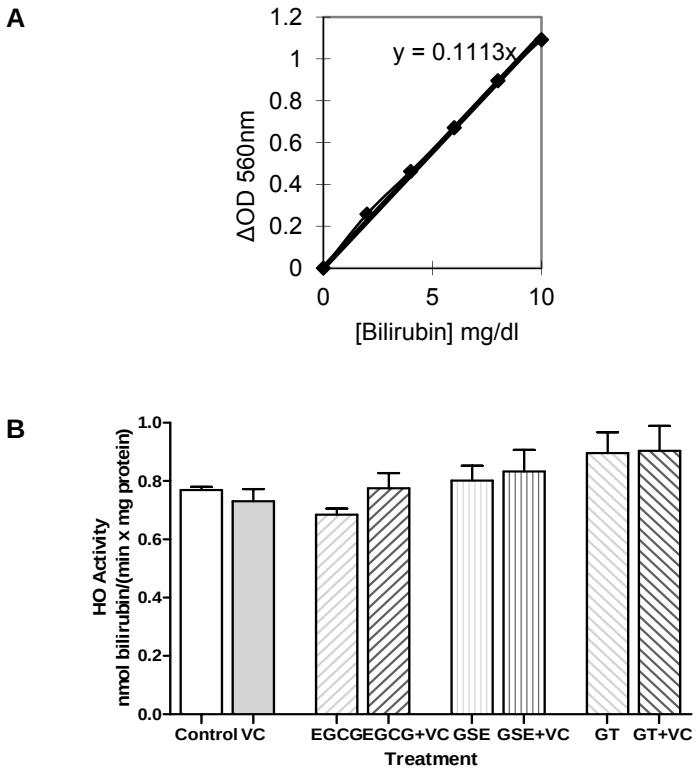

Figure 4-7. Effects of polyphenols and ascorbic acid on the expression of proteins involved in intestinal heme iron absorption and metabolism

Representative western blots of HO-1, HO-2, FPN-1, Heph, TfR1, Ft protein expression from Caco-2 cells treated with 1μM heme in the uptake buffer containing 46 mg/L EGCG, GSE, GT and/or 100 μM ascorbic acid for 7 h. Calnexin was used as a loading control.

DISCUSSION

Dietary heme iron is principally provided by meat, blood-derived products and other animal tissues. Heme iron absorption is relatively unaffected by other dietary factors that are common inhibitors of mineral absorption, such as phytates and fiber (Torre *et al.* 1991). The best known dietary factors that affect intestinal heme iron absorption are meat factor and calcium (Lopez *et al.* 2004). Heme iron absorption is increased by the presence of meat factor in animal tissues (Carpenter *et al.* 1992). In contrast, calcium is known to inhibit heme iron absorption in the same fashion that it inhibits non-heme iron at high concentrations (Hallberg *et al.* 1991). Although most research on the relationship between dietary factors and iron absorption has focused on non-heme iron, few studies have been conducted on heme iron.

We found that EGCG and GSE significantly inhibited the transepithelial transport of heme iron across the fully-differentiated Caco-2 cell monolayer. A significant decrease of heme iron absorption was also observed in humans when Hb was given with tea instead of water (Disler *et al.* 1975). Heme iron absorption was decreased by 60% and it was suggested that the decreased heme iron absorption might be due to the formation of insoluble heme-polyphenol complexes in the gastrointestinal lumen (Disler *et al.* 1975). Our study showed that the cellular assimilation of heme ^{55}Fe was decreased by 18 ± 3.0% in cells treated with EGCG. However, our data do not support the possibility that EGCG inhibits the apical heme uptake by forming an insoluble EGCG-heme complex. We observed that the solubility of heme was not decreased by EGCG or GSE. The inhibitory effect of EGCG on the transepithelial transport of heme-derived iron is similar to that for non-heme iron. We previously reported that EGCG markedly decreased the transepithelial non-heme iron transport but increased the apical uptake of iron and suggested that the decreased iron transport is due to the reduced basolateral iron export (Kim *et al.* 2008). The

reason for this discrepancy for the apical uptake might be related to the different apical uptake pathways for heme and non-heme iron.

The recent discovery of a heme/folate transporter called proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1), located on the apical membrane of enterocytes (Shayeghi *et al.* 2005; Qiu *et al.* 2006), suggests that heme and folate may share at least partially for the intestinal apical uptake pathway. Interestingly, the inhibitory effect of bioactive Polyphenols on folate uptake were previously shown in intestinal cells (Lemos *et al.* 2007). Addition of EGCG, green tea, and red wine decreased the apical uptake of folate in Caco-2 cells and it was suggested that EGCG and other polyphenolic compounds in green tea and red wine decreased folate uptake, possibly by blocking folate transport pathway (Lemos *et al.* 2007). Thus, EGCG and other polyphenolic compounds may share with heme and folate at least partially the proton-coupled folate transporter/HCP1-mediated uptake step and have competitive inhibitory effect on heme and folate apical uptake in intestinal absorptive cells. The mechanisms of intestinal absorption of EGCG and other polyphenols remain to be elucidated.

GSE also inhibited the transepithelial transport of heme iron across the cell monolayer. GSE, however, increased the cellular assimilation of heme ^{55}Fe as previously shown for non-heme iron (Kim *et al.* 2008). The increased apical heme uptake suggests that GSE reduces heme iron absorption by decreasing the basolateral transport of heme iron. It is possible that some polyphenols in GSE might enter the cell, chelate iron released from heme in the cytoplasm, and prevent iron release across the basolateral membrane of the enterocyte. It is not clear how polyphenols in GSE enhance the apical heme uptake in the enterocyte.

One possible mechanism for the inhibitory action of EGCG and GSE on the basolateral iron release is that addition of EGCG and GSE might decrease an iron exporter FPN protein level. To test this, we assessed FPN protein level by western blot analysis. FPN protein level was not changed by either EGCG or GSE (Figure 4-2B). The Heph protein level was also not changed by

these polyphenolic compounds. Both FPN and Heph are key proteins involved in the basolateral iron export in intestinal enterocytes. Together, these data support that EGCG and GSE might form non-transportable complexes with heme-derived iron in cells and prevent iron release across the intestinal basolateral membrane. This hypothesis was further confirmed by another data set. The assimilation of cellular iron generally increases cellular ferritin level by inducing ferritin protein production. To test if the assimilated cellular heme ^{55}Fe was able to induce expression of ferritin protein in cells treated with GSE, cellular ferritin protein level was also measured. Our western blot data (Fig. 4.2B) suggest that the assimilated ^{55}Fe might be not utilized by cells, because cellular ferritin level was not increased by the assimilated iron in GSE-treated cells.

During iron deficiency, degradation of heme is increased by up-regulation of HO-1, thus increasing the amount of iron available for absorption (Raffin *et al.* 1974). The HO-1 is present in many tissues, including the intestine, with the highest levels observed in the duodenum. HO-1 activity can be increased 10- to 100-fold by various conditions, including hyperthermia, heme, and heavy metals such as cobalt and cadmium (Cable *et al.* 1993; Ny *et al.* 1997). It was suggested that increased HO-1 activity and expression might be responsible for the increased heme iron absorption and a rapid export of heme iron (Follett *et al.* 2002; Arredondo *et al.* 2008). Heme iron transport was increased in cells treated with cadmium and the increased heme iron transport was associated with the increased HO-1 protein by heme in Caco-2 cell culture. Therefore, it was speculated that bioactive polyphenols might downregulate HO-1 protein and/or activity and decrease the amount of iron available for the basolateral efflux. However, neither HO-1 nor HO-2 proteins were decreased by EGCG and GSE (Figure 4-2A), suggesting that EGCG and GSE possibly inhibit the basolateral release of heme-derived iron by forming complexes with iron in the intestinal cells. Although some studies suggested that heme iron can be absorbed intact across enterocytes, we did not observe that in our current study. Only a negligible portion (<3%) of iron was transported as heme into the basolateral chamber,

suggesting that the decreased basolateral iron release is not due to dysfunction of the heme exporter FLVCR activity in cells treated with EGCG and GSE.

An early study indicated that heme iron absorption decreased due to the consumption of tea in humans (Disler *et al.* 1975), and this finding was confirmed by our recent study (Figure 4-1). We found that dietary polyphenolic compounds (such as EGCG, a major polyphenolic compound in green tea, and GSE) markedly inhibited heme iron absorption when polyphenolic compounds were added at the concentration of 46 mg/L. Because many dietary factors modulate iron absorption in a dose-dependent manner (Hallberg *et al.* 1989; Tuntawiroon *et al.* 1991), we next investigated whether these selected bioactive polyphenolic compounds retained the ability to reduce heme iron absorption when added at lower concentrations. Our data clearly indicated that EGCG and GSE inhibit heme iron absorption in a dose-dependent manner in human intestinal cells (Figure 4-3). Similarly, GT, the natural source of EGCG, also significantly decreased heme iron absorption in a dose-dependent way. Even at very low concentrations (0.46 mg/L), EGCG, GSE, and GT significantly reduced heme iron transport across the cell monolayer during a 7-h transport assay. According to the IC50 values, the transepithelial heme iron transport across the cell monolayer can be reduced 50% by 3.6, 3.0, and 5.1 mg/L of EGCG, GSE, and GT, respectively.

Because the inhibitory effect of dietary factors on iron absorption can be offset or reversed by ascorbic acid, the most prominent dietary factor that enhances iron absorption, we next examined whether the inhibitory action of polyphenols on heme iron absorption can be counteracted by ascorbic acid. The addition of 100 μM ascorbic acid completely reversed the inhibitory action of dietary polyphenols on heme iron absorption when the polyphenolic compounds were added at 0.46 mg/L. When the concentrations of polyphenols were increased to 4.6 mg/L, ascorbic acid was not able to counteract the inhibitory action of polyphenols on heme iron absorption, although the inhibition was reduced. However, ascorbic acid failed to have any

positive effect on heme iron absorption when the polyphenols were added at a high (but still within physiological) level of 46 mg/L. These results imply that, while the inhibitory effect of low concentrations of bioactive polyphenols on heme iron absorption can be easily counteracted by ascorbic acid, the inhibitory action of high concentrations of polyphenolic compounds cannot be offset by regular consumption of dietary ascorbic acid. Because the addition of ascorbic acid enhanced heme iron absorption above the control in the presence of the low level of polyphenolic compounds, we investigated the effect of ascorbic acid on heme iron absorption in the absence of dietary polyphenols. We found that ascorbic acid markedly enhanced heme iron transport across the cell monolayer without altering the apical uptake of heme. The applied ^{55}Fe-labeled heme remained intact because the free iron was not detected in solution containing heme-^{55}Fe. This finding is similar to a previous study on the effect of soy protein on heme iron absorption. Lynch and others showed a significant increase in heme iron absorption by soy protein in human subjects (Lynch *et al.* 1985). However, the mechanism of the soy protein-mediated increase of heme iron absorption still remains to be explored.

To examine the possible mechanism by which ascorbic acid enhances heme iron absorption, we initially analyzed the expression of proteins involved in heme iron absorption and metabolism. Because these test solutions modulate heme transport across the cell monolayer without altering the apical heme uptake, we assessed the proteins involved in heme splitting, cellular iron metabolism, and basolateral iron transport. Our western blot analysis data indicated that neither ascorbic acid nor selected polyphenolic compounds changed the expression of proteins involved in heme iron absorption and metabolism.

After heme enters the cell across the apical membrane of the enterocyte, it is then degraded by HOs to release ferrous iron and bilirubin, and the released iron enters into the soluble cytoplasmic pool of the enterocyte (Raffin *et al.* 1974). It is likely that, after heme iron is disassembled by HOs, the liberated iron enters the same storage and export pathways as non-

heme iron does. Duodenal HO activity was previously proposed as a limiting factor for heme iron absorption and shown to be linearly associated with heme iron absorption (Wheby *et al.* 1981). Therefore, to determine whether ascorbic acid and/or polyphenols modulate heme iron absorption by affecting the release of iron from heme, we assessed HO activity in cells treated with ascorbic acid and/or polyphenolic compounds. In the conventional HO assay, enzyme activity is measured by the rate of bilirubin formation, as iron release from heme is linearly associated with production of bilirubin. As indicated by Figure 4-7, HO activity was not changed by ascorbic acid and polyphenolic compounds, suggesting that the addition of ascorbic acid and polyphenols modulated heme iron absorption without changing heme splitting in Caco-2 cells.

Our data clearly indicate that ascorbic acid enhances heme iron absorption across the cell monolayer without modulating the apical heme uptake, indicating that ascorbic acid may affect cellular or basolateral events that increase heme-derived free iron export. The addition of ascorbic acid may enhance heme iron absorption by modulating several different steps in the cell, such as (1) by increasing the release of iron from heme, (2) by inducing basolateral iron export through an increase in FPN-1 expression, and (3) by facilitating the transfer of iron to the basolateral membrane. Because our western blot analysis data (Figure 4-6) and HO activity data (Figure 4-7) do not support the first 2 possible mechanisms, it is possible that ascorbic acid may increase the available iron pool for basolateral iron transporter or improve iron transport to the basolateral membrane. It is believed that ascorbic acid mainly enhances non-heme iron absorption by reducing ferric to ferrous iron, a substrate of DMT1 in the gastrointestinal lumen, and many studies have confirmed this conclusion (Raja *et al.* 1992). A study previously demonstrated that ascorbic acid enhanced non-heme iron absorption by reducing ferric to ferrous iron and then increasing the apical iron uptake. However, the iron transported across the basolateral membrane was also enhanced (Han *et al.* 1995). Although the transepithelial iron transport across the Caco-2 cell monolayer was increased by 3.5-fold, the apical iron uptake was only elevated less than 2-

fold compared with the control, indicating that ascorbic acid enhanced not only the apical uptake but also the basolateral transport. It is clear how ascorbic acid increases the apical uptake of non-heme iron, but it is unknown how ascorbic acid enhances the basolateral transport of iron. One proposed explanation is that ascorbic acid increases the basolateral iron transport by increasing the assimilation of iron into the cell (Han *et al.* 1995). In the current study, ascorbic acid enhanced the transepithelial iron transport without changing the apical heme uptake, HO activity or the level of FPN-1, suggesting that ascorbic acid may facilitate iron transfer to the basolateral membrane, leaving more ^{55}Fe available for the iron exporter FPN-1. Together, our results indicate that small amounts of polyphenolic compounds in foods are capable of reducing heme iron transport across the intestinal enterocyte. However, these inhibitory effect of dietary polyphenolic compounds on heme iron absorption can be offset by ascorbic acid, and they can possibly be avoided by decreasing consumption of polyphenols while simultaneously consuming ascorbic acid.

Conclusions

In conclusion, our study shows that bioactive dietary polyphenols inhibit heme iron absorption in human intestinal cells as previously reported in humans (Disler *et al.* 1975). EGCG and GSE decrease heme iron absorption mainly by reducing basolateral release of heme-derived iron. This is the first report, to our knowledge, demonstrating that bioactive dietary polyphenols inhibit heme iron transport across the enterocyte by decreasing basolateral iron export, possibly by forming non-transportable complexes with iron in the cell. The precise mechanism by which bioactive dietary polyphenolic compounds inhibit heme iron absorption remains to be elucidated. In addition, bioactive dietary polyphenols (including EGCG, GSE, and GT) inhibit heme iron absorption in a dose-dependent manner in human intestinal Caco-2 cells. Ascorbic acid cannot

offset the inhibitory effect of high concentrations (46 mg/L) of polyphenols but can reverse the effects of lower concentrations of polyphenols.

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Chapter 5

ABSORPTION OF HEME AND NON-HEME IRON ARE SYNERGISTIC VIA TRANSLOCATION OF FPN1 IN CACO-2 CELLS

ABSTRACT

Background

It is well known that animal tissues are often associated with enhanced intestinal iron absorption. The factors in animal tissues responsible for increased intestinal iron absorption are named as meat factors. However, the identities and mechanism of the meat factors are not fully-elucidated. The aim of this study was to gain new insight into the mechanism of increased iron absorption promoted by meat and whether and how the absorption of heme and non-heme iron interfere with each other.

Design and Methods

Cellular uptake and transepithelial absorption of non-heme ^{55}Fe were analyzed in human intestine-like Caco-2 cells in response to different doses of heme or protoporphyrin treatment. Expression patterns and localizations of non-heme iron transporters, divalent metal transporter-1 (DMT1) and ferroportin-1 (FPN1) were examined in Caco-2 cells treated by heme and/or non-heme iron using western blot analysis and confirmed by confocal microscopy. Enzyme activity of duodenal cytochrome b (Dcytb) was also assessed using ferrireductase assay.

Results

We found that non-heme ^{55}Fe absorption across Caco-2 cell monolayer was enhanced by heme via redistributing DMT1 from cytosol to apical membrane and FPN1 from cytosol to basolateral membrane. Interestingly, heme ^{55}Fe absorption was also increased by treatment of non-heme iron. Although non-heme iron alone did not affect FPN1 localization, heme and non-heme iron together synergistically redistributed FPN1 to basolateral membrane. In addition, the protoporphyrin ring of heme was sufficient to induce translocation of DMT1 and FPN1 as well as non-heme iron absorption, while the competition between the iron released from the heme moiety and non-heme iron was negligible compared to compensatory increase of FPN1 on basolateral membrane. Finally, ferrireductase activity was increased by heme treatment for 7 h.

Conclusions

Intestinal absorption of heme and non-heme iron are reciprocal of each other via translocation of DMT1 and FPN1 to cell membrane. Therapies that target intestinal DMT1 and FPN1 might be beneficial for patients with iron-related disorders.

Keywords

Heme, Non-heme iron, Intestinal iron absorption, FPN1, DMT1

INTRODUCTION

Iron is an essential micronutrient required for survival and proliferation for almost all living organisms. As there is no regulated way to eliminate iron from the body, iron levels in the body are mainly controlled by dietary iron absorption. Intestinal iron absorption is comprised of three sequential steps: apical iron uptake from intestinal lumen, intracellular iron transfer and basolateral iron export into blood.

There are two forms of dietary iron, non-heme iron and heme iron. The apical uptake for non-heme iron is facilitated by divalent metal transporter-1 (DMT1), which transports dietary ferrous iron (Fleming *et al.* 1997; Gunshin *et al.* 1997), while dietary ferric iron must first be reduced to its ferrous state before it can be absorbed into enterocytes. Heme iron derived from hemoglobin or myoglobin is supposed to be taken up via proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) on the brush border (Shayeghi *et al.* 2005) and split to free ferrous iron by heme oxygenases (HOs) within enterocytes (Weintraub *et al.* 1968). All free ferrous iron in enterocytes ultimately joins the labile iron pool, the chemical identity of which is still poorly characterized due to the multiplicity of iron ligands present in cells (Kakhlon *et al.* 2002). Iron from labile iron pool can then be incorporated to iron storage protein ferritin or other iron-requiring proteins. Basolateral iron release is mediated by ferroportin (FPN1), the only iron exporter known to date (Donovan *et al.* 2000; McKie *et al.* 2000). Iron released into blood is subsequently oxidized by hephaestin, a multicopper ferroxidase homologous to ceruloplasmin. The ferric iron is then loaded onto transferrin (Tf) in the circulation for delivery into tissues.

Intestinal iron absorption is regulated by both pathophysiological conditions and dietary factors. Regulation of intestinal iron absorption in response to systemic cues is mediated by hepcidin, a small peptide hormone secreted by liver according to iron status, erythropoietic needs, hypoxia and inflammation (Ganz 2011). High level of iron or inflammation in the body elicits

secretion of hepcidin from hepatocytes, which, in turn, acts on its downstream targets including but not limited to small intestine, macrophages and liver via post-translational modification of FPN1 to down-regulate iron release into the plasma. In contrast, hypoxia condition or erythropoietic signal reduces liver hepcidin production and thereby increases intestinal iron absorption via FPN1. In addition to systemic regulation by hepcidin levels, intestinal iron absorption is also adjusted by mucosa itself by modulating expression of DMT1, duodenal cytochrome b (Dcytb) and FPN1 via iron-regulatory proteins (IRPs) and hypoxia-inducible factor 2α in response to local iron status and hypoxia (Shah *et al.* 2009).

Apart from the pathophysiological regulators, intestinal iron absorption is known to be affected by several dietary factors, such as ascorbic acid, phytates and some polyphenols. In western diets, non-heme iron constitutes approximately two thirds of dietary iron. Intestinal absorption of non-heme iron in humans is promoted by animal products including pork, beef, poultry and fish (Cook *et al.* 1976). Single-meal radioisotope absorption studies in both animal and human subjects consistently revealed that pork, beef, fish, chicken and calf thymus all increased non-heme iron absorption 2 – 3-fold (Bjorn-Rasmussen *et al.* 1979; Gordon *et al.* 1989; Navas-Carretero *et al.* 2008). The factors in animal products that promote iron absorption are coined as “meat factor”, the nature of which remains unresolved. Most studies in pursuit of meat factor focused on the protein fraction of meat, but it is well possible that other components of animal tissues are involved (Hurrell *et al.* 2006). Although heme is a component in animal products, no studies has investigated into the effect of heme on intestinal non-heme iron absorption yet. It is possible that heme itself may be a component in meat that enhances non-heme iron absorption.

It was assumed that intestinal absorption of heme iron and non-heme iron compete with each other as iron from them share the basolateral exporter FPN1 for exit into blood; and if so, it would be reasonable to only study the characteristics and regulation of them individually and

separately. However, whether and how intestinal absorption of heme and non-heme iron affect each other has never been elucidated yet. Based on our preliminary data, it is possible that dietary absorption of heme and non-heme iron boost each other in a synergistic way instead of compete. In this case, they may well be bundled together as a dietary strategy to enhance iron absorption for people with iron deficiency and the characteristics and mechanism of their absorption deserve being studied jointly. Under the hypothesis that heme and non-heme iron may boost the absorption of each other via regulation of intestinal iron transporters, we studied whether intestine balances between absorption of the two forms of dietary iron and how each form of iron achieved these effects.

MATERIALS AND METHODS

Reagents

The ^{55}Fe (in the form of $^{55}FeCl_3$) was obtained from Perkin–Elmer Life Sciences (Boston, MA). Hanks' balanced salts solution (HBSS), glutamine, nonessential amino acids and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Unless otherwise noted, all other reagents were obtained from Sigma Chemical (St. Louis, MO), VWR (West Chester, PA), or Fisher Scientific (Springfield, NJ).

Caco-2 Cell Culture

The human Caco-2 cell line HTB37 was purchased from American Type Culture Collection (Rockville, MD). Stock cultures were maintained at 37°C in complete medium in a humidified atmosphere of 95% air and 5% CO_2 and used for experiments within 20 serial passages. The complete culture medium contained Dulbecco's Modified Essential Medium (DMEM) supplemented with 25 mM glucose, 2 mM glutamine, 100 μM non-essential amino acids, 100 U/l penicillin G, 100 mg/l streptomycin and 10% FBS. For experiments, 5.0×10^4 cells/cm^2 in 1.5 ml complete DMEM were seeded on 3 μm microporous membrane inserts (4.9 cm^2, BD Biosciences, Bedford, MA) coated with collagen type I (5 μg/cm^2) on 6-well plates. The basolateral chamber was filled with 2.5 ml complete DMEM. The culture medium was changed every 2 days, and cells were used after 17-day post-confluence for experiments. The Caco-2 cell monolayer was routinely checked for tight junctions at 17-d postconfluence with transepithelial electrical resistance (TEER) >250 Ω/cm^2, which indicates full differentiation under normal cell culture conditions.

^{55}Fe Transport Study

Transepithelial heme-derived ^{55}Fe transfer from apical to basolateral compartment were determined by scintillation (Han *et al.* 1994). After washing the cell monolayer three times with Ca^{2+}- and Mg^{2+}-free Hank's balanced saline solution (HBSS) containing 137 mM NaCl, 5.36 mM KCl, 1.3 mM $CaCl_2$, 410 μM $MgSO_4$, 490 μM $MgCl_2$, 337 μM Na_2HPO_4, 440 μM KH_2PO_4, 4.17 μM $NaHCO_3$ and 5.55 mM dextrose at 37°C, cells were incubated at 37°C with 1.5 ml of ^{55}Fe/Fe(NTA)$_2$ or ^{55}Fe/Fe-heme in iron-uptake buffer in the apical compartment and 2.5 ml DMEM in the basolateral compartment. The uptake buffer contained 130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES, pH 7.0. Transepithelial iron transfer from apical to basolateral chamber and cellular iron accumulation were determined. An aliquot of 200 μL was removed from the basolateral chamber at the indicated time points and replaced with an equivalent volume of pre-warmed DMEM; time course data were corrected to account for this sample replacement. To measure the cellular levels of ^{55}Fe, cell monolayers were washed three times with ice-cold wash buffer containing 150 mM NaCl, 10 mM HEPES, pH 7.0, and 1 mM EDTA to remove any nonspecifically bound radioisotope. Cells were homogenized in PBS containing 0.3% Triton X-100, and ^{55}Fe was quantified by liquid scintillation counting in glass vials. Protein levels were assessed using Bio-Rad protein assay kit (BioRad Laboratory Inc., Hercules, CA). All ^{55}Fe transport studies were repeated for at least 3 times.

Western Blot

Western blot was performed to determine protein levels of DMT1, Dcytb, FPN1, TfR1, hephaestin, HO-1, HO-2 and calnexin in Caco-2 cells. Protein samples extracted from the same cells were used for all Western blot analyses. Cell lyses (20 μg or 40 μg) were solubilized in Laemmli buffer, boiled for 10 min, and separated by a 7.5% SDS–PAGE. Proteins were transferred by electroblotting to nitrocellulose membranes. The membranes were first blocked by

5% non-fat dry milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) at room temperature for 1 h, and then incubated for 2 h at room temperature with the primary antibodies (1:3,000) in TBS containing 0.05% Tween 20 (TBST). The membranes were washed for several times with TBST and then incubated for 1 h at room temperature with peroxidase-linked goat anti-rabbit IgG (1:3,000). The visualized antigens by ECL were detected ChemiDoc XRS system (Bio-Rad, Hercules, CA).

Dcytb Activity Assay

Ferrireductase activity was assessed by the production of a tris-Fe^{2+}-ferrozine complex from the membrane-impermeable ferrozine (Fz). Fully-differentiated Caco-2 cell monolayers grown on microporous membrane inserts were incubated at 37°C for 7 h with 1.5 ml of 100 μM Fz and 10 μM Fe^{3+} $(NTA)_2$ and in absence or presence of 10 μM heme in iron uptake buffer added to the apical chamber and with 2.5 ml complete DMEM in the basolateral chamber. After the 7h incubation, the apical medium was collected and centrifuged at 2000 rpm for 5 min to remove any cell debris. The concentration of Fe^{2+} in the resulting supernatant was immediately determined by measuring the absorbance at 562 nm for the formation of the Fe^{2+}-Fz complex. Standards were prepared by adding 100 μM ascorbic acid to uptake buffer containing both Fe^{3+} $(NTA)_2$ at the various concentrations (0, 0.001, 0.01, 0.1, 1 and 10 μM) and 100 μM Fz, followed by incubation for 20 min at room temperature. The standard curve for the absorbance at 562 nm and the concentration of Fe^{2+}-Fz product was linear for the above concentrations of substrates. The amount of proteins for cells growing on each insert was determined by protein assay and is approximately 1 mg for all inserts. The Dcytb activity was expressed as nmol Fe^{2+}-Fz complex formed per mg protein per hour.

HO Activity Assay

HO activity was assessed on the basis of the rate of bilirubin production (Maines 1996). HO activity reflects the total activity of both HO-1 and HO-2 in the cell. Caco-2 cells treated with 1 μM heme in the absence or presence of 10 μM Fe^{3+} $(NTA)_2$ for 7 h were harvested in ice-cold 0.3 mL PBS with 1% Triton X-100 and homogenized. The cell lysates were incubated with 10 μM hemin at 37 °C with shaking for 30 min. The reaction was terminated by placing the samples on ice. The samples were centrifuged at 4 °C for 10 min to remove the cell debris. The amount of bilirubin in the supernatant was determined using the QuantiChrom™ bilirubin assay kit (BioAssay Systems, Hayward, CA). All procedures of the assay were conducted under dim light. The HO activity was expressed as nmol bilirubin formed per minute per mg protein.

Cell Fractionation by Sucrose Gradient

Cell monolayers were washed with ice-cold PBS for three times, collected in 0.5 ml PBS and then centrifuged for 10 min at 2000 rpm. The cell pellets were suspended in 0.3 ml breaking buffer (pH 7.4) containing 25 mM Hepes, 85 mM sucrose, 100 mM KCl and 20 μM EGTA and homogenized by passing through a syringe for several times. The cell lysates were centrifuged for 5 min at 2000 rpm. The post-nuclear supernatant was transferred to a new microcentrifuge tube and centrifuged again for 20 min at 14000 rpm. The resulting supernatant was the cytosol fraction and transferred to a new tube. The pellet was the crude membrane fraction and re-suspended in 0.1 ml breaking buffer containing 1% Triton X-100 and protease inhibitor. Both fractions were stored at -20°C and used for preparing protein samples for western blot analysis.

Cell Fixing & Immuno-staining

The fully-differentiated Caco-2 cells grown on microporous membrane inserts was first fixed with 2% formaldehyde and permeabilized with 0.3% Triton X-100 for 30 min. Cells were

then incubated with indicated primary antibodies (anti-FPN1 1:200, anti-DTM1 1:200) with 1% BSA in PBS for 2 hours at room temperature. After several washes, Caco-2 cells were incubated with either Alexa Fluor 546 (red) conjugated goat anti-rabbit IgG or Alexa Fluor 488 conjugated goat anti-rabbit IgG for 1 h at room temperature. To stain the plasma cell membrane, the fixed cells were incubated with Alexa Fluor 594 conjugated WGA for 10 min at room temperature. The inserts were placed on glass slides and mounted. Since the inserts were placed on the microscope stage with their bases on top of a glass slide, the direction of the laser beam was basal to apical. To determine the cellular location of proteins, the fluorescence image was taken at every 1.0 mm from the basal end to the apical side. The images for XY view were taken at 5 mm from the basal side. To analyze fluorescence intensity in the basal to apical axis from cells, the lateral view (xz) is made up by the addition of consecutive pixels in the y-axis. Cells were analyzed at 60x magnification on a laser scanning confocal microscope (Fluoview 1000 Confocal Laser Scanning Microscope, Olympus).

MEL Cell Culture and Synthesis of ^{55}Fe-Hemoglobin (Hb)

The murine erythroleukemia (MEL) cell line was a generous gift from Dr. Robert Paulson at Pennsylvania State University (University Park, PA). The MEL cells were grown in the same conditions as described above for the Caco-2 cells. As previously reported, Hb was synthesized using MEL cells as previously reported (Ma *et al.* 2010). Briefly, to induce Hb synthesis, erythroid differentiation of cells was induced by adding dimethyl sulfoxide (DMSO). $(^{55}Fe)_2$-Tf was prepared from apo-Tf and ^{55}Fe and purified. The level of Tf saturation was estimated from the A465/A280 ratio, which was routinely found to be 0.046, consistent with complete saturation on both sites of Tf for iron binding (Huebers *et al.* 1978). To produce ^{55}Fe-Hb, MEL cells were seeded at 10,000 cells/cm^3 and were treated with 2 μmol/L $(^{55}Fe)_2$-Tf and 2%

DMSO. After a 6-d treatment, the cells were harvested and washed three times with phosphate-buffered saline (PBS, pH 7.0) and then collected by centrifugation for 5 min at 800 xg, 4°C.

Spectrophotometric Quantification of Hb and Hb Digestion

Hb concentrations in MEL cell lysates were measured by the benzidine assay as previously described (Ma *et al.* 2011). Briefly, pellets of MEL cells (70 x 10^6 of cells) harvested after 6 d were resuspended in 0.5 mL distilled water and lysed by 4 cycles of freezing (in dry ice/ethanol for 3 min) and thawing (in a water bath at 37°C for 3 min). The cell lysate were centrifuged at 14,000 xg for 15 min at 40°C using an Eppendorf 5402 centrifuge (Hamburg, Germany). Bezidine assay was performed in duplicate by adding the reagents in the following order: 100 µL supernatant, 900 µL deionized water and 100 µL freshly prepared benzidine-HCl (10 mg/ml in 0.5% acetic acid). The reaction was started by the addition of 40 µL 30% H_2O_2. The contents were mixed well and after exactly 90 sec the absorbance was measured at 604 nm. The Hb concentration was then calculated based on the measured absorbance using a calibration curve obtained with purified Hb standard (Sigma, Saint Louise, MO) measured in the same way as the supernatant. Both Hb-^{55}Fe prepared from MEL cell lysis and commercially bought bovine Hb were digested with 0.1% pepsin at pH 2.0 overnight at 37°C. The solution was then centrifuged for 5 min at 14,000 xg at 4°C. The digestion rate was estimated by measuring the remaining Hb content in the supernatant using benzidine assay as described above. Pellets of ^{55}Fe-heme and heme were dissolved in 10 mmol/L of NaOH and further diluted in iron uptake buffer (final pH 7). The ^{55}Fe-heme specific activity was between 0.4 Ci/mol and 0.45 Ci/mol heme.

Statistical Analysis

Values are expressed as non-transformed means ± SEM (n = 3 – 6). The experiments were repeated at least three times for each treatment. Data were analyzed by 1-way ANOVA with

the following post-hoc tests for multiple comparisons using Prism 5.0 software (GraphPad). Differences were considered significant when $p < 0.05$.

RESULTS

5.1 Heme Enhances Non-Heme Absorption in Caco-2 Cells

To understand if heme is a non-protein substance in meat that enhances dietary non-heme iron absorption, we asked ourselves whether heme regulates intestinal non-heme ^{55}Fe absorption in Caco-2 cells. Since heme consists of protoporphyrin ring and the atomic ferrous iron, we treated Caco-2 cells with either 10 μM heme or protoporphyrin IX (PPIX), a heme precursor that does not contain iron atoms, accompanying the non-heme ^{55}Fe transport study for 7h. Our results showed that heme itself increased the rate of non-heme ^{55}Fe absorption across the Caco-2 cell monolayer by 24.4 ± 7.41% (Table 5-1). Apical non-heme ^{55}Fe uptake in Caco-2 cells was also increased by 45.7 ± 3.66% with the treatment of heme (Figure 5-1B). This finding suggests that heme could possibly be a meat factor that mediates dietary non-heme iron absorption. Interestingly, treatment with identical concentrations of PPIX showed a comparable effect to heme, indicating that the response of increased non-heme iron absorption is independent of iron release from heme by activities of heme oxygenases (Figure 5-1C & D). Meanwhile, as hemoglobin is the major protein that contains heme in animals and a significant source of heme supply from meat products, to determine if the effect is heme-specific, we also examined the effect of hemoglobin on non-heme iron absorption. We found that heme and PPIX, but not Hb, enhanced non-heme ^{55}Fe absorption across the Caco-2 cell monolayer (Figure 5-1G). Transepithelial ^{55}Fe transport across the Caco-2 cell monolayer at 7 h were increased by the treatment of 10 μM heme or PPIX, but remained the same with 2.5 μM Hb. Taken together these results suggest an active role of the protoporphyrin ring in the heme-dependent control of intestinal non-heme iron absorption.

As the level of heme iron used above (10 μM) is similar to that obtained from a meal containing 100 g of cooked beef (Hazell *et al.* 1982), we are concerned whether smaller amounts of heme would be capable of enhancing intestinal non-heme iron absorption and if the effect is dose-dependent. We found heme increased non-heme ^{55}Fe uptake and transport in Caco-2 cells in a dose-dependent manner when the concentration of heme was between 1 and 25 μM (Figure 5-1E & F). Interestingly, even 1 μM of heme enhanced non-heme ^{55}Fe uptake and transport significantly by 18.3 ± 3.86% and 14.1 ± 8.33%, respectively, suggesting that a small amount of heme is able to enhance intestinal non-heme iron absorption. Heme at the concentration of 0.5 μM tended to enhance non-heme ^{55}Fe uptake and transport, although the effect was not significant.

5.2 Non-Heme Iron Markedly Induces Intestinal Heme Iron Absorption

To further explore how intestinal absorption of heme and non-heme iron affect each other and to exclude the possibility that dietary absorption of heme and non-heme iron compete with each other, we investigated whether non-heme iron interferes with intestinal heme iron absorption. Our data revealed that both transepithelial heme-derived ^{55}Fe transport across the Caco-2 cell monolayer and cellular heme-^{55}Fe accumulation at 7 h were increased by the treatment of non-heme iron (Figure 5-2). Specifically, although the apical heme-^{55}Fe uptake was only slightly increased by 12.2 ± 0.17% ($p < 0.05$), transepithelial heme-derived ^{55}Fe transport across the Caco-2 cell monolayer was drastically enhanced by 5.18 ± 0.25 fold with the treatment of 10 μM $Fe(NTA)_2$, suggesting that non-heme iron induces intestinal heme iron absorption mainly through increasing basolateral heme-derived iron release rather than elevating apical heme uptake. The overall data in Figure 5-1 & 2 suggest that the absorption of heme and non-heme iron boost each other in a synergistic way instead of competing.

5.3 Heme Redistributes DMT1 & FPN1 to Membrane Fractions of Caco-2 Cells

To investigate how heme regulates intestinal non-heme iron absorption, we first analyzed the total expression levels of those proteins involved in intestinal iron absorption and metabolism, including apical ferrireductase Dcytb, apical iron importer DMT1, basolateral iron exporter FPN1 and ferroxidase hephaestin (Heph). The total expression levels of none of these proteins, however, were altered in response to the treatment of 10 μM heme for up to 7h in Caco-2 cells (Figure 5-3A & B). This could be attributable to the fact that total protein expression and degradations are not affected much within such a brief period.

However, when it came to the fractionated crude membrane of the cells, our western blot data demonstrated that 10 μM heme enhanced DMT1 and FPN1 levels on crude membrane by 43.6 ± 12.7% and 65.5 ± 1.77%, respectively ($p < 0.05$) (Figure 5-3C & D), suggesting that heme induces translocation of DMT1 and FPN1 from cytosol to their respective apical and basolateral membrane fraction for functional purposes. The increase of DMT1 on crude membrane with the treatment of heme partially explained how heme enhanced apical non-heme ^{55}Fe uptake and transepithelial ^{55}Fe transport, while the increment of FPN1 on crude membrane with heme treatment justified the elevated basolateral non-heme ^{55}Fe release in Figure 5-1A. We further localized FPN1 protein by integrating fluorescence intensity of FPN1 staining in the basal-to-apical axis of Caco-2 cells. Our immunostaining data also supported that treatment of heme enhanced FPN1 expression on the basolateral membrane in Caco-2 cells (Figure 5-4).

Apart from these, although non-heme iron did not alter FPN1 expression levels on crude membrane, simultaneous treatments of same concentrations of heme and non-heme iron augmented FPN1 expression levels on crude membrane by 152.7 ± 18.1% compared to the control, which is much more than the increment of FPN1 expression by 65.5 ± 1.77% with heme treatment alone (Figure 5-3D). This suggests that not only heme alone enhances FPN1

translocation to crude membrane, but also heme and non-heme iron together synergistically boost FPN1 translocation to crude membrane further.

To explore the mechanism how non-heme iron drastically induces intestinal absorption of 1 μM heme ^{55}Fe, we first analyzed the total expression of HO-1, HO-2, FPN1 and Heph proteins, which play crucial roles in intestinal heme iron absorption. Like those proteins involved in non-heme iron absorption as discussed above, the total expression levels of these proteins remained unaltered in response to 10 μM $Fe(NTA)_2$ treatment for up to 7h in Caco-2 cells (Figure 5-3A & B). We then analyzed the expression of these proteins on crude membrane fraction of cells. Similar to the results in Figure 5-3D, our data revealed that although non-heme iron itself did not alter FPN1 expression levels on crude membrane, 1 μM heme together with 10 μM non-heme iron enhanced FPN1 levels on crude membrane by 27.6 ± 2.55% ($p < 0.05$) compared the group treated with 1 μM heme alone (Figure 5-3C & E), suggesting that heme and non-heme iron enhance FPN1 translocation to crude membrane in a synergistic way. This partially explains why heme iron absorption is induced by non-heme iron treatment in Figure 5-2.

5.4 Heme Increases Dcytb Ferrireductase Activity in Caco-2 Cells

As non-heme iron in foods primarily exists in its ferric form, which needs to be reduced to ferrous iron before it can be absorbed, the level of ferrireductase activity is important for intestinal non-heme iron absorption. To further investigate how heme enhances intestinal non-heme iron absorption, we examined whether heme affects the activity of Dcytb ferrireductase. We found that treatment of 10 μM heme for 7h in apical chamber of Caco-2 cells increased Dcytb ferrireductase activity in apical medium by 58.6 ± 0.79% compared to the control group ($p < 0.05$) (Figure 5-5), indicating that heme renders more non-heme Fe^{3+} being reduced to Fe^{2+},

which facilitates apical non-heme iron uptake. This also partially justify how heme enhances apical uptake and transepithelial transport of non-heme ^{55}Fe in Figure 5-1A.

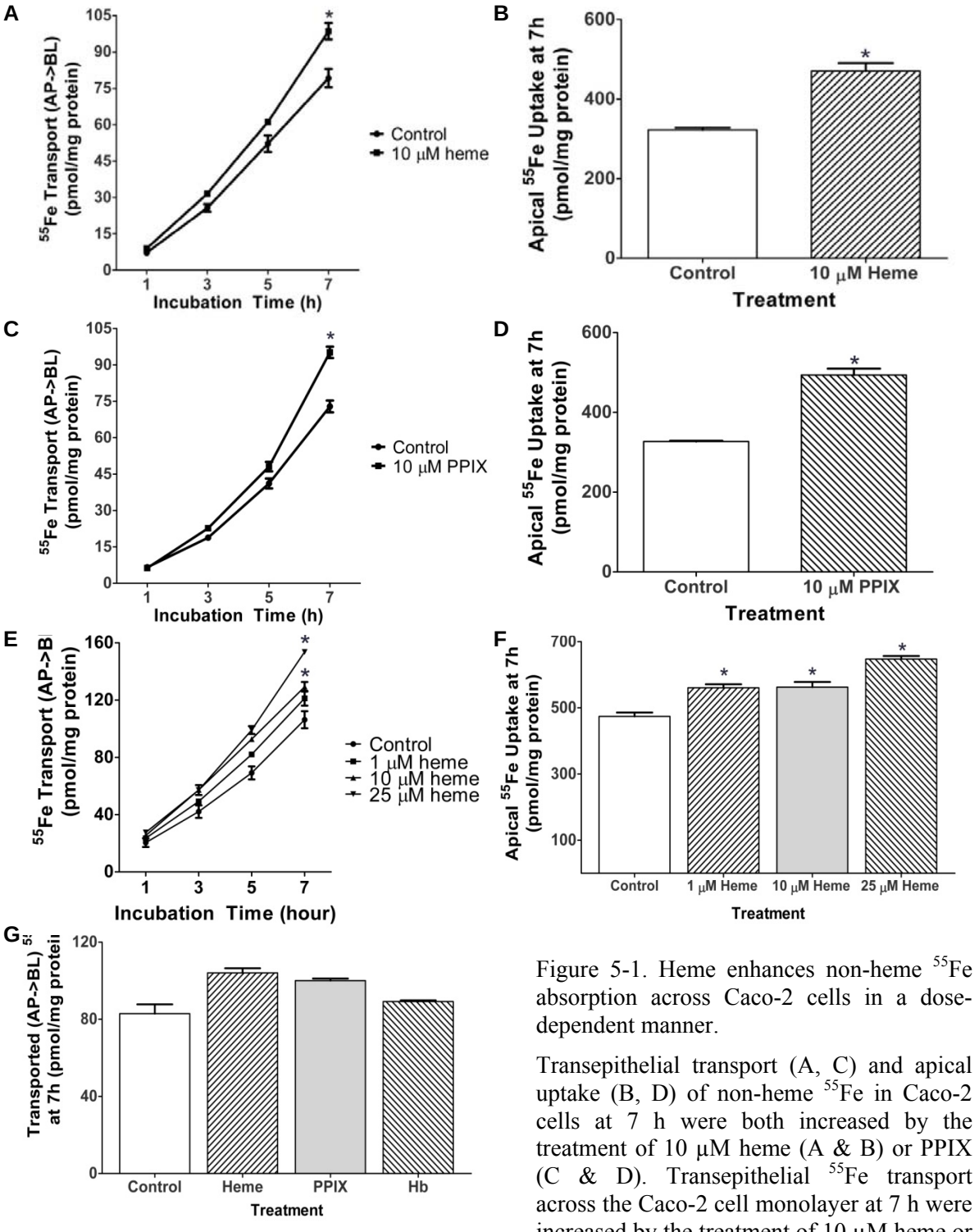


Figure 5-1. Heme enhances non-heme ^{55}Fe absorption across Caco-2 cells in a dose-dependent manner.

Transepithelial transport (A, C) and apical uptake (B, D) of non-heme ^{55}Fe in Caco-2 cells at 7 h were both increased by the treatment of 10 μM heme (A & B) or PPIX (C & D). Transepithelial ^{55}Fe transport across the Caco-2 cell monolayer at 7 h were increased by the treatment of 10 μM heme or PPIX, but remained unchanged with 2.5 μM Hb (G). Heme also increased non-heme ^{55}Fe transport and uptake in a dose-dependent manner (E & F). Values are means ± SEM, n = 3. Means with * indicate that the treatment has a significant effect, $p < 0.05$.

Table 5-1. Rate of ^{55}Fe Transfer across the Caco-2 Cell Monolayer[1]

Treatment	Rate of ^{55}Fe transfer (AP to BL chamber), pmol/(hr•well)
Control	11.31 ± 0.9361
Heme	14.07 ± 0.8386*
PPIX	14.77 ± 0.8199*

1 Data are means ± SEM, n = 3. Means with * differ, p < 0.05; the rate of transepithelial transport of non-heme ^{55}Fe across the differentiated Caco-2 cell monolayer was calculated during 7 h incubation by linear regression analysis (control, r = 0.999; EGCG, r = 0.943; GSE, r = 0.984).

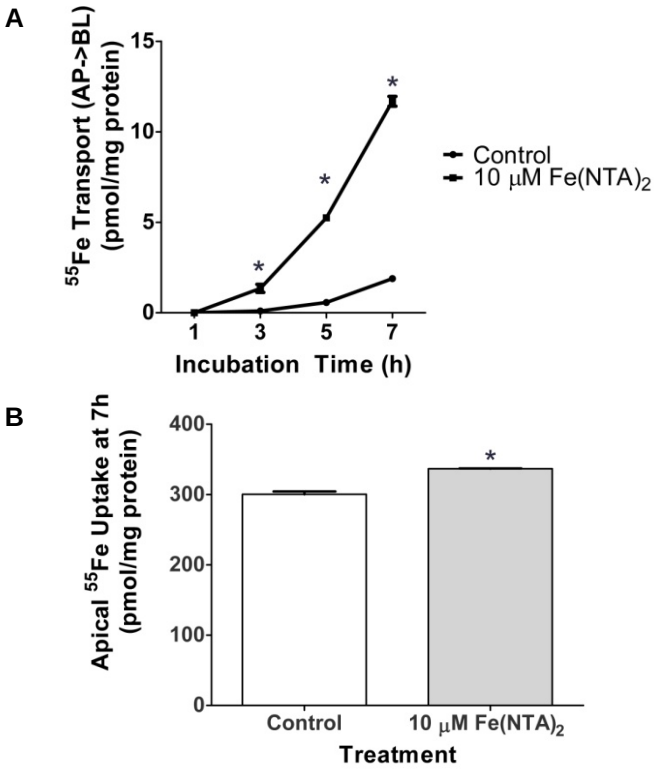
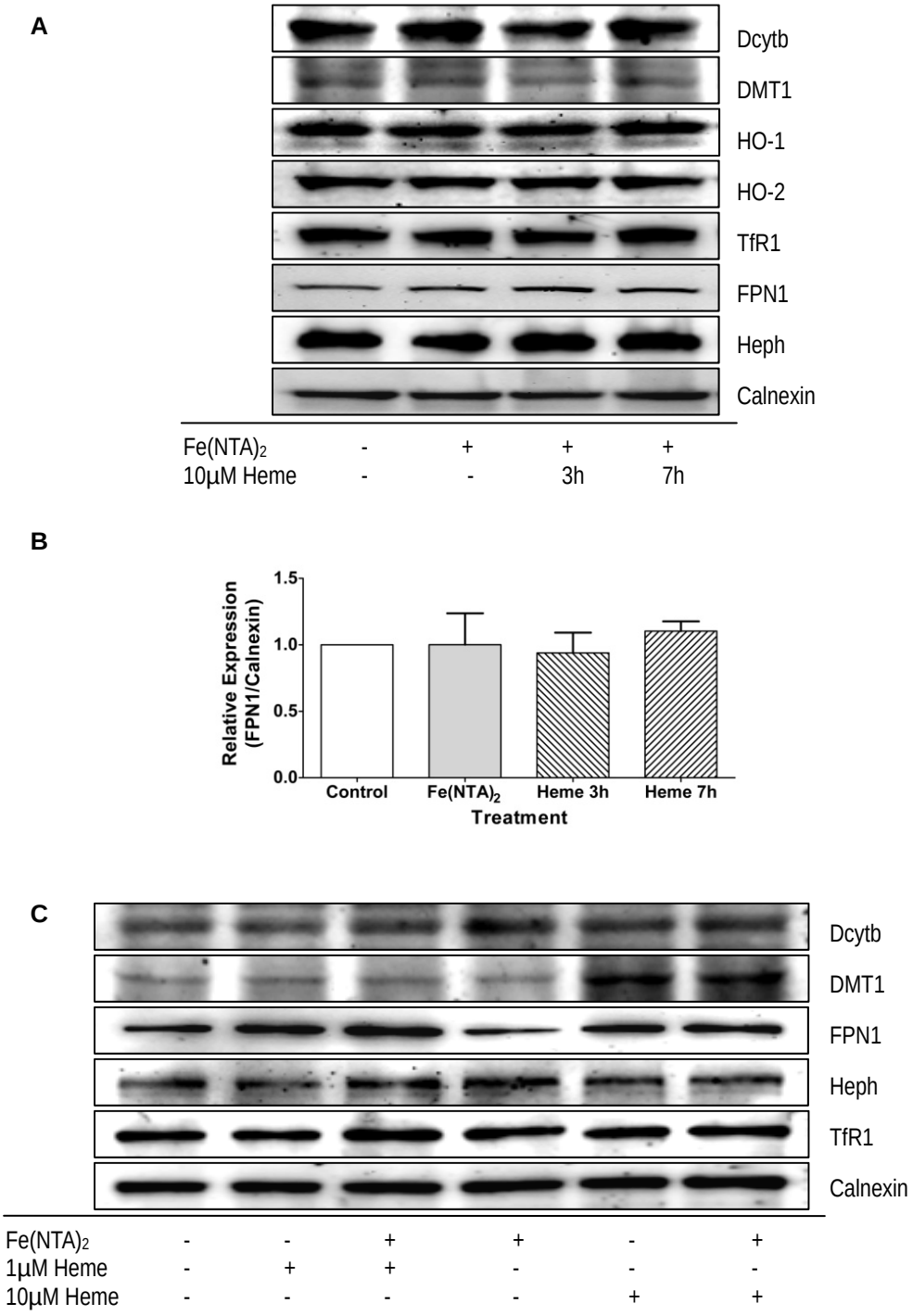


Figure 5-2. Non-heme iron enhances heme-^{55}Fe absorption in Caco-2 cells

Transepithelial heme-derived ^{55}Fe transport across the Caco-2 cell monolayer (A) was drastically increased with the treatment of 10 μM Fe(NTA)$_2$. Cellular ^{55}Fe accumulation at 7 h (B) was slightly increased by 10 μM Fe(NTA)$_2$. Values are means ± SEM, n = 6. Means with * indicate that the treatment has a significant effect, $p < 0.05$.


A
Dcytb
DMT1
HO-1
HO-2
TfR1
FPN1
Heph
Calnexin
Fe(NTA)2 - + + +
10μM Heme - - 3h 7h
B
Relative Expression (FPN1/Calnexin)
1.5
1.0
0.5
0.0
Control
Fe(NTA)2
Heme 3h
Heme 7h
Treatment
C
Dcytb
DMT1
FPN1
Heph
TfR1
Calnexin
Fe(NTA)2 - - + + - +
1μM Heme - + + - - -
10μM Heme - - - - + +

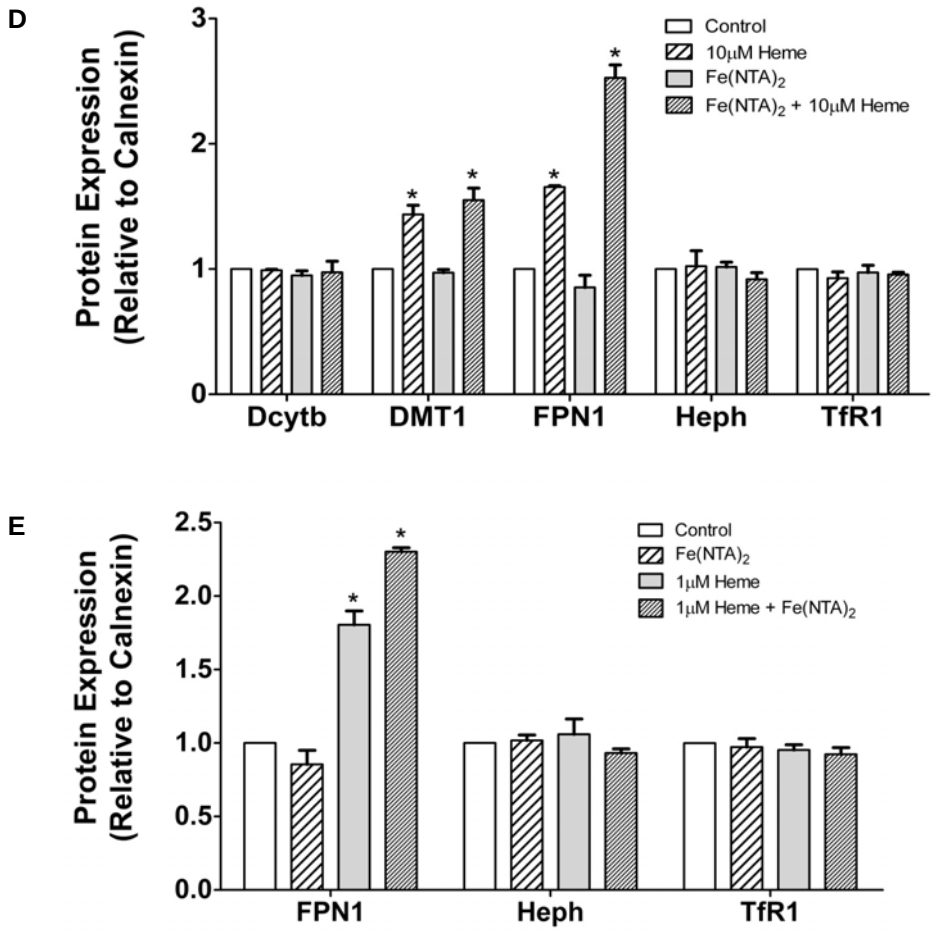


Figure 5-3. Heme redistributes DMT1 and FPN1 to crude membrane of Caco-2 cells

Representative western blots of Dcytb, DMT1, HO-1, HO-2, FPN1, Heph, TfR1 and calnexin from total cell lysates treated with 10 μM heme and/or 10 μM $Fe(NTA)_2$ for up to 7h (A). FPN1 protein levels from total cell lysates were quantified using Quantity One software and normalized by calnexin protein levels (B). Representative western blots of DMT1, FPN1, Heph, TfR1 and calnexin from crude membrane fraction of cells treated with 10 μM $Fe(NTA)_2$ ± 10 μM heme or 1 μM heme ± 10 μM $Fe(NTA)_2$ for 7h (C). Protein levels from crude membrane fraction of cells treated with 10 μM $Fe(NTA)_2$ ± 10 μM heme were quantified using Quantity One software and normalized to calnexin levels, $n = 3$, $p < 0.05$; TfR1 was used as a control (D). Protein levels from crude membrane fraction of cells treated with 1 μM heme ± 10 μM $Fe(NTA)_2$ were quantified using Quantity One software and normalized to calnexin levels, $n = 3$, $p < 0.05$ (E).

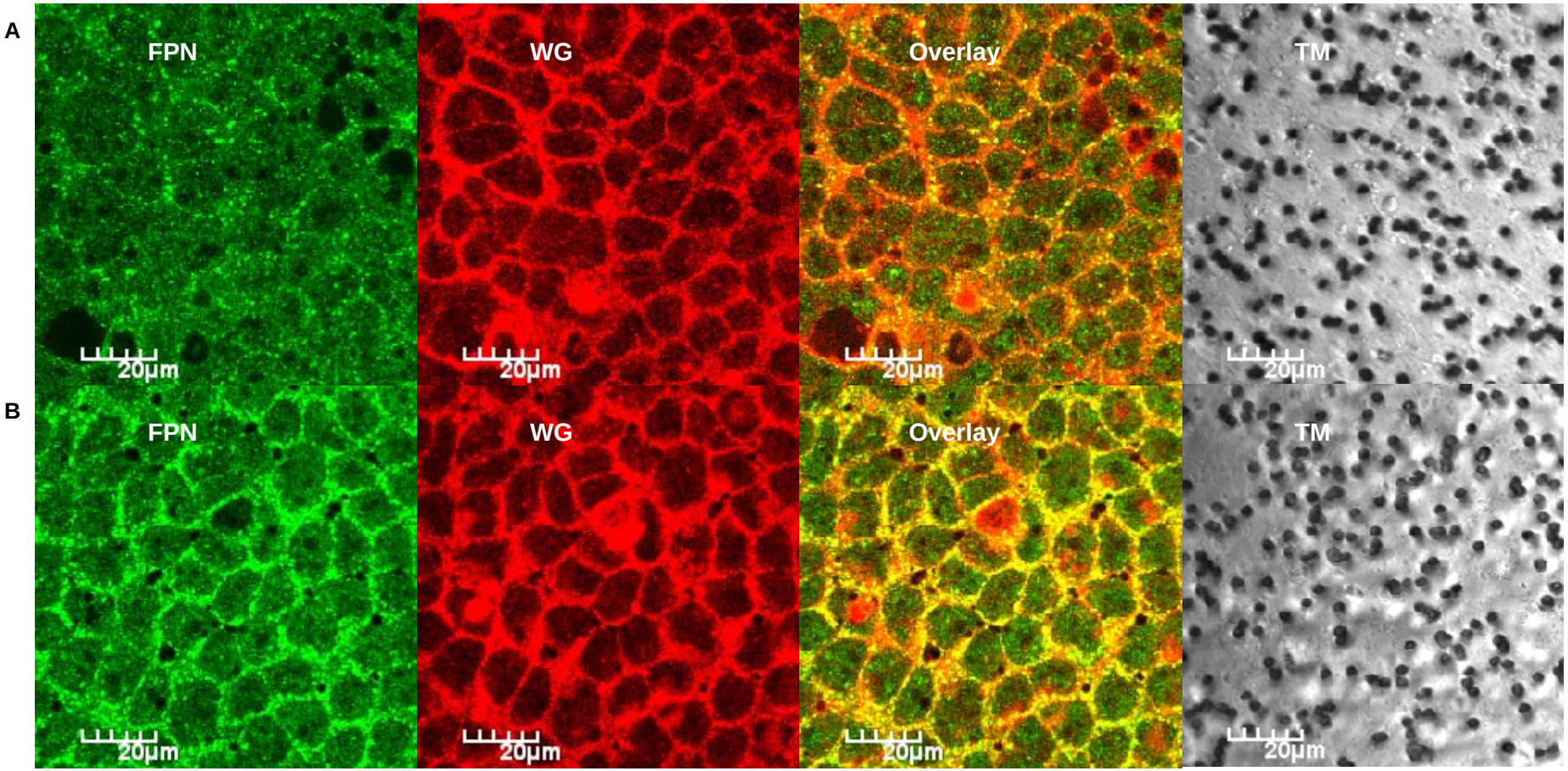


Figure 5-4. Heme increased expression of FPN1 on basolateral membrane of fully-differentiated Caco-2 cells

The fully-differentiated Caco-2 cells grown on microporous membrane inserts were treated without (A) or with heme (B) for 7h. After 7-h treatment, the Caco-2 cells were first fixed and incubated with Alexa 594-conjugated WGA in the bottom chamber to stain the basolateral membrane of cells. The cells were then permeabilized and incubated with FPN1 antibody. FPN1 staining is represented in green and WGA is shown as red. Yellow in the overlay image indicates localization of FPN1 on the basolateral membrane. TM: view of cells.

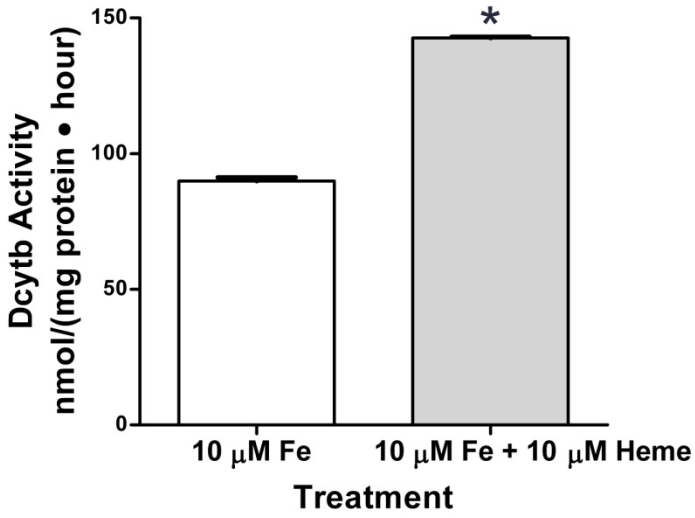


Figure 5-5. Heme increases Dcytb ferrireductase activity in Caco-2 cells

Dcytb ferrireductase activity was assessed by the production of a colored Fe^{2+}-Fz complex. The Dcytb activity was expressed as nmol of Fe^{2+}-Fz complex formed per mg protein per hour. Cells treated without and with 10 μM heme were incubated at 37°C with 1.5 ml of 100 μM Fz and 10 μM Fe^{3+} $(NTA)_2$ in iron uptake buffer added to the apical chamber. After 7-h incubation, the cell-conditioned medium was collected and centrifuged at 2000 rpm for 5 min at 4°C to remove debris. The concentration of Fe^{2+}-Fz complex was measured as described in Materials and Methods to determine the reduction of Fe^{3+} to Fe^{2+}. Values are means ± SEM, n = 3. Mean with * indicate that the treatment has a significant effect, $p < 0.05$.

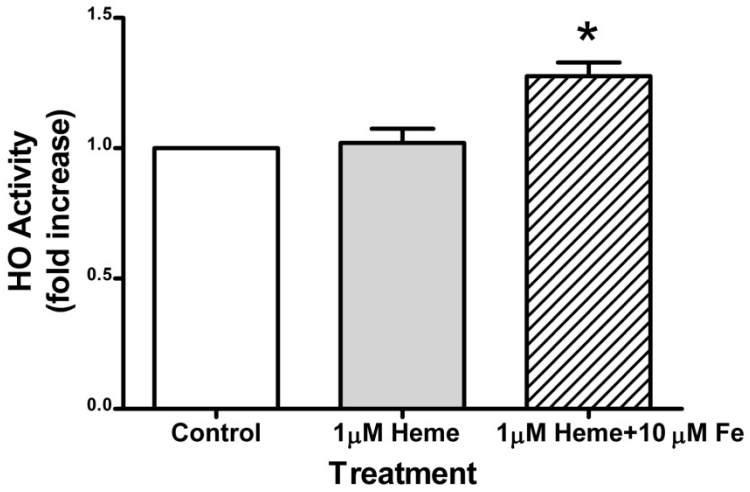


Figure 5-6. Non-heme iron increases HO activity in Caco-2 cells

Cells treated without and with 10 μM Fe^{3+} $(NTA)_2$ were incubated at 37°C in iron uptake buffer containing 1 μM heme added to the apical chamber. After 7-hour incubation, cells were collected for HO activity assay. The uptake buffer alone without any treatment was used as a negative control. Values are means ± SEM, n = 3. Mean with * indicate that the treatment has a significant effect, $p < 0.05$.

DISCUSSION

In this chapter, we analyzed the immediate interactions between intestinal absorption of heme and non-heme iron in human intestine-like Caco-2 cells. Previous studies on iron absorption only characterized absorption of heme and non-heme iron separately. However, we analyzed the absorption of heme and non-heme iron together. The most important finding of our study is that in contrary to the common assumption that heme and non-heme iron compete for absorption, they actually boost the absorption of each other, mainly by translocation of FPN1. This nominates heme as one of the long-sought meat factors of animal tissues that enhance intestinal non-heme iron absorption and explains how heme itself regulates intestinal iron absorption.

Iron homeostasis in the humans is regulated by absorption only because there is no physiologic mechanism for excretion. Intestinal non-heme iron absorption in humans is known to be promoted by animal tissues including pork, beef, poultry and fish (Cook *et al.* 1976). Single-meal radioisotope absorption studies consistently revealed that beef, fish, chicken and calf thymus all increased non-heme iron absorption 2 – 3-fold, while egg albumin and the water extracts of beef had no effect on iron absorption (Bjorn-Rasmussen *et al.* 1979). Animal studies also demonstrated the enhancing effect of meat on iron absorption (Gordon *et al.* 1989). Substituting washed beef for lactalbumin increased the changes in hemoglobin concentration of rats fed with bran-derived iron significantly to 4 folds. More recently, a randomized cross-over trial in 21 young women with low iron stores (ferritin < 30 μg/l) showed that the addition of fish to the bean meal significantly increased iron absorption (Navas-Carretero *et al.* 2008). A randomized cross-over study in 19 healthy female subjects showed that non-heme iron absorption in the group with the addition of pork meat was significantly higher compared to those with vegetarian diet (Bach Kristensen *et al.* 2005). The factors in animal products that promote iron absorption are coined as "meat factor", the nature of which remains unresolved. Although some evidence indicates that

some proteins or peptide in meat may fulfill this function, it is still possible that some non-protein substances are involved (Hurrell *et al.* 2006). It seems that some cysteine-containing peptides enhance iron absorption due to their reducing and solubilizing capability (Taylor *et al.* 1986). Studies in Caco-2 cells indicated that carbohydrate fractions from cooked fish, especially glycosaminoglycans, contribute to the enhancing effect of meat on iron uptake by the enterocyte (Huh *et al.* 2004). In addition, L-alpha-glycerophosphocholine might also play a role in the enhancement of non-heme iron absorption by meat (Armah *et al.* 2008). However, there are also human studies with controversial results showing that neither cysteine nor glycosaminoglycans increases iron absorption (Bjorn-Rasmussen *et al.* 1979; Storcksdieck genannt Bonsmann *et al.* 2007). We postulated that heme is a factor in meat that can enhances non-heme iron absorption. Although heme is a component in animal products, no studies had investigated into the effect of heme on intestinal non-heme iron absorption yet. Our result in Caco-2 cells showed that heme could be a non-protein substance in meat that enhances intestinal non-heme iron absorption (Figure 1). The fact that the same concentration of PPIX itself also increased non-heme iron absorption in Caco-2 cells excludes the possibility that the heme-dependent increase of non-heme iron absorption was due to the atomic iron released from heme. Although hemoglobin is a major heme-containing protein, it failed to elicit any positive influence on non-heme iron absorption in Caco-2 cells, suggesting that the response of enhanced non-heme iron absorption is specific to heme instead of the whole protein. This could be attributable to the requisite duration of proteolysis before heme became available. Remarkably, heme at a very low concentration of even 1 μM, equivalent to 10 g of cooked beef, significantly increased non-heme iron uptake and transport in Caco-2 cells, indicating that even a tiny bit of meat could enhance intestinal non-heme iron absorption. Heme at the concentrations between 1 and 25 μM increased non-heme iron absorption in Caco-2 cells in a dose-dependent manner, suggesting that non-heme iron absorption could be enhanced further by intake of more animal tissues.

Regarding the effect of dietary factors on intestinal heme iron absorption, it is only established that heme iron absorption is enhanced by the presence of animal tissues or soy proteins, while it is inhibited by calcium or polyphenols, although the underlying mechanisms have not been completely elucidated. Animal tissues promote not only non-heme iron absorption, but also intestinal heme iron absorption (Hallberg *et al.* 1979). Soy proteins reduced non-heme iron absorption significantly, although it improved heme iron absorption (Lynch *et al.* 1985). Phytate is a major inhibitory factors in soy-protein isolates, which decreases non-heme iron absorption but does not affect heme iron absorption (Hurrell *et al.* 1992). Polyphenols from green tea and grape seed extracts inhibit both non-heme iron absorption and heme iron absorption by blocking basolateral iron exit from enterocytes to blood circulation (Ma *et al.* 2010; Kim *et al.* 2011). No studies had previously analyzed the effect of non-heme iron on heme iron absorption. As heme and non-heme iron share the basolateral exporter FPN1, it was assumed that intestinal absorption of the two types of iron compete with each other at the basolateral level. However, no previous study had examined whether and how intestinal absorption of heme and non-heme iron affect each other. Interestingly, our results showed that heme ^{55}Fe absorption was dramatically enhanced by non-heme iron, whereas non-heme ^{55}Fe was also increased by heme iron (Figure 5-1 & 5-2). Importantly, non-heme iron induced intestinal heme ^{55}Fe absorption mainly through increasing basolateral heme-derived ^{55}Fe release rather than elevating apical heme uptake. These results, taken together, suggest that dietary absorption of heme and non-heme iron boost each other in a synergistic way instead of competing.

We found that intestinal absorption of heme and non-heme iron are synergistic of each other, in that heme enhanced absorption of non-heme iron across the Caco-2 cell monolayer and vice versa. Increased basolateral iron export correlated with increased level of functional FPN1 on basolateral membrane. The level of DMT1 on membrane fractions was also enhanced with the treatment of heme. Intestinal iron absorption is regulated by both pathophysiological factors

(body iron status, hypoxia, increased erythropoiesis needs, inflammation and infection) and dietary factors through modulating expression levels as well as cellular localizations of those proteins and activities of those enzymes that are relevant to intestinal iron absorption. For example, low iron status and hypoxia condition in mucosa stimulates intestinal iron absorption by inducing expression levels of Dcytb, DMT1 and FPN1 (Mastrogiannaki *et al.* 2009; Shah *et al.* 2009); and most recently, it is suggested that iron feeding regulates intestinal iron absorption by redistributing DMT1 and FPN1 proteins between membrane and intracellular compartments (Nunez *et al.* 2010). These three mechanisms, altered expression levels of relevant proteins, cellular localizations of iron transporters and changed enzymatic activity, were all explored in this study to elucidate the possible mechanism underlying the absorption of heme and non-heme iron.

While multiple pathways control apical iron uptake in small intestine, iron export appears to be the converging step that accurately reflects the amount of iron absorbed. As FPN1 is the only identified iron exporter in mammals, the regulation of FPN1 expression is of major importance for the control of iron absorption. At the posttranslational level, FPN1 expression is controlled by the small peptide hormone hepcidin that binds FPN1 to cause its internalization and degradation (Nemeth *et al.* 2004; De Domenico *et al.* 2009). For example, chronically elevated hepcidin level causes decreased intestinal iron absorption by eliminating functional FPN1 in small intestine. At the translational level, FPN1 is regulated via the iron regulatory proteins (IRP) and iron-responsive element (IRE) system (Galy *et al.* 2008). Binding of IRPs to the FPN1 5'-IRE blocks FPN1 translation under iron deprivation. In small intestine, there is an alternate splicing variant of FPN1 that lacks the 5'-IRE, which skips the regulation by IRP/IRE system (Zhang *et al.* 2009). At the transcriptional level, it has recently been found that FPN1 mRNA expression is enhanced in macrophages with heme treatment in a similar manner as HO-1 (Marro *et al.* 2010).

While the above control mechanisms of FPN1 expression are increasingly well understood, comparatively little is known about how FPN1 localization is controlled. Movement of FPN1 was demonstrated in the enterocytes in response to iron feeding. In the iron-starved rat enterocyte, FPN was seen in vesicles in the apical half of the cell primarily above the nucleus, and with iron feeding, FPN moved to the basolateral surface (Ma *et al.* 2006).

Translocation of transporters between the plasma membrane and intracellular domains is a crucial mechanism to regulate influx of ions and metabolites. The most paradigmatic example is the GLUT4 transporter. GLUT4 is predominantly positioned in the cytosol compartment in absence of insulin, whereas it is markedly exocytosed to plasma membrane upon insulin stimulation (Kanzaki *et al.* 2003). Another example of transporters regulated by this translocation mechanism is the transient receptor potential (TRP) family of ion channels (Bezzerides *et al.* 2004). Growth factor initiates the rapid translocation of the TRP ion channel, TRPC5, from vesicles held in reserve to just under the plasma membrane, resulting in tight spatial-temporal control of these Ca^{2+}-permeable nonselective channels (Bezzerides *et al.* 2004). Therefore, the sequestration of a transporter to the plasma membrane provides a fast and effective mechanism to regulate its function.

In regard to our experiments, increased levels of membrane-bound DMT1 and FPN1 coincided with increased non-heme iron absorption in Caco-2 cells with treatment of heme, while enhanced levels of membrane-associated FPN1 concurred with enhanced heme iron absorption with treatment of non-heme iron. In accordance with the above translocation mechanism, these data revealed that treatment of heme is a signal for translocation of both DMT1 and FPN1 designated to increase intestinal iron absorption, while heme and non-heme iron together are a synergistic signal for FPN1 translocation to enhance intestinal iron absorption.

Dcytb is the only iron-regulated ferrireductase expressed in duodenal enterocytes (McKie *et al.* 2001). Its ferrireductase activity has been shown to correlate positively with apical iron

uptake in Caco-2 cells (Nunez *et al.* 1994). Our showed that although Dcytb expression level was not altered during the transport study, heme treatment induced Dcytb activity, resulting in increased non-heme iron uptake and transport in turn by promoting the availability of ferrous iron for uptake by DMT1 into cells. As heme readily participates in antioxidant enzymes like peroxidase and iron released from heme is in the reduced form, it is possible that heme changes the redox environment that favor enhanced Dcytb activity (Battistuzzi *et al.* 2010).

Conclusions

In summary, our study revealed that intestinal absorption of heme and non-heme iron are synergistic of each other in Caco-2 cells mainly through the apical/basolateral membrane translocation of DMT1 and FPN, which could be a fast physiological mechanism to regulate intestinal iron absorption. Intestinal DMT1 and FPN1 are therefore major targets of therapies for patients with iron-related disorders. The fact that heme readily increases non-heme iron absorption may partially explain how animal tissues promote intestinal non-heme iron absorption. This provides a new perspective to characterize intestinal heme and non-heme iron absorption jointly and could serve a basis for supplementing heme and non-heme iron together.

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Chapter 6

HEME BOOSTS INTESTINAL NON-HEME IRON ABSORPTION ADAPTIVELY IN CACO-2 CELLS

ABSTRACT

The bioavailability of non-heme iron varies greatly depending on dietary composition. Our previous study revealed that the heme component of animal products immediately enhanced intestinal non-heme iron absorption in Caco-2 cells (as shown in Chapter 5). Most studies regarding intestinal non-heme iron absorption, however, only used single-meal radioisotope tests. It would therefore be timely to determine how non-heme iron absorption is affected adaptively. The objective of this study was to explore adaptive effect of heme consumption on intestinal non-heme iron absorption and metabolism using Caco-2 cell model and to investigate possible mechanisms behind this. Our results showed that pretreatment of heme for 2 to 3 days boosted non-heme ^{55}Fe absorption significantly by 28.97 ± 0.86% ($p < 0.05$) in Caco-2 cells without considerable alterations in apical uptake of ^{55}Fe, indicating that heme pretreatment enhanced non-heme iron absorption mainly by increasing basolateral ^{55}Fe release. This could be explained by our RT-PCR and western blot data, showing that heme pretreatment for 2 to 3 days increased expression of ferroportin-1 (FPN1). The enhancing effect of heme on intestinal non-heme iron absorption was dose-dependent, although heme at the concentration of ≤1 μM could not exert any significant effect, suggesting that consumption of large amounts of heme adaptively increased non-heme iron absorption. Overall, our data suggest that pretreatment of heme adaptively increased intestinal non-heme iron absorption in Caco-2 cells by inducing FPN1 expression.

Keywords

Heme, Non-heme iron, Intestinal iron absorption, FPN1, Adaptive effect

INTRODUCTION

Iron deficiency is the most common public health problem worldwide. The numbers are prodigious: 66-80% of the world's population (up to 4-5 billion people) may be iron deficient, while over 30% of the world's population (2 billion people) are anemic, mainly due to iron deficiency (World Health Organization 2007). It not only affects almost half the population of women of child-bearing age in developing countries, but also is the only nutrient deficiency which is also significantly prevalent in industrialized countries. Iron deficiency is often associated with diets of low iron bioavailability (Coad *et al.* 2011). Iron supplementation is the most effective way to treat iron deficiency anemia, but it is less effective in preventing iron deficiency due to problems with poor compliance, long-term acceptability, cost effectiveness and risks of iron overload. Preventing iron deficiency through diet is one of the main targets in WHO.

Dietary absorption of non-heme iron in humans is known to be enhanced by various animal tissues (Cook *et al.* 1976). Single-meal radioisotope absorption studies in both animal and human subjects consistently revealed that animal tissues including pork, beef, fish, chicken and calf thymus all increased non-heme iron absorption 2 – 3-fold (Bjorn-Rasmussen *et al.* 1979; Gordon *et al.* 1989; Navas-Carretero *et al.* 2008). The factors in animal tissues that promote intestinal iron absorption are collectively coined as "meat factor", although the precise identity and mechanism of meat factor are not fully understood. Our previous study revealed that the heme component of animal products readily enhanced intestinal non-heme iron absorption in Caco-2 cells. Several studies have demonstrated enhanced iron absorption by animal tissues using single-meal tests, but few have focused on the adaptive effect of animal tissues on intestinal iron absorption. To our knowledge, no study has assessed the adaptive influence of the heme component of animal tissues on intestinal non-heme iron absorption yet.

Intestinal non-heme iron absorption is accomplished by trans-membrane iron transporters. The iron importer divalent metal transporter-1 (DMT1) transfers ferrous iron from intestinal lumen following reduction of ferric iron by the membrane-bound ferroxidase duodenal cytochrome b (Dcytb) (Gunshin *et al.* 1997; McKie *et al.* 2001). While an undefined amount of iron entering the enterocyte is retained in the labile iron pool or temporarily stored in ferritin, iron is primarily subjected to basolateral export (Hentze *et al.* 2004). The only known iron exporter, ferroportin 1 (FPN1) transfers intracellular ferrous iron across the basolateral membrane, where iron is oxidized by ferroxidase hephaestin (Heph) before being incorporated into transferrin for transport in the circulation (Vulpe *et al.* 1999; Donovan *et al.* 2000). In contrast, intestinal heme iron absorption starts by being taken up as a whole metalloporphyrin. Within enterocyte, heme iron is supposed to be degraded to release free ferrous iron, which can then be exported into blood circulation via FPN1 in the same manner as non-heme iron does. The processes of intestinal heme and non-heme iron absorption converge at the point of iron export into blood and share the only iron exporter, FPN1. Although it was suggested that heme and non-heme iron may compete for basolateral export through FPN1, heme could possibly adaptively induce expression of FPN1 and subsequently increases absorption of non-heme iron.

The expression of iron transporters are balanced at three levels, post-translational, post-transcription and transcriptional. While key events of intestinal iron absorption are regulated post-translationally by hepcidin and transcriptionally by HIFs, the IRE/IRP system acts at the post-transcriptional level to maintain proper intestinal iron absorption (Nemeth *et al.* 2004; Muckenthaler *et al.* 2008; Shah *et al.* 2009). Much effort has been given to elucidate the mechanism of the action of meat factor on iron bioavailability but no pathway has been verified. There is emerging evidence suggesting that some nutrients directly modulate the expression of enterocyte iron transporters. Recent studies using murine macrophages have revealed that the

expression of iron exporter FPN1 is enhanced at the transcriptional level (Marro *et al.* 2010). It is possible that FPN1 in small intestine is regulated by heme in the same way as in macrophages.

The objective of this study was to explore the adaptive effect of heme consumption on intestinal non-heme iron absorption and metabolism using Caco-2 cell model and to investigate possible mechanisms behind this. We examined how pretreatment of heme for 2 – 3 days affect succeeding non-heme iron absorption and FPN1 expression.

MATERIALS AND METHODS

Reagents

The ^{55}Fe (in the form of $^{55}FeCl_3$) was obtained from Perkin–Elmer Life Sciences (Boston, MA). Hanks' balanced salts solution (HBSS), glutamine, nonessential amino acids and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Unless otherwise noted, all other reagents were obtained from Sigma Chemical (St. Louis, MO), VWR (West Chester, PA), or Fisher Scientific (Springfield, NJ).

Caco-2 Cell Culture

The human Caco-2 cell line HTB37 was purchased from American Type Culture Collection (Rockville, MD). Stock cultures were maintained at 37°C in complete medium in a humidified atmosphere of 95% air and 5% CO_2 and used for experiments within 20 serial passages. The complete culture medium contained Dulbecco's Modified Essential Medium (DMEM) supplemented with 25 mM glucose, 2 mM glutamine, 100 μM non-essential amino acids, 100 U/l penicillin G, 100 mg/l streptomycin and 10% FBS. For experiments, 5.0×10^4 cells/cm^2 in 1.5 ml complete DMEM were seeded on 3 μm microporous membrane inserts (4.9 cm^2, BD Biosciences, Bedford, MA) coated with collagen type I (5 μg/cm^2) on 6-well plates. The basolateral chamber was filled with 2.5 ml complete DMEM. The culture medium was changed every 2 days, and cells were used after 17-day post-confluence for experiments. The Caco-2 cell monolayer was routinely checked for tight junctions at 17-d postconfluence with transepithelial electrical resistance (TEER) >250 Ω/cm^2, which indicates full differentiation under normal cell culture conditions.

^{55}Fe Transport Study

Transepithelial heme-derived ^{55}Fe transfer from apical to basolateral compartment were determined by scintillation (Han *et al.* 1994). After washing the cell monolayer three times with Ca^{2+}- and Mg^{2+}-free Hank's balanced saline solution (HBSS) containing 137 mM NaCl, 5.36 mM KCl, 1.3 mM $CaCl_2$, 410 μM $MgSO_4$, 490 μM $MgCl_2$, 337 μM Na_2HPO_4, 440 μM KH_2PO_4, 4.17 μM $NaHCO_3$ and 5.55 mM dextrose at 37°C, cells were incubated at 37°C with 1.5 ml of ^{55}Fe/Fe(NTA)$_2$ or ^{55}Fe/Fe-heme in iron-uptake buffer in the apical compartment and 2.5 ml DMEM in the basolateral compartment. The uptake buffer contained 130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES, pH 7.0. Transepithelial iron transfer from apical to basolateral chamber and cellular iron accumulation were determined. An aliquot of 200 μL was removed from the basolateral chamber at the indicated time points and replaced with an equivalent volume of pre-warmed DMEM; time course data were corrected to account for this sample replacement. To measure the cellular levels of ^{55}Fe, cell monolayers were washed three times with ice-cold wash buffer containing 150 mM NaCl, 10 mM HEPES, pH 7.0, and 1 mM EDTA to remove any nonspecifically bound radioisotope. Cells were homogenized in PBS containing 0.3% Triton X-100, and ^{55}Fe was quantified by liquid scintillation counting in glass vials. Protein levels were assessed using Bio-Rad protein assay kit (BioRad Laboratory Inc., Hercules, CA). All ^{55}Fe transport studies were repeated for at least 3 times.

Western Blot

Western blot was performed to determine protein levels of FPN1, TfR1, Ft, HO-1 and calnexin in Caco-2 cells. Protein samples extracted from the same cells were used for all Western blot analyses. Cell lyses (20 μg or 40 μg) were solubilized in Laemmli buffer, boiled for 10 min, and separated by a 7.5% SDS–PAGE. Proteins were transferred by electroblotting to nitrocellulose membranes. The membranes were first blocked by 5% non-fat dry milk in 10 mM

Tris-HCl, pH 7.4, 150 mM NaCl (TBS) at room temperature for 1 h, and then incubated for 2 h at room temperature with the primary antibodies (1:3,000) in TBS containing 0.05% Tween 20 (TBST). The membranes were washed for several times with TBST and then incubated for 1 h at room temperature with peroxidase-linked goat anti-rabbit IgG (1:3,000). The visualized antigens by ECL were detected ChemiDoc XRS system (Bio-Rad, Hercules, CA).

RNA Isolation

The Caco-2 cells from each insert were collected in 1 mL TRIzol (Invitrogen, CA). Total RNA was isolated by phenol-chloroform extraction. Concentration and purity of the RNA was determined by OD260/280 reading. Quality of the RNA was assessed by gel electrophoresis and ethidium bromide staining.

Reverse transcription and real-time PCR analysis

Two micrograms of total RNA were reverse transcribed using 10 μM dNTP, 10 nM oligo dT, 0.1 mg/ml BSA, 16 units of RNase inhibitor, 5x buffer and 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, WI) in an 80 μL reaction for 10 min at 37°C. Real-time polymerase chain reaction (PCR) was performed using the MJ Research® Opticon 2 system (Bio-Rad, CA). Amplification reactions were carried out in a final volume of 20 μL using SYBR Green supermix (Bio-rad). PCR conditions were optimized for denaturing at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min for 35 cycles. For regular PCR, the products were analyzed by electrophoresis via 1% agarose gel containing ethidium bromide. The DMT1, Dcytb, FPN1, HO-1, HO-2, 18S and β-actin primers used for PCR are listed in Table 6-1. The mRNA/cDNA abundance of each gene was calculated relative to the expression of the housekeeping 18s ribosome RNA.

Statistical Analysis

Values were expressed as non-transformed means ± SEM (n = 3 – 6). The experiments were repeated at least three times for each treatment. Data were analyzed by 1-way ANOVA with the following post-hoc tests for multiple comparisons using Prism 5.0 software (GraphPad). Differences were considered significant when p-value is < 0.05.

Table 6-1. Primer Sequences Used for PCR

Gene	Forward Sequence	Reverse Sequence
dmt1	GTTTGTCATGGAGGGATTCCT	CATTCATCCCTGTTAGATGCT
fpn1	CCACAATACGAAGGATTGACC	GACGTACTCCACGCACATG
cytb1	GTCACCGGCTTCGTCTTCA	TACAGACTGCCGTGGACCTG
hmox1	TGTGGCAGCTGTCTCAAACCTCCA	TTGAGGCTGAGCCAGGAACAGAGT
hmox2	GCTGACCAAGGACATGGAGTA	GGTAGAGCTGCTTGAACTGCT
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
actb	ACCGAGCGCGGCTACAG	CTTAATGTCACGCACGATTTCC

RESULTS

6.1 Heme Pretreatment Adaptively Enhances Non-Heme Iron Absorption in Caco-2 Cells

Using human intestine-like Caco-2 cells, we have previously demonstrated that heme immediately enhances non-heme iron absorption (Chapter 5). Here, we performed similar experiments using Caco-2 cells pretreated with heme in order to investigate the adaptive effect of heme on non-heme iron absorption. Our results showed that pretreatment of 10 μM of heme for 72 h significantly enhanced succeeding non-heme ^{55}Fe transport by 28.97 ± 0.86% at 7 h ($p < 0.05$), suggesting that heme not only readily boosts non-heme iron absorption immediately, but also adaptively increases non-heme iron absorption (Figure 6-1). In addition, pretreatment of 10 μM of the heme precursor PPIX for 72 h in substitution for heme even increased subsequent non-heme ^{55}Fe transport further by 31.84 ± 0.67% at 7 h-point of transport ($p < 0.05$) (Figure 6-1A). Given that heme is comprised of protoporphyrin ring and the atomic ferrous iron, it is possible that for heme pretreatment, the non-heme ferrous iron released from heme alleviates the enhancing effect of heme/PPIX on non-heme iron absorption. Pretreatment of 10 μM of heme for 48 h also enhanced non-heme ^{55}Fe transport significantly by 16.12 ± 3.02% ($p < 0.05$), although pretreatment of 10 μM of heme for 24 h did not affect non-heme ^{55}Fe transport across Caco-2 cell monolayer (Figure 6-1B). Apart from these, apical uptakes of non-heme ^{55}Fe into Caco-2 cells were not significantly altered for any of these pretreatment, indicating that pretreatment of heme/PPIX for 2 – 3 days increased non-heme ^{55}Fe absorption mainly by enhancing basolateral ^{55}Fe export rather than affecting its apical uptake. Take together these results imply that heme/PPIX could adaptively boost non-heme iron absorption by increasing their basolateral release.

6.2 Pretreatment of Heme Induces Non-heme Iron Absorption Dose-dependently

Because 10 μM of heme that we used is similar to that obtained from a meal containing 100 g of cooked beef (Hazell *et al.* 1982), it is interesting to test whether the adaptive effect of heme on non-heme iron absorption is dose-dependent. Our result showed that pretreatment of heme for 72 h increased non-heme ^{55}Fe transport in Caco-2 cells in a dose-dependent manner when the concentration of heme was between 10 and 25 μM despite of unaltered apical ^{55}Fe uptake (Figure 6-2). However, pretreatment of 1 μM heme for 72 h did not change non-heme ^{55}Fe transport, suggesting that a small amount of heme consumption would not affect succeeding non-heme iron absorption. The overall data in Figure 6-1 & 2 suggest that pretreatment of a relatively large amount of heme for 2 – 3 days would enhance subsequent non-heme iron absorption by increasing its basolateral exit.

6.3 Heme Induces Expression of FPN1 in Caco-2 Cells

Heme pretreatment increases non-heme iron absorption by enhancing basolateral iron export. We therefore wondered whether heme itself could be directly involved in regulating the expression of the only iron exporter, FPN1. To test this hypothesis, we first evaluated the expression levels of FPN1 in Caco-2 cells pretreated with heme. For this purpose, western blots were performed on Caco-2 cells pretreated with 10 μM of heme for up to 72 h. Enhanced expression of FPN1 was detected for cells pretreated with heme for 48 and 72 h, compared with control (Figure 6-3).

Recent studies have reported that heme activates FPN1 transcription in macrophages (Marro *et al.* 2010; Harada *et al.* 2011). However, because duodenum and macrophages utilize an alternative upstream promoter to express FPN1 transcript (Zhang *et al.* 2009), it is prudent to

understand how heme affects FPN1 expression in duodenum. We then tested whether FPN1 mRNA is induced by heme in human intestine-like Caco-2 cells. Caco-2 cells were pretreated with 10 μM of heme for 12, 18, 24, 36, 42, 48, 60, 66 and 72 h and then processed for RT-PCR (Figure 6-4). As a positive control, we also examined HO-1 gene transcription, which is known to be induced by heme in various cell types, including duodenal enterocytes. We observed that heme pretreatment for 42, 48, 60 and 66 h significantly enhanced FPN1 gene expression. The enhancing effect of heme on FPN1 and HO-1 mRNA levels could reflect either induction of transcription of these genes or stabilization of preexisting mRNAs. To clarify this point, Caco-2 cells pretreated with heme were incubated together with the transcriptional inhibitor actinomycin D (actD) for the same period of time (Figure 6-4B). Interestingly, the induction of FPN1 and HO-1 mRNAs by heme was inhibited in the presence of 1 μg/ml actD, supporting the hypothesis that the effect of heme on these two genes in Caco-2 cells is attributable to transcriptional activation.

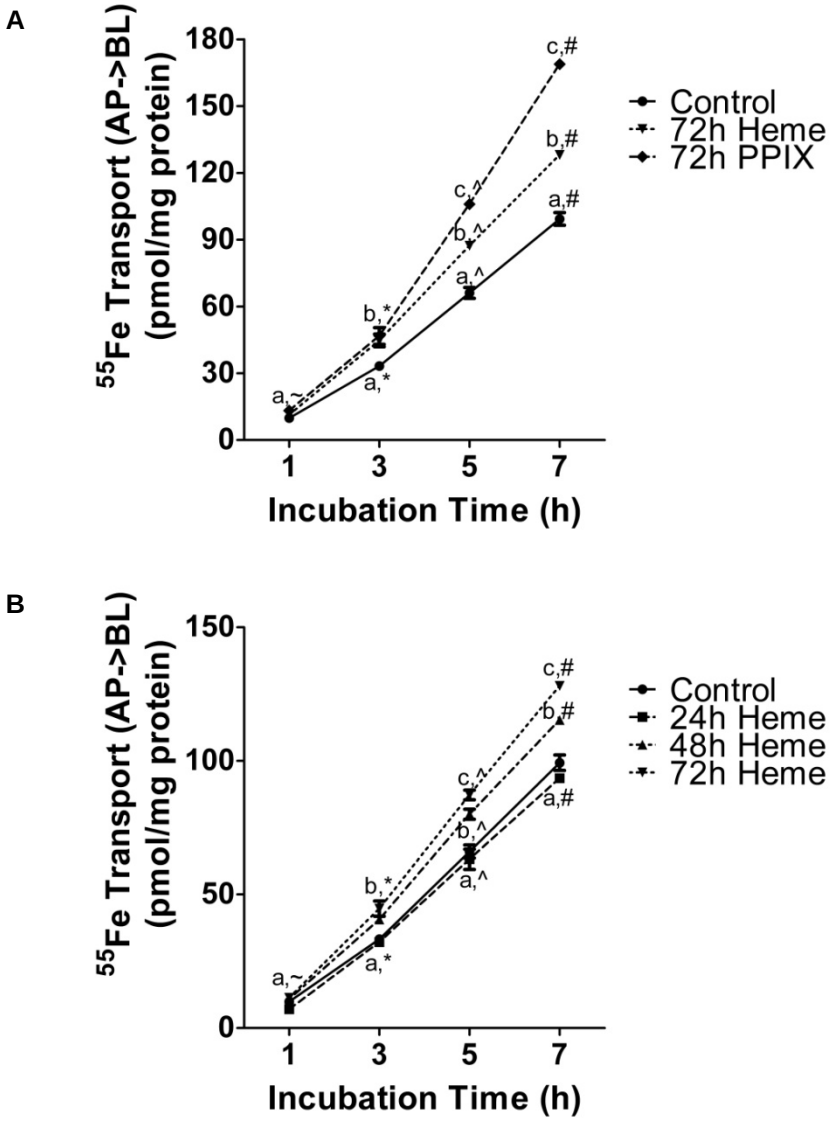


Figure 6-1. Pretreatment of heme adaptively enhances non-heme ^{55}Fe absorption across Caco-2 cells.

Transepithelial transport of non-heme ^{55}Fe across Caco-2 cell monolayer at 7 h was increased adaptively by pretreatment of both 10 μM heme and PPIX for 72h (A). Transepithelial non-heme ^{55}Fe transport at 7 h was enhanced adaptively by pretreatment of 10 μM heme for 48 and 72h, but remained unchanged with heme pretreatment for 24h (B). Values are means ± SEM, n = 3. Means with different letters indicate that the treatment has a significant effect, $p < 0.05$; Means with different symbols indicate that time has a significant effect, $p < 0.05$.

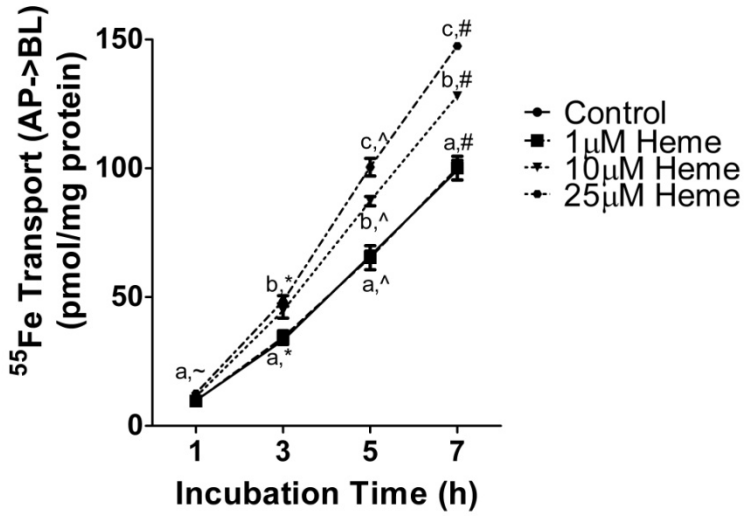


Figure 6-2. The adaptive enhancing effect of heme pretreatment on non-heme ^{55}Fe absorption is dose-dependent.

Transepithelial non-heme ^{55}Fe transport across the Caco-2 cell monolayer at 7 h was adaptively enhanced by heme pretreatment for 72h in a dose-dependent manner, although pretreatment of heme at the concentration of 1 μM didn't enhance non-heme ^{55}Fe absorption. Values are means ± SEM, n = 3. Means with different letters indicate that the treatment has a significant effect, $p < 0.05$; Means with different symbols indicate that time has a significant effect, $p < 0.05$.

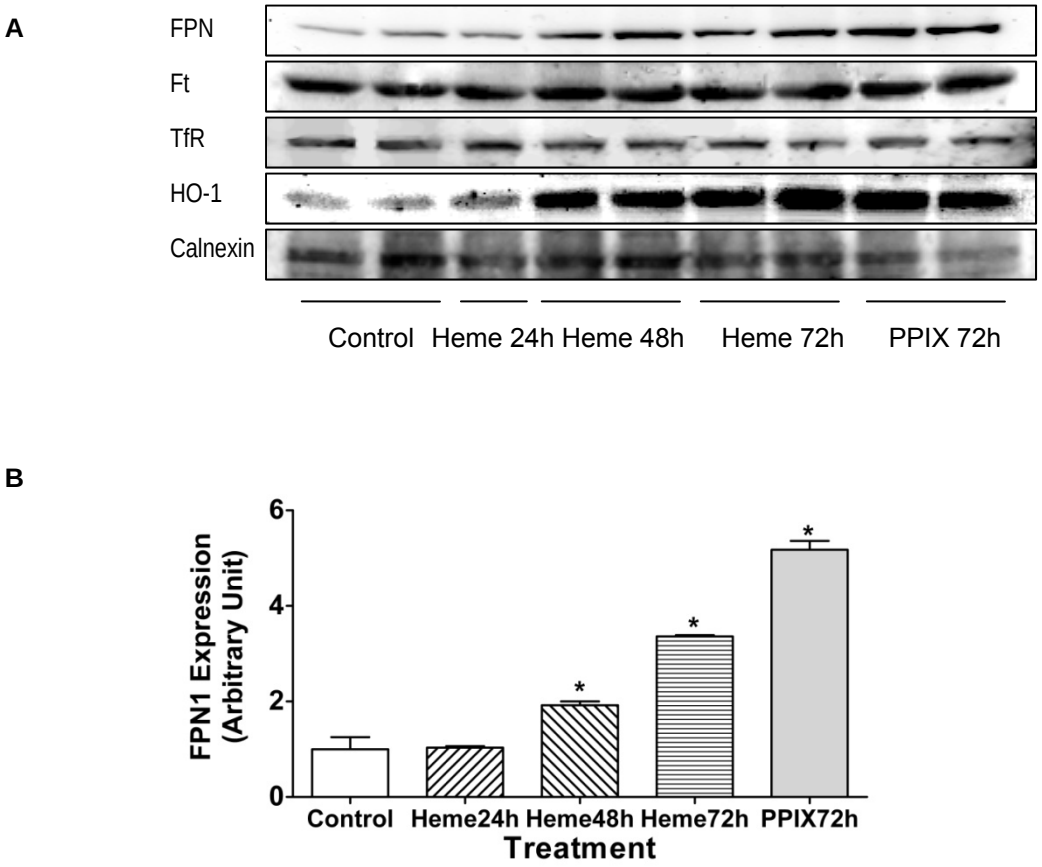
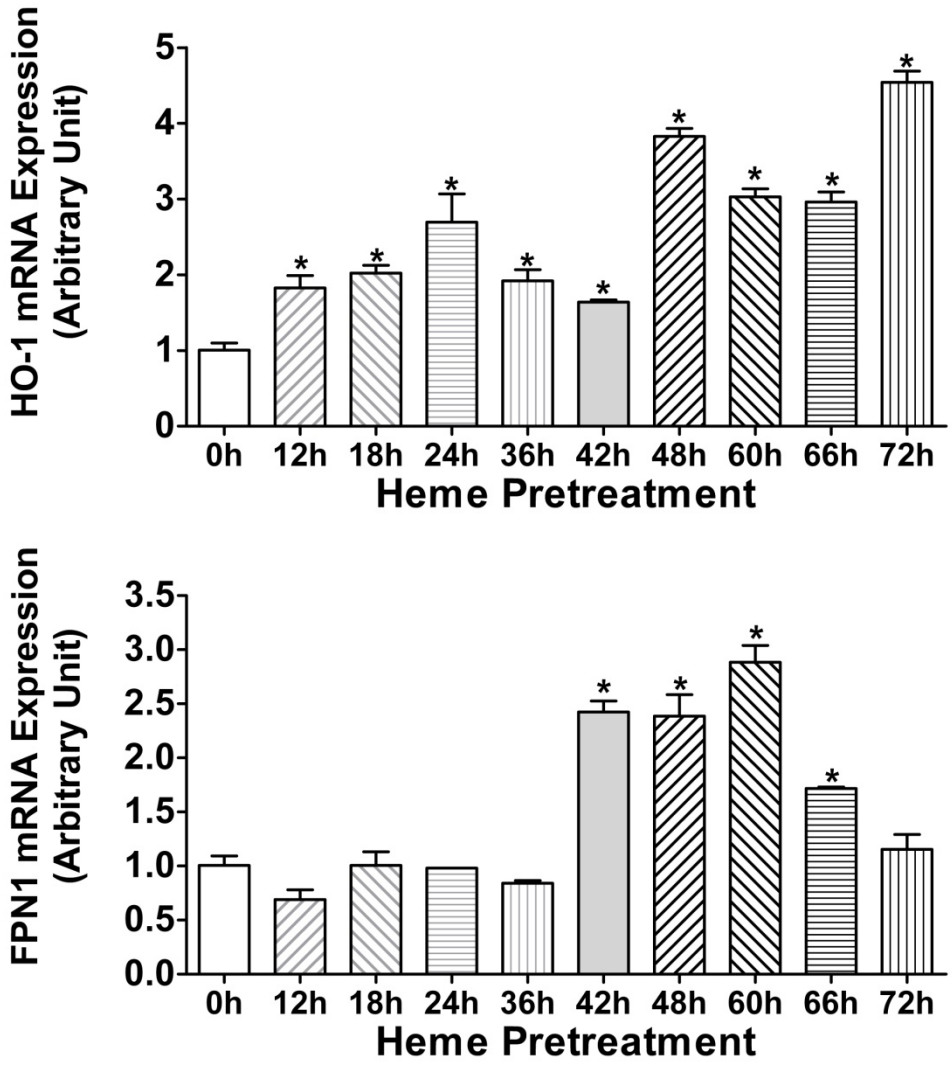


Figure 6-3. Pretreatment of heme adaptively enhances FPN1 protein levels in Caco-2 cells.

Representative western blots of FPN1, Ft, TfR1, HO-1 and calnexin from total cell lysates treated with 10 μM heme or 10 μM PPIX for up to 72 h (A). FPN1 protein levels from total cell lysates were quantified using Quantity One software and normalized by calnexin protein levels. Values are means ± SEM, n = 3. *, $p < 0.05$ (B).

A


HO-1 mRNA Expression
(Arbitrary Unit)
0
1
2
3
4
5
0h
12h
18h
24h
36h
42h
48h
60h
66h
72h
Heme Pretreatment
FPN1 mRNA Expression
(Arbitrary Unit)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0h
12h
18h
24h
36h
42h
48h
60h
66h
72h
Heme Pretreatment

B

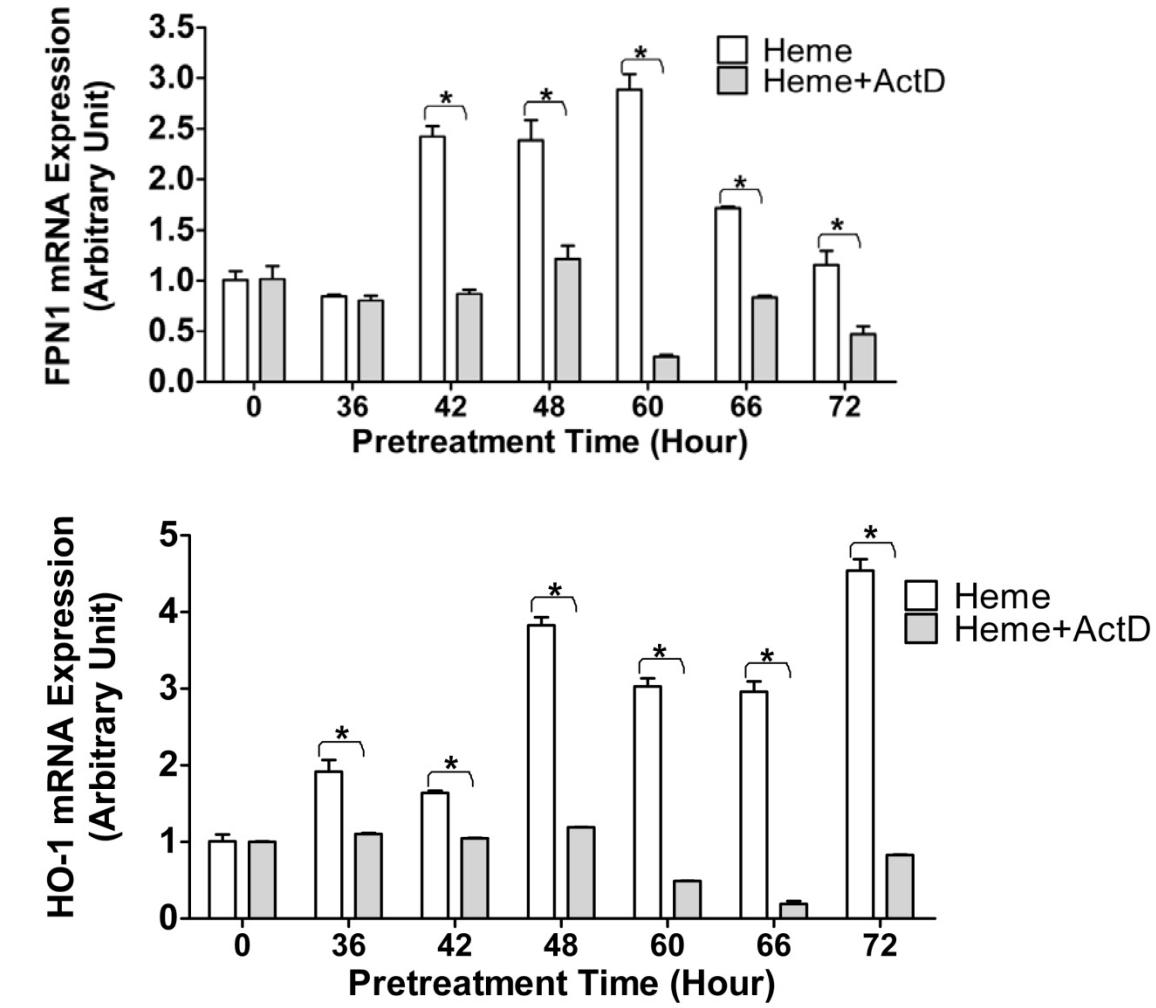


Figure 6-4. Pretreatment of heme adaptively induces FPN1 mRNA expression in Caco-2 cells.

RT-PCR for FPN1 and HO-1 with mRNA isolated from Caco-2 cells treat with 10 μM heme (A) or 10 μM heme ± 1 μg/ml actD (B) for up to 72h. 18s was used as the control. Values are means ± SEM, n = 3. *, $p < 0.05$.

DISCUSSION

In this chapter, we analyzed the adaptive effect of heme on intestinal non-heme iron absorption and its possible mechanism. The major finding is that pretreatment of heme for 2 – 3 days adaptively increased succeeding non-heme iron absorption in Caco-2 cells via stimulating transcription of FPN. This is the first report to show how heme regulates intestinal iron absorption adaptively.

Intestinal absorption of non-heme iron in humans is known to be promoted by various animal products (Cook *et al.* 1976). Single-meal radioisotope absorption studies in both animal and human subjects consistently revealed that animal tissues increased non-heme iron absorption 2 – 3-fold (Bjorn-Rasmussen *et al.* 1979; Gordon *et al.* 1989; Navas-Carretero *et al.* 2008). Since the bioavailability of non-heme iron varies greatly depending on dietary composition, it would be important to determine not only how non-heme iron absorption is influenced immediately by consumption together, but also how it is affected adaptively by pretreatment of specific dietary factor. Most studies focusing on the effect of animal products on non-heme iron absorption only used simultaneous consumption of heme and non-heme iron by a single-meal radioisotope study to conclude that non-heme iron absorption is enhanced by animal tissues immediately. Our previous data also demonstrated that heme as a meat factor of animal tissues readily increases non-heme iron absorption. We demonstrated here that pretreatment of heme for 2 – 3 days significantly enhances non-heme iron absorption in an adaptive way in human intestine-like Caco-2 cells, suggesting that the enhancing effect of animal products on non-heme iron absorption could be adaptive.

There are several findings that demonstrate the transcriptional activation of the FPN1 gene by heme pretreatment in Caco-2 cells. Pretreatment of heme or PPIX induced FPN1 mRNA and protein expression. The transcription of FPN1 was activated by either heme or the

protoporphyrin ring alone, suggesting that the protoporphyrin itself is capable of stimulating FPN1 mRNAs expression and that iron released from heme by HO-1 activity is unlikely to be involved in this process. Consistently, previous studies revealed that high iron level down-regulates FPN1 transcription in small intestine (McKie *et al.* 2000; Zhang *et al.* 2009). Our study also demonstrated that pretreatment of Caco-2 cells with an identical amount of iron did not activate FPN1 transcription. The deviation may be explained by the point that much higher concentrations of iron were applied in the previous studies. In addition, pretreatment to the cells with actinomycin D (an inhibitor of RNA polymerase II) blocked the heme-mediated induction of FPN1 and HO-1 mRNAs, confirming that heme regulates FPN1 expression at the transcriptional level.

Heme plays vital roles in mammals not only as the prosthetic group of various hemoproteins including hemoglobin, myoglobin and cytochromes, but also as a sensor molecule regulating the expression of several genes (Furuyama *et al.* 2007). For example, in erythrocytes, heme deficiency activates heme-regulated translational inhibitor (HRI), thereby limiting protein synthesis and especially the production of globin chains (Ranu *et al.* 1976). Importantly, heme determines its own fate by self-regulating its synthesis and breakdown. Accumulation of intracellular uncommitted heme reduces heme synthesis by repressing the mRNA stability and mitochondrial translocation of 5-aminolevulinate synthase 1, the first and rate-limiting enzyme of the heme biosynthetic pathway (Hamilton *et al.* 1991; Lathrop *et al.* 1993). Heme also stimulates its catabolism by inducing expression of HO-1 (Sun *et al.* 2002). Under heme-rich condition, heme binds to cysteine-proline motifs of the transcriptional repressor Bach1 and replaces it with the transcription activator Nrf2 on the antioxidant response element of HO-1 gene, thereby activating its transcription. Heme is a potent transcriptional activator of HO-1 gene in various organism, including macrophages and small intestine (Shibahara *et al.* 1979). It has recently been found in macrophages that FPN1 gene is induced by heme at the transcriptional level in a similar

mechanism as HO-1 (Marro *et al.* 2010). It is tempting to speculate that similar regulation could occur in small intestine. Even though FPN1 is regulated differently in macrophages and small intestine due to the alternate splicing, it is still possible that FPN1 is directly regulated by heme at the transcriptional level in small intestine. Our data showed that the pattern of induction of FPN-1 gene is similar as that of HO-1 in Caco-2 cells with heme pretreatment.

FPN1 is the only iron exporter in small intestine identified to date (McKie *et al.* 2000). Thus regulation of FPN1 expression in small intestine is of major importance for the control of iron efflux into the body. Several studies documented changes in mRNA level and transcription rate of FPN1 under conditions of both iron deficiency and iron overload. For example, there was a substantial increase in duodenal FPN1 mRNA expression when mice were fed an iron-deficient diet and a significant decrease in duodenal FPN1 mRNA expression when mice were fed an iron-supplemented diet (Dupic *et al.* 2002). Consistently, FPN1 transcription was increased in desferrioxamine-treated Caco-2 cells, while iron treatment decreased FPN1 expression (Zoller *et al.* 2002). While translational and posttranslational control mechanisms of FPN1 are relatively well understood, comparatively little is known about how FPN1 expression is controlled at the transcriptional level in small intestine.

In regard to our experiment, the transcriptional activation of FPN1 mediated by heme pretreatment in Caco-2 cells is consistent with the recent demonstration of heme-induced FPN1 mRNA expression murine and cultured macrophages (Marro *et al.* 2010; Harada *et al.* 2011). In haptoglobin-null mice with chronic systemic hemoglobin overload, FPN1 mRNA expression is increased in duodenal enterocyte, liver and spleen macrophages compared to that in wide type mice (Marro *et al.* 2007). It is speculated that FPN1 transcription is increased as a consequence of increased levels of free hemoglobin in circulation resulting from inefficient clear up without haptoglobin. Similarly, in mice with β-thalassemia, FPN1 mRNA level is clearly augmented in response to the hemolysis (Gardenghi *et al.* 2007). In addition, for mice with phenylhydrazine-

induced hemolysis, FPN1 mRNA is increased in duodenum, liver and spleen (Latunde-Dada *et al.* 2004). Apart from these, in mice with hemolytic anemia resulting from superoxide dismutase-1 (SOD1)-knockout, expression of HO-1 and FPN1 were both up-regulated in liver macrophages (Starzynski *et al.* 2009). These combined data suggest that heme-induced FPN1 transcription may well enhance duodenal iron absorption as well as systemic iron availability to sustain erythropoiesis in cases of hemolytic anemia.

Conclusions

In summary, we found that FPN1 is up-regulated adaptively at the transcriptional level by heme pretreatment in Caco-2 cells, which represents a new mechanism for regulating intestinal iron absorption. More importantly, our data substantiate how heme as a meat factor of animal products could adaptively enhance intestinal non-heme iron absorption, resolving the missing piece in understanding the long-term effect of meat on iron absorption. Animal studies are needed to further confirm this result.

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Chapter 7

CONCLUSION

SUMMARY

These studies were designed to explore the effect of components of certain dietary factors on intestinal heme and non-heme iron absorption. We have discovered two novel types of dietary factors that regulate intestinal iron absorption in vitro, including bioactive polyphenols as inhibitors for intestinal heme iron absorption and heme as an enhancer for intestinal non-heme iron absorption.

The first aim focused on characterizing intestinal heme iron absorption using human intestine-like Caco-2 cells. As studies regarding the mechanism and regulation of intestinal heme iron absorption are very limited, these results provided the basis for the rest of the study as well as future studies in this area. Using ^{55}Fe-labeled heme, we found that the apical uptake and transepithelial transport of heme iron were dose-dependent and saturable. The rate of heme-^{55}Fe absorption decreased with increased amount of heme-^{55}Fe in apical chamber when the concentration of heme-^{55}Fe was above 10 μM. In addition, we found that the apical uptake and transepithelial transport of heme-^{55}Fe were both temperature-dependent in Caco-2 cells. The decrease in the transepithelial transport of heme-^{55}Fe at 4°C was possibly due to reduced apical heme uptake. As apical uptake of heme is both saturable and temperature-dependent, it could occur through a receptor-mediated endocytic pathway. Finally, our experiment revealed that the overwhelming majority of heme iron was exported into basolateral chamber as heme-free iron, suggesting that heme was largely degraded within Caco-2 cells to release free ferrous iron.

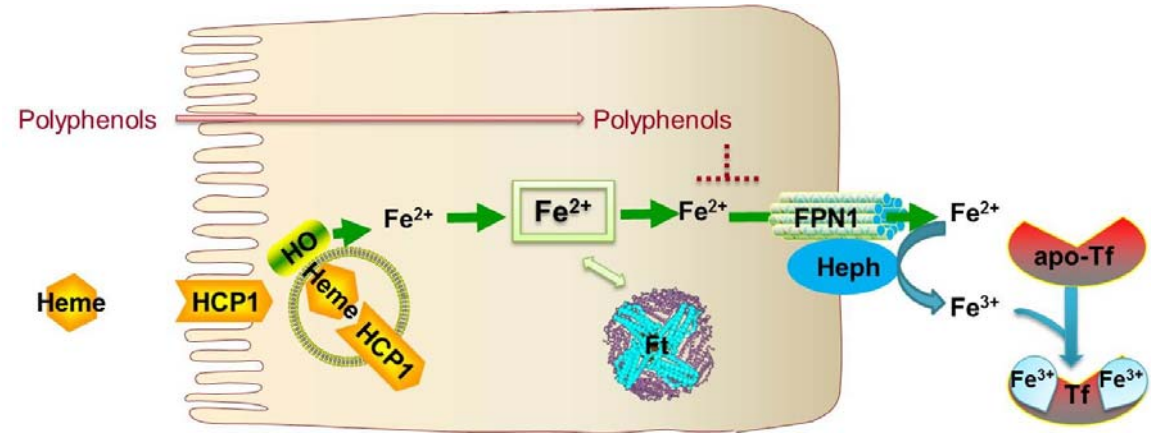

Figure 7-1. Inhibitory Effect of Bioactive Polyphenols on Intestinal Heme Iron Absorption

Polyphenols inhibit heme iron absorption by blocking basolateral release of heme-derived iron, possibly via forming a complex with iron within the enterocyte. Heme is taken up into enterocyte as an intact metalloporphyrin ring possibly via HCP1. Within enterocyte, heme is supposed to be split by HOs to release free ferrous iron. All free ferrous iron ultimately joins the labile iron pool. In absence of polyphenols, heme-derived iron can be exported across basolateral membrane via FPN and subsequently be oxidized by Heph. In presence of polyphenols, it is withheld in the enterocyte by the polyphenols.

7.1 Polyphenols as Inhibitors for Intestinal Heme Iron Absorption

The second aim was designed to explore the influences of bioactive polyphenolic ingredients of green tea and grade seed extract on intestinal heme iron absorption using Caco-2 cells. Our study showed that bioactive dietary polyphenols including EGCG, GSE and GT all inhibited iron absorption in Caco-2 cells. EGCG and GSE decreased heme iron absorption mainly by reducing basolateral release of heme-derived iron. This was the first report, to our knowledge, demonstrating that bioactive dietary polyphenols inhibit heme iron transport across the enterocyte by decreasing basolateral iron export, possibly by forming non-transportable complexes with iron in the cell (Figure 7-1). In addition, we found that these bioactive dietary polyphenols inhibited heme iron absorption in a dose-dependent manner in Caco-2 cells. Even a tiny bit of these polyphenolic compounds inhibited heme and non-heme iron absorption significantly, albeit to a lesser extent. Ascorbic acid could not offset the inhibitory effect of higher concentrations (46 mg/L) of polyphenols but reversed the effects of very low concentrations of polyphenols.

These results highlighted a new class of dietary factor, bioactive polyphenols, that impair intestinal absorption of both heme and non-heme iron and identified the components of green tea and grape seed that inhibit iron transport across the enterocyte.

7.2 Heme as an Enhancer for Intestinal Non-heme Iron Absorption

The third and final aims were designed to determine the immediate and adaptive effect of heme on non-heme iron absorption using intestinal Caco-2 cells and clarify whether heme could possibly be a meat factor that enhances intestinal iron absorption. Our study revealed that intestinal absorption of heme and non-heme iron were synergistic of each other in Caco-2 cells mainly through the apical/basolateral membrane translocation of DMT1 and FPN1, which could

be a fast physiological mechanism to regulate intestinal iron absorption (Figure 7-2). Intestinal DMT1 and FPN1 are therefore major targets of therapies for patients with iron-related disorders. The fact that heme readily increased non-heme iron absorption may partially explain how animal tissues promote intestinal non-heme iron absorption.

These results provide a new perspective to characterize intestinal heme and non-heme iron absorption jointly and serve as a basis for supplementing heme and non-heme iron together. In addition, our data showed that heme also adaptively enhanced non-heme iron absorption by inducing FPN1 expression, suggesting that overall heme increases intestinal non-heme iron absorption.

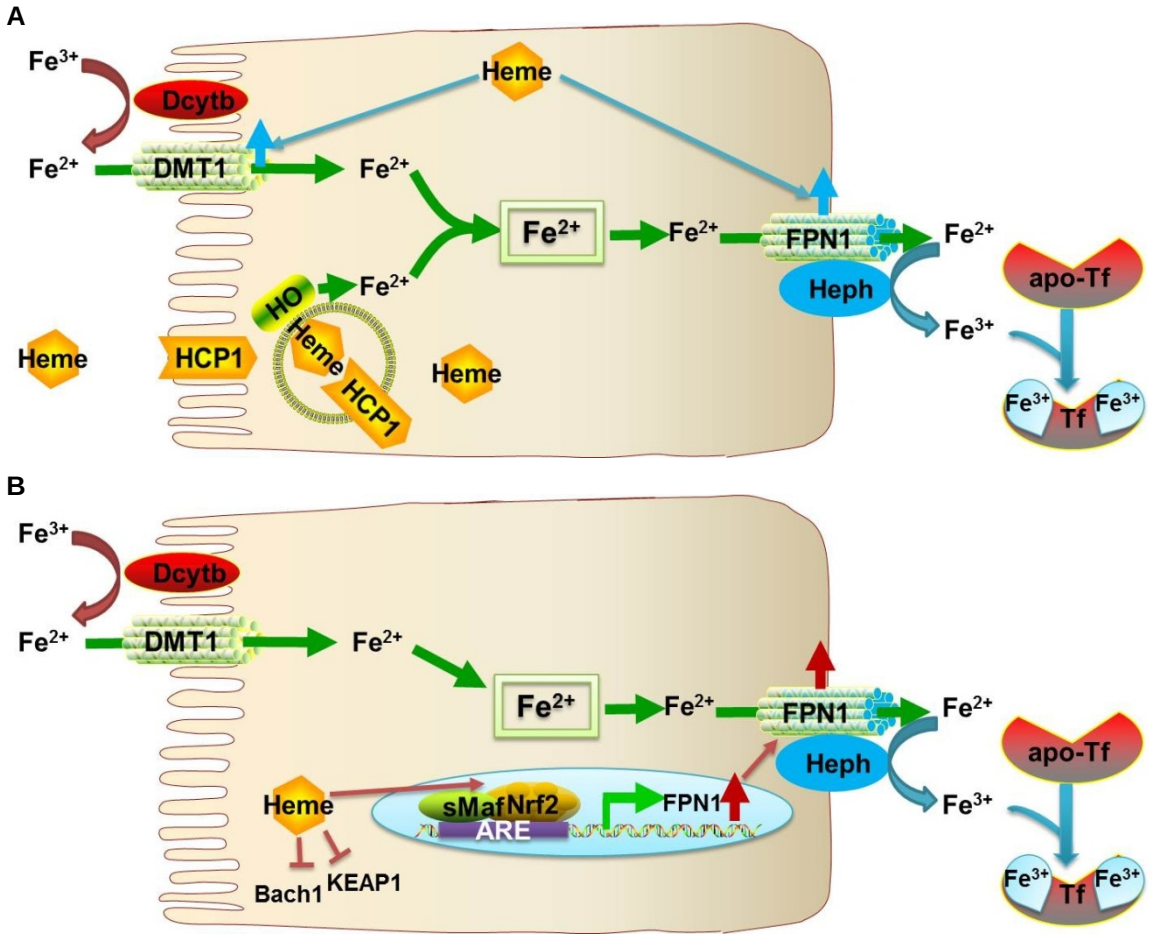

Figure 7-2. Enhancing Effect of Heme on Intestinal Non-heme Iron Absorption

Heme immediately enhances non-heme iron absorption by translocating DMT1 and FPN1 from cytosol to plasma membrane (A). Pretreatment of heme adaptively enhances succeeding non-heme iron absorption by inducing FPN1 mRNA expression (B). Dietary non-heme ferric iron is firstly reduced to ferrous iron by Dcytb and then absorbed via iron importer DMT1. Cellular iron can be exported to circulation via iron exporter FPN1 and subsequently be oxidized by Heph. Heme binds to cysteine-proline motif of transcription inhibitor Bach1 and replaces it with the transcription activator Nrf2 by inhibiting the activity of KEAP1. The thick up arrows indicate those determined in this dissertation.

FUTURE DIRECTIONS

This work has demonstrated that intestinal heme iron absorption can be blocked by some bioactive polyphenols such as EGCG, GSE and GT and that intestinal non-heme iron absorption can be boosted by heme in vitro. However, further work is needed to elucidate the following questions.

Firstly, it has been demonstrated using human intestine-like Caco-2 cells that these polyphenols inhibit both heme and non-heme iron absorption, while heme and non-heme iron boost the absorption of each other synergistically. However, the effect of these polyphenolic compounds and the interactions between heme and non-heme iron needs to be better understood by in vivo studies. We have demonstrated that the concentration of EGCG that almost completely blocked intestinal iron absorption in human intestine-like Caco-2 cells was 46 mg/l, which equals drinking of approximately 1 cup of green tea in humans. If this is also true for in vivo studies, it would be of great importance to separate tea drinking from taking iron supplementations. In addition, rather than the homogeneity of in-vitro cell culture, the iron status of the individual and other host factors, such as infectious diseases and obesity, may affect the extent of inhibitory effect of these polyphenols on iron absorption as well as the interactions between heme and non-heme iron during their absorptive process. It would therefore be important to assess the influences of dietary factors based on subject characteristics in the future.

Secondly, our studies only established the inhibitory effect of three polyphenols, EGCG, GSE, and GT, on iron absorption. Not all polyphenols, however, decreases intestinal iron absorption. Our preliminary data (data not shown) imply that some types of polyphenolic compounds enhances intestinal iron absorption by reducing ferric iron to ferrous iron instead of chelating iron. Further exploration regarding the effect of other common polyphenolic compounds on intestinal iron absorption will help make a more comprehensive conclusion on the

relationship between bioactive polyphenols and iron absorption. Moreover, since our study regarding the effect of polyphenolic compounds on iron absorption was based on single-treatment isotope studies, pretreatment studies analyzing the adaptive effect of polyphenols as well as multi-treatment studies with multiple inhibitors and enhancers can take place. This could ultimately lead to greater understanding of how dietary polyphenols affect intestinal iron absorption.

Finally, the molecular mechanisms behind the effect of polyphenols and heme deserve further exploration. Our study showed that dietary polyphenols blocked intestinal heme iron absorption mainly by decreasing basolateral iron exit, possibly by forming non-transportable complexes with iron within the cell, while the precise mechanism remains to be resolved. Apart from that, we found that heme and non-heme iron synergistically boost the absorption of each other via translocation of DMT1 and FPN in Caco-2 cells. However, for humans, it might be regulated differently when the iron status of the body differs. In addition, intestinal iron absorption is regulated in response to different conditions, such as hypoxia and inflammation. It would therefore be interesting to combine these different conditions together and examine how our findings apply to each condition.

In conclusion, the research presented in this dissertation has provided valuable insight into specific bioactive polyphenols and meat factor related to intestinal iron absorption. Our findings have contributed to the knowledge base of dietary factors and regulatory mechanism for intestinal iron absorption and have elucidated distinct areas for future research.

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