The Hong Kong Polytechnic University

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THE ROLE OF HEPCIDIN IN IRON TRANSPORT OF MACROPHAGES

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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CERTIFICATE OF ORIGINALITY

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ABSTRACT

The existence of a sensor for iron homeostasis has been suspected for a long time. The sensor is thought to be a soluble component of the plasma that would signal between different organs and tissues such as the intestine (iron absorption), liver (iron store), bone marrow (iron utilization) and reticuloendothelial (RE) macrophage (iron recycling). Recent studies showed that hepcidin might fulfill this important role. However, the biological roles of this peptide and the associated mechanisms have not been determined.

The experiments in this thesis were designed to investigate the effect of hepcidin on iron uptake and release in the rat peritoneal macrophages and the possible

mechanisms involved. It was found that the cells treated with hepcidin were significant lower in iron release as well as higher in iron uptake than those treated without hepcidin. Western blot analysis demonstrated that hepcidin has the ability to decrease the expression of the ferroportin1 (FPN1) protein and increase the divalent metal transporter 1 (DMT1) and transferrin receptor (TfR1) protein. These indicate that hepcidin regulates iron metabolism in macrophage through the regulation of the iron transporters.

To compare the differences of hepcidin regulation *in vivo* with that *in vitro,* we used LPS to induce hepcidin expression in rat and examined the iron metabolism changes in the peritoneal macrophages. Our results showed that iron release was decreased at 6 hours after LPS i.p, and the iron uptake was increased at 6 hours after LPS i.p. These data were consistent with changes of the expression of FPN1 and TfR1 *in vivo***.**

In addition, the regulation of iron uptake and release, and iron transporters (DMT1, TfR1, FPN1, CP and hephaestin (Heph)) expression by hepcidin in heart H9C2 cells were investigated. Hepcidin decreased the iron uptake and release of H9C2 cells through regulating the TfR1 and FPN1 expression in these cells.

Studies in this thesis also demonstrated the synthesis and distribution of hepcidin in different regions of the rat brain. On the other hand, we studied the role of hepcidin on iron metabolism of C6 glioma cells *in vitro*. After treating with hepcidin, the iron uptake and release of C6 glioma cells were both decreased when compared to that of the control groups, which had not been treated with hepcidin. Western blot results showed that the levels of DMT1-IRE, TfR1 and FPN1 were all decreased at the same time.

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CONTENTS

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTORY STATEMENT

The aim of this chapter is to provide a general introduction to this thesis. Hepcidin (or the liver-expressed antimicrobial peptide, LEAP-1) was first isolated from human urine and blood by Krause in 2000 (Krause et al., 2000). The bioactive form of hepcidin, a 25-amino-acid peptide with approximately 2-3 kDa molecular mass, is produced by hepatocytes in the liver and secreted into the serum. In contrast to that of humans, the mouse genome contains two hepcidin genes. Hepcidin-1 is expressed in the liver, while hepcidin-2 is expressed in the liver and pancreas. It is widely accepted that hepcidin functions as a homeostatic regulator of iron metabolism between the intestine (iron absorption), liver (iron store), bone marrow (iron utilization) and reticuloendothelial (RE) macrophage (iron recycling). Recently, there is some evidence showing that hepcidin presumably acts by regulating the expression or activity of one or more iron transporters on cell membrane, such as divalent metal transport 1 (DMT1), transferrin receptor 1 (TfR1), ferroportin 1 (FPN1), hephaestin

(Heph) and ceruloplasmin (CP). Although the negative effect of hepcidin on iron absorption and efflux in enterocytes has been reported, the effects and mechanisms of hepcidin in controlling iron metabolism of macrophages, heart and neural cells are still not well understood.

1.2 GENERAL DESCRIPTION OF HEPCIDIN

Hepcidin is a recently discovered peptide, which was isolated from human urine by Park and was named on the basis of the site of synthesis (hep-) and the antibacterial properties *in vitro* (-cidin)(Park et al., 2001). Independently, Krause et al. also isolated the same peptide from plasma ultrafiltrate (Krause et al., 2000), and named it LEAP-1 (liver-expressed antimicrobial peptide). Hepcidin is a β-defensin-like peptide hormone that functions both as a homeostatic regulator of iron metabolism and as a mediator of host defense and inflammation. Loss-of-function mutations in the hepcidin gene will induce hereditary hemochromatosis (HH). Until now the effect of hepcidin on iron metabolism has not been fully established.

1.2.1 The Characterization of Hepcidin

1.2.1.1 Constitution and Molecular Structure of Hepcidin

The major hepcidin form is a cationic peptide with 25 amino acid residues, 2-3KDa in size, and has an overall charge of +3 at neutral pH. As judged by mass spectrometry, electrophoretic migration in acid-urea PAGE, retention on C18 reverse-phase HPLC columns and reactivity with anti-hepcidin antibody, the natural and synthetic forms were identified. Hunter et al. analyzed the synthetic forms by nuclear magnetic resonance spectrometry (Hunter et al., 2002) and established their connectivity and structure in solution (Fig 1-1). The amino acid sequence of human hepcidin is DTHFPICIFCCGCCHRSKCGMCCKT, which has 8 cysteines and appears to be engaged in four intramolecular disulfide bonds. The molecular structure is a simple hairpin whose two arms are linked by disulfide bridges in a ladder-like configuration. The symmetric arrangement of pairs of cysteines around a cationic segment (HRSK) resembles that of antimicrobial protegrins and tachyplesins, peptides that have a two-strand β-sheet structure stabilized by interstrand disulfide bonds. The cysteine connectivity pattern is: 1-4, 2-8, 3-7 and 5-6 (Fig 1-1)(Ganz, 2003; Park et al., 2001). One highly unusual feature of the molecule is the presence of a disulfide linkage between two adjacent cysteines near the turn of the hairpin. Compared to most disulfide bonds, those formed between adjacent cysteines are

stressed and have a greater chemical reactivity. Like other antimicrobial peptides, hepcidin displays spatial separation of its positively charged hydrophilic side chains from the hydrophobic ones, a characteristic of peptides that disrupts bacterial membranes (Ganz, 2003).

Compared with other forms, only hepcidin-25 has biological activity. The NMR solution structures of both peptides Hepc-25 and Hepc-20 have been determined and reveal a similar structural topology consisting of a distorted β-sheet stabilized by four disulfide bridges. The major structural differences between the two peptides are present at the N-terminal region. NMR diffusion studies indicated that hepcidin-20 exists as a monomer in solution, whereas hepcidin-25 readily aggregates (Hunter et al., 2002). At the N-terminus region of the hepcidin-25, there is a putative metal-binding site corresponding to the first three amino acids NH_2-Asp-Thr-His, which is named as 'ATCUN motif'. This metal-binding property at the N-terminus of hepcidin-25 seems to be necessary for its biological activity as an iron-regulatory hormone (Fleming and Sly, 2001b; Nicolas et al., 2001; Nicolas et al., 2002a). Deletion of the 5 amino acids of N-terminal sequence leads to total loss of the biological activity (Nemeth et al., 2006). Moreover, the metal binding can inhibit the aggregation propensity of both the whole hepcidin-25 and its N-terminal fragments,

probably including a conformational change leading to the stabilization of the N-terminal region.

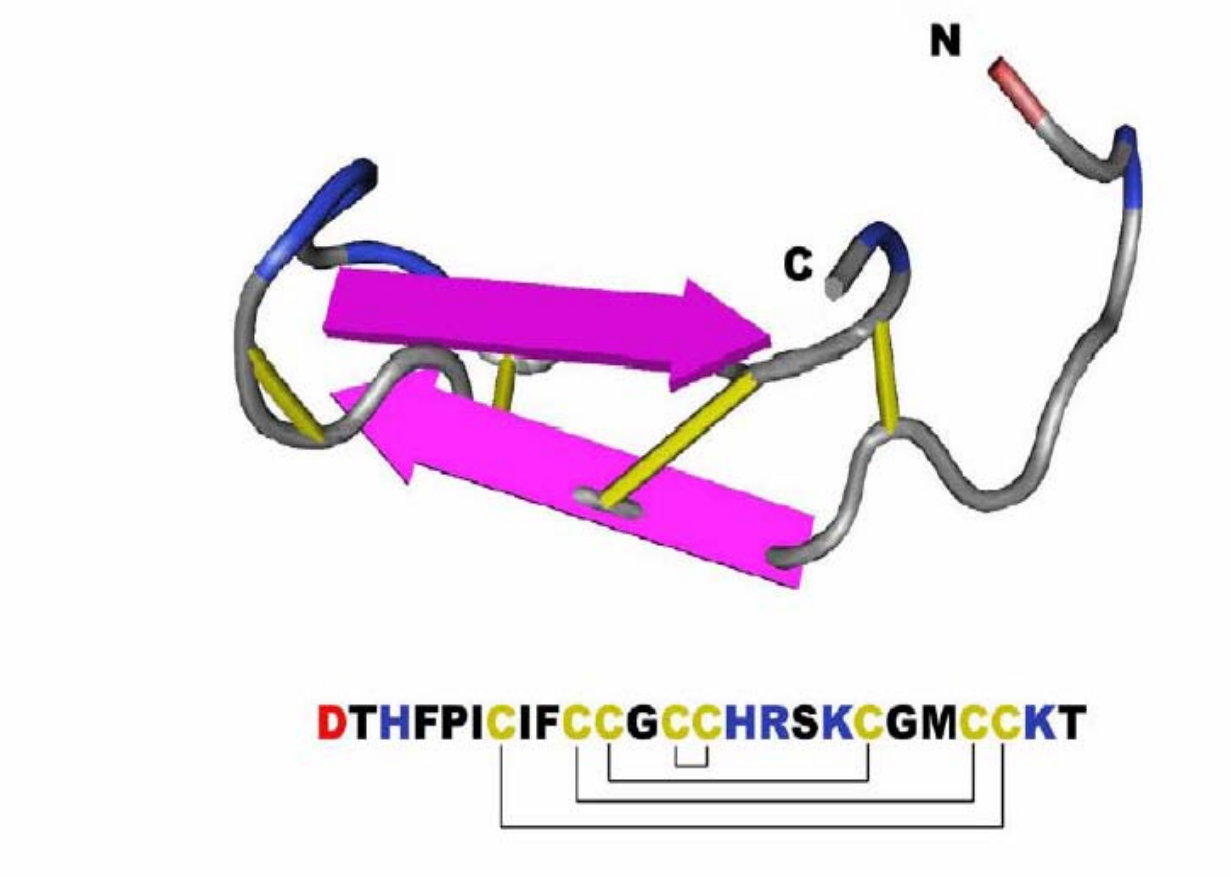


Figure 1-1. Amino acid sequence and a cartoon model of the major form of human hepcidin. The amino and carboxy termini are labeled as N and C respectively. Disulfide bridges are in yellow, basic amino acids in blue and acidic in red. The pattern of disulfide linkages between the 8 cysteines is also shown in the amino acid sequence (Ganz, 2003).

1.2.1.2 Forms of Hepcidin

Human hepcidin isolated from urine exhibits three predominant forms: 20- (hepc-20), 22- (hepc-22) and 25- (hepc-25) amino acids and the predominant form is hepc-25 (Fig1-2)(Park et al., 2001). All these peptides are derived from a precursor peptide of 84 amino acids. In humans, the cDNA sequence of hepcidin exhibits an open reading frame encoding an 84-residue precursor protein which contains the isolated peptide at the COOH-terminus. An NH_2-terminal signal sequence (residues 124) characteristic of secretory proteins was identified. In addition, a basic penta-arginyl segment directly adjacent to the NH_2-terminus of the isolated peptide is contained within the precursor protein. It appears that the hepcidin precursor is a secretory protein that is proteolytically processed at the polybasic proteolysis site whereby the isolated peptide hepcidin is released. A potential signal-peptide cleavage site between Gly24 and Ser25 would generate a 60-amino acid propeptide species. Consensus sites for myristylation sites and phosphorylation sites occur on Gly44 and Thr24, respectively. In addition, a potential furin cleavage site is found at Arg59. The 20- to 25-amino acid peptides are derived from the C-terminus of the 84-amino-acid prepropeptide by an amino-terminally process (Krause et al., 2000).

A search of the protein database revealed no homology to hepcidin in vertebrates and invertebrates. Surprisingly, and unlike other antimicrobial peptides, hepcidin sequences were remarkably similar among various mammalian species. Search of the EST database revealed liver cDNA homologues in pig, rat, mouse, flounder and the long-jawed mudsucker (Fig1-3). All 8 cysteine residues are conserved with particularly strong sequence similarity in residues 60–84 that encompass the processed forms isolated from urine. It is noteworthy that the putative propeptide convertase cleavage sites are also conserved. In humans, the paired basic residues are Arg-Arg, whereas the other homologues have the same recognition sequence or an alternative recognition sequence of Lys-Arg (Park et al., 2001).

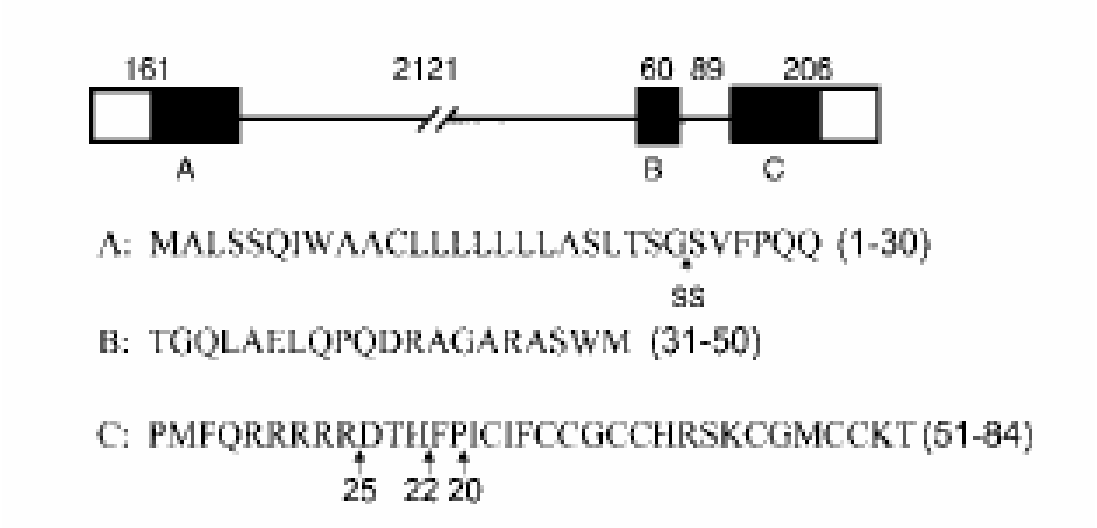


Figure 1-2. Hepcidin gene and precursor peptide**.**

An intron-exon diagram and the peptide sequences encoded by the three exons A, B and C are shown. The exons encode an 84-amino acid prepropeptide. *Arrows* denote three processed forms isolated from urine. An *arrow* indicates the putative signal sequence (*SS*) cleavage site (Park et al., 2001).

Figure 1-3. Comparison of hepcidin sequences from various species.

A BLAST search of GenBankTM EST entries revealed cDNA homologues of human hepcidin (*hHEPC*) in pig (*pHEPC*), rat (*rHEPC*), mouse (*mHEPC*), flounder (*fHEPC*) and the long-jawed mudsucker *Gillichthys mirabilis* (*gHEPC*). Putative peptide sequences were translated from the cDNA sequences isolated from the liver of each species. The positions of cysteine residues (outlined in *gray boxes*) are conserved (Park et al., 2001).

1.2.2 Hepcidin Synthesis and Distribution

1.2.2.1 The Genetic Expression of Hepcidin

The human genome contains one hepcidin gene, which is located on the chromosome 19. The 2.5 kb hepcidin gene consists of three exons and two introns and is predominantly expressed in the liver. A 0.4 kb mRNA generated from these exons encodes the 84 amino acids preprohepcidin (Park et al., 2001). The human hepcidin gene lies in close proximity to the USF2 gene, and contains potential binding sites for HNF3β, C/EBPβ and NF-κB in the upstream regulatory region (Pigeon et al., 2001).

In contrast to humans, the mouse genome contains two hepcidin genes which are located on chromosome 7. Hepcidin-1 is expressed in the liver, while hepcidin-2 is expressed in the liver and pancreas. They also consist of three exons and two introns and are located close to the USF2 gene. Comparisons between hepcidin-2 and hepcidin-1 show that they share great similiarities with hepcidin-1 both at the nucleotide and at the amino acid level in the corresponding translated regions (92% and 89% identity, respectively). However, the 25-aa C-terminal peptide

corresponding to the putative mature chain of the mouse hepcidin-2 shares with hepcidin-1 only 68% identity (8 out of 25 aa are different). It is noteworthy that all 8 cysteine residues in the 25-aa mature hepcidin-1 and hepcidin-2 peptides are conserved (Ilyin et al., 2003). Hepcidin-1 is more closely related to the human peptide than hepcidin-2 (76% identity between mouse hepcidin-1 and human hepc-25 vs only 58% for hepcidin-2)(Nicolas et al., 2002b).

1.2.2.2 The Distribution of Hepcidin

In mammals, the hepcidin gene is predominantly expressed in the adult liver (Krause et al., 2000; Park et al., 2001; Pigeon et al., 2001), in which hepcidin mRNA represents one of the most abundant messengers. Hepcidin expression in the liver is restricted to the hepatocytes and appears to be dependent on hepatocyte differentiation status (Courselaud et al., 2002; Pigeon et al., 2001). But to a much lesser extent, it is also detectable in the heart and brain. In addition, hepcidin is found in the expressed sequence tag databases from lung, testis, stomach and pancreas. However, the functional role of hepcidin in these tissues has not been investigated. In rodent, hepcidin gene expression is developmentally regulated (Courselaud et al., 2002; Nicolas et al., 2002c). Hepcidin mRNAs are undetectable at the end of

gestation, increase around birth and reach high level only at the adult stage. It is noteworthy that in mouse, hepcidin gene expression is highly dependent upon the genetic background (Weinstein et al., 2002).

1.3 REGULATION OF HEPCIDIN

1.3.1 Factors Responsible to the Regulation of Hepcidin Synthesis

Different studies have now shown that hepcidin expression can be altered by three factors: inflammation, anemia and hypoxia, iron levels (Nicolas et al., 2002b)**.**

1.3.1.1 Regulation of Hepcidin Synthesis by Infection and Inflammatory Factor

It has been shown for many decades that inflammation alters iron metabolism by decreasing the iron content of plasma (hypoferremia of inflammation). The recently elucidated molecular basis of these changes centered on the regulation of hepcidin by inflammation. Shike et al (Shike et al., 2002) showed that in white bass liver, infection with the fish pathogen *Streptococcusiniae* increased hepcidin mRNA

expression 4500-fold. In another study by Nicolas et al (Nicolas et al., 2002c), injections of turpentine, a standard inflammatory stimulus, into mice induced hepcidin mRNA 4-fold expression and led to a 2-fold decrease in serum iron. The hypoferremic response to turpentine-induced inflammation was absent in the USF2/hepcidin-deficient mice, indicating that this response was fully dependent on hepcidin. A reduction of serum iron levels was also observed during chronic hepcidin overproduction in the murine liver (Nicolas et al., 2002a) or after peritoneal injection of hepcidin peptide (Rivera et al., 2005a). Nemeth et al (Nemeth et al., 2003) assayed urinary hepcidin peptide in patients with anemia due to chronic infections or severe inflammatory diseases, and observed as much as 100-fold increase in hepcidin excretion (adjusted for urinary creatinine), with smaller increases in patients with less severe inflammatory disorders. Interestingly, the excreted hepcidin levels gradually decreased with the resolution of infection in a patient with epididymitis and sepsis, providing evidence that hepcidin expression correlates with the infection state (Nemeth et al., 2003). Urinary hepcidin was also increased about 100-fold in patients with iron overload from transfusions for sickle cell anemia or myelodysplasia. Hepcidin excretion correlated well with serum ferritin, which is also increased by both iron loading and inflammation.

The cytokine IL-6 is the key inducer of hepcidin synthesis during inflammation (Lee et al., 2005). Studies on the effect of iron or cytokines on isolated primary human hepatocytes (Nemeth et al., 2003) revealed that hepcidin mRNA expression was induced by lipopolysaccharide and strongly induced by monokines from monocytes exposed to lipopolysaccharide. Among the cytokines, interleukin-6 (IL-6), but not IL-β or tumor necrosis factor α (TNF-α), strongly induced hepcidin mRNA. The anti-IL-6 antibodies can block the induction of hepcidin mRNA in human primary hepatocytes treated with LPS or peptidoglycan, and block the induction of hepcidin mRNA in human hepatocyte cell lines treated with supernatants of LPS- or peptidoglycan-stimulated macrophages. Cytokines have all sorts of effects on cultured cells, and it is important to show that IL-6 induction of hepcidin occurrs in vivo and triggers hypoferremia as predicted. First, Nemeth et al used turpentine injection to cause inflammatory abscesses in wild-type and IL-6 knockout mice and analyzed the responses (Nemeth et al., 2004a). The wildtype mice had increased hepcidin expression and a substantial decrease in serum iron levels. In contrast, the IL-6 knockout mice showed no increase in hepcidin expression and no decrease in serum iron. A complementary experiment carried out in human volunteers showed that IL-6 infusion stimulated urinary hepcidin excretion within 2 hours and induced hypoferremia. In humans injected with LPS, there was an early and transient

induction of TNF-α and interferon-γ, boosted by a dramatic increase in IL-6 which peaked at 3–4 h. This correlated with both the maximal hepcidin excretion at 6 h and the onset of hypoferraemia (Kemna et al., 2005). Taken together, these data provide strong support for the conclusions that IL-6 is a primary inducer of hepcidin expression and that increased hepcidin expression results in hypoferremia. This is gratifyingly consistent with clinical observations that hypoferremia occurs very quickly after the onset of inflammation (Andrews, 2004).

1.3.1.2 Regulation of Hepcidin Synthesis by Anemia or Hypoxia

In addition to inflammation, anemia and hypoxia also affect the iron metabolism. These stimuli would be expected to decrease hepcidin production and remove the inhibitory effect on iron absorption from enterocytes and iron release from macrophages so that more iron is available for compensatory erythropoiesis. Several investigators (Weinstein et al., 2002) (Nicolas et al., 2002b) confirmed that these effects indeed take place. The physiological response of hepcidin to experimentally induced acute anemia in mice was studied in two models, acute hemolysis provoked by phenylhydrazine (PHZ), and bleeding provoked by repeated phlebotomies. In both cases, hepcidin gene expression was shown to be dramatically decreased.

Interestingly, the suppressive effect of hemolytic anemia was seen even in iron-overloaded mice, suggesting that the suppression of hepcidin by anemia has a stronger effect than the stimulation of hepcidin by iron overload. This hierarchy of effects could explain why iron overload commonly develops with certain hemolytic disorders (Ganz, 2003). Thalassaemic mice with iron overload had significantly lower hepcidin expression compared with the controls, which also supports the dominant force of anaemia over hepatic iron in hepcidin induction (Adamsky et al., 2004). Hepcidin inhibition in this scenario can be seen as an inappropriate physiological response, which leads to a worsening of iron overload. This is supported by a recent study of urinary hepcidin levels in humans with iron overload (Papanikolaou et al., 2005). Very low urinary hepcidin levels (11 patients) or normal hepcidin levels (four patients) were found in 15 patients with thalassaemia despite high ferritin levels. Hepcidin was undetectable in two patients with congenital dyserythropoietic anaemia type 1 despite iron overload, which was consistent with the dominance of the erythropoietic drive secondary to hypoxia or anaemia on hepcidin expression. Finally, Weinstein et al found that hepcidin gene expression was markedly decreased in the livers of iron-deficient anemic mutant mice (Weinstein et al., 2002). In the case of the *mk* and *sla* mice (with defective DMT1 and hephaestin genes, respectively), the genetic defects interrupted iron transport at

the duodenal level, precluding any iron replenishment. This was not the case of the *hpx* mice (with aberrant splicing of the transferrin gene) where a decreased iron uptake occurred with systemic iron overload. This result again suggested that anemia-induced suppression of hepcidin gene expression occurred even in the setting of abundant iron stores (Weinstein et al., 2002).

Downregulation of hepcidin gene expression was also reported in mice housed in hypobaric hypoxia chambers and in human HepG2 and Hep3B hepatoma cells under hypoxic conditions. In conditions of hypoxia and anemia, renal erythropoietin synthesis is stimulated by the transcription factor HIF (hypoxia-inducible factor), which leads to increased production of erythropoietin that in turn stimulates erythropoiesis. The decreased level of hepcidin will allow iron uptake from the intestine and recirculation from the macrophages; thus iron will be delivered to the bone marrow to sustain the erythropoiesis, resulting in increased oxygen supply. Increased intestinal absorption is also seen in hypoxia (Raja et al., 1990). Indeed, injection of EPO was found to dramatically decrease liver hepcidin gene expression in mice (Nicolas et al., 2002b). However the exact mechanism by which anemia and hypoxia down-regulate hepcidin gene is unknown.

1.3.1.3 Regulation of Hepcidin Synthesis by Iron

The connection between hepcidin and iron metabolism was first made by Pigeon et al. (Pigeon et al., 2001), who were searching for new genes upregulated during iron excess. Hepcidin was isolated by suppressive subtractive hybridization performed between livers from carbonyl iron-loaded and control mice. Hepcidin mRNA was found to be increased in the liver of the experimentally (carbonyl iron) and spontaneously (β_2-microglobulin knockout mice) iron-overloaded mice. The amount of mRNA was directly linked to the liver iron concentration as evidenced by the dose dependence of mRNA induction in the carbonyl-iron–treated mice and the decreased expression of hepcidin when the β_2-microglobulin knockout mice were fed a low-iron diet. Thus, hepcidin expression increases with iron overload and decreases with iron depletion. The cellular and molecular mechanisms regulating hepcidin gene expression in response to iron status are not yet understood. Because no iron-responsive element (McKie et al., 2001) has been identified in the transcript, hepcidin expression is not regulated by the IRE/IRP system. The transcription factor CCAAT/enhancer-binding protein (C/EBP-α) has been proposed to be involved in this iron-mediated increase in hepcidin expression (Nicolas et al., 2002b). Sequence analysis of the 5-flanking region of the human and mouse hepcidin gene identified

several binding sites for the liver-enriched transcription factors (C/EBP) and the hepatocyte nuclear factor 4 (HNF4). Studies in hepatic C/EBP-α null mice and cell culture assays indicated that C/EBP-α induces and HNF4-α reduces hepcidin mRNA expression. Iron overload results in an increase in both C/EBP protein and hepcidin transcripts. In addition, hepcidin expression has also been shown to be dependent on hepatocyte differentiation status (Courselaud et al., 2002; Pigeon et al., 2001). It decreases spontaneously in conventional mouse hepatocyte culture, is undetectable in hepatic cell lines, and its expression is higher in adult liver as compared to fetal liver. Whether alterations in hepcidin expression during developmental process are caused by altered iron or oxygen availability is currently unclear. Furthermore, female mice express more hepcidin mRNA than male mice and an elevated hepcidin mRNA expression in different mouse strains is correlated with the increased systemic iron level.

In contrast to in vivo situation where iron overload stimulates hepcidin mRNA expression (Muckenthaler et al., 2003; Pigeon et al., 2001), iron treatment of primary human hepatocytes and hepatocyte-like cell lines decreases hepcidin mRNA expression (Nemeth et al., 2003; Pigeon et al., 2001). This is commonly interpreted as an indication that it is the hepatic cell types and not hepatocytes which may be

involved in the iron-sensing and hepcidin regulation (Nemeth et al., 2004a).

1.3.2 Molecules Involved in the Regulation of Hepcidin Expression

The exact mechanism by which the body senses the iron status and inflammation stimuli, then regulates hepcidin expression in hepatocytes is unknown. The discovery of different forms of hereditary hemochromatosis (HH) caused by mutations of HFE, trasferrin receptor 2 (TfR2) and hemojuvelin (HJV) offers some possible answers for this problem. These diseases are characterized by hepcidin deficiency in spite of a massive iron overload, indicating that all these molecules act as direct or indirect regulators.

1.3.2.1 Molecules Involved in Iron Regulation of Hepcidin Expression

The iron-sensitive mechanism for up-regulating hepcidin expression in response to iron overload appears to involve HFE, TfR2 and HJV (Kawabata et al., 2005; Nemeth et al., 2005). Body iron status may be sensed through diferric transferrin, which has been proposed to regulate the interactions of HFE with either TfR1 or TfR2 and the subsequent downstream signaling to hepcidin (Goswami and Andrews,

2006).

1.3.2.1.1 HFE and Hepcidin

The HFE gene encodes an atypical major histocompatibility (MHC) class I protein, which is a 343 amino acids cell surface protein with homology to major histocompatibility complex (MHC) class I molecules (Feder et al., 1996). The structure includes a large extracellular domain consisting of three loops (α1 to α3), a single transmembrane domain and a short cytoplasmic tail. Similar to other MHC class I molecules, HFE physically associates with β_2-microglobulin (β_2M) via its α3 loop (Feder et al., 1997). HFE is broadly expressed in many tissues (Feder et al., 1996), however, its expression in the liver is high relative to other tissues. Hereditary hemochromatosis is a common autosomal recessive disorder characterized by increased intestinal iron absorption and iron deposition in various organs, primarily the liver and heart. The most frequent HH mutation disrupts the HFE secondary structure, blocks its interaction with β_2-microglobulin (β_2M) and subsequently its presentation at the cell surface.

A direct link between HFE and iron metabolism was provided by the finding that

HFE binds to transferrin receptor 1 (TfR1) at the cell surface and competes for transferrin binding. Despite numerous studies in tissue culture cells, it is currently unclear how the TfR1/HFE interaction can be reconciled with the pathophysiology of HH. Affected patients have increased iron absorption despite adequate or high body iron stores, indicating an inability to appropriately limit absorption. This observation, coupled with the widespread support for the crypt programming model, led most researchers focus on the intestinal crypts as the site of HFE action. However, in 2003, Bridle et al (Bridle et al., 2003) reported that mutations in HFE led to inappropriately low hepcidin levels in both humans and mice, suggesting that HFE is involved in the regulation of hepcidin expression. Because hepcidin expression is essentially restricted to the liver, this provided strong evidence that HFE was exerting its effects in the hepatic cells and not in the intestinal crypts. Furthermore, Nicolas et al. (Nicolas et al., 2003) showed that iron loading in HFE-null mice could be corrected by constitutively expressing hepcidin. Taken together, these data suggested that HFE could act as a sensor for Tf saturation and a subsequent hepcidin expression regulator. Subsequent studies showed that HFE is most strongly expressed in hepatocytes, as indeed is hepcidin, suggesting that it is in these cells that HFE exerts its effect on hepcidin expression (Zhang et al., 2004).

1.3.2.1.2 TfR2 and Hepcidin

Also strongly expressed in hepatocytes is transferrin receptor 2 (TfR2). This protein is a homolog of the classical TfR1 and can take up transferrin-bound iron from the circulation via receptor-mediated endocytosis. In comparison to TfR1, TfR2 binds diferric transferrin with an approximately 30-fold lower affinity. TfR2 protein is stabilized upon the binding of diferric transferrin but not by apo-transferrin in the hepatocyte-derived cell lines (Johnson and Enns, 2004). In mice and rats manipulated by diet or genetics, TfR2 protein levels reflect the available holotransferrin levels regardless of the concurrent presence of anaemia or total hepatic iron content (Robb and Wessling-Resnick, 2004). Hepcidin levels are reduced in patients with HH type 3 which is caused by mutations in TfR2, similar to observations in HH type 1. Only patients with concomitant inflammatory conditions presented with increased hepcidin expression (Nemeth et al., 2005). Likewise, TfR2 deficient mice did not induce hepcidin mRNA expression upon iron loading (Kawabata et al., 2005). These data suggest that TfR2, like HFE, controls the appropriate production of hepcidin and it is therefore likely that both proteins are involved in the same or converging regulatory pathways. Current models suggest that hepcidin expression is under the dual control of TfR2 and HFE/TfR1, both of which sense the amount of iron bound

transferrin available in the serum. A signal of unknown nature is then transmitted to the nucleus to control hepcidin expression. Mutations in TfR2 and HFE most likely interfere with this sensing mechanism for systemic iron levels and result in pathologically low hepcidin expression (Frazer and Anderson, 2003). Contrary to previous suggestions (Townsend and Drakesmith, 2002), iron mediated hepcidin regulation may be achieved by hepatocytes only or may depend on the communication of hepatocytes with cell types other than Kupffer cells (Lou et al., 2005; Montosi et al., 2005).

1.3.2.1.3 A Model for the Molecular Basis of Hepcidin Regulation

Precisely how these molecules monitor body iron requirements and direct the regulation of hepcidin expression is not yet known. Any regulatory pathway must allow the hepatocytes to detect events occurring at distant sites in the body, such as alterations in iron demands of developing erythroid cells in the bone marrow. Transferrin saturation has previously been suggested as a signal for body iron status, and recent studies suggest that diferric transferrin in the circulation may relay body iron demand back to the liver.

Frazer et al (Frazer and Anderson, 2003) have recently proposed a detection mechanism for diferric transferrin that involves both HFE and TfR2 on the hepatocyte plasma membrane. In this model, they proposed that diferric transferrin would outcompete HFE for TfR1 binding such that higher diferric transferrin levels would lead to an increased amount of free HFE on the cell surface. The unbound HFE on the cell surface is able to stimulate a signal transduction pathway that leads to an increase in the expression of hepcidin. This would explain the decrease in hepcidin expression that occurs when HFE is disrupted in hemochromatosis.

TfR2 could play a similar role in regulating the expression of hepcidin in response to circulating diferric transferrin levels. In this case, however, there is evidence suggesting that the TfR2 protein is stabilized by the binding of diferric transferrin (Johnson and Enns, 2004). If a signal to increase hepcidin expression is produced by TfR2, the stabilization of this molecule by diferric transferrin would maintain this signal. A lowering of diferric transferrin level would decrease this signal, reducing hepcidin expression and increasing iron absorption. Hepatic iron stores could also play part in the regulation of hepcidin by altering the surface expression of TfR1 in hepatocytes (Frazer and Anderson, 2003). Low intracellular iron levels increase the level of TfR1 expression at the cell surface, making more of this molecule available

to interact with HFE. The increased levels of TfR1 would also effectively outcompete TfR2 for diferric transferrin binding because TfR2 has a ~25-fold lower affinity for diferric transferrin than TfR1. The combined effect would be a decrease in the signal to produce hepcidin when hepatocyte iron stores are low. The opposite would occur when iron stores are high as the cell surface expression of TfR1 decreases. The expression of hepcidin, therefore, would be regulated by the combined effects of diferric transferrin levels, which indicate body iron usage and hepatocyte iron stores.

The presence of two parallel pathways (one requiring HFE and the other TfR2) for the regulation of hepcidin expression explains the phenotypes observed when the various molecules are disrupted. Mutations in either HFE or TfR2 produce a relatively mild iron overload, with hepcidin levels lower than expected but still detectable. Frazer et al (Frazer and Anderson, 2003) previously predicted from their model that mutations in both HFE and TfR2 would result in a more severe phenotype, similar to that seen with mutations in hepcidin or hemojuvelin. Recently, a patient with juvenile hemochromatosis due to mutations in both HFE and TfR2 was reported, which supported the hypothesis (Pietrangelo et al., 2005).

1.3.2.1.4 Hemojuvelin and Hepcidin

Recently, Papanikolau et al cloned a novel gene responsible for the Ch1q-linked form of the HH，called *HFE2* or *HJV*, which encodes the protein hemojuvelin (Papanikolaou et al., 2004). The *HJV* gene is 4,265 bp long and contains four exons. It is transcribed into a full-length messenger-RNA with five spliced isoforms. Hemojuvelin, the protein product of the *HJV* gene, exists in three isoforms of 426, 313 and 200 amino acids. The putative full-length protein from the longest transcript (transcript 1) is 426 amino acids (Rodriguez Martinez et al., 2004), consisting of a signal peptide, a large von Willebrand D-like domain and a C-terminal transmembrane domain with a likely GPI anchor (Papanikolaou et al., 2004).

Hemojuvelin (Papanikolaou et al., 2004) belongs to the family of repulsive guidance molecules (RGM), which is involved in neuronal differentiation, migration and apoptosis (Brinks et al., 2004; Matsunaga and Chedotal, 2004; Niederkofler et al., 2004; Samad et al., 2004). However, unlike other RGMs which are expressed in neural tissue, hemojuvelin is expressed predominantly in the skeletal muscle, liver and heart. One form of hemojuvelin is GPI-linked to the membrane (Zhang et al.,

2005), with a molecular weight of 46KDa, and this form was shown to undergo cleavage in cell culture, resulting in the release of soluble hemojuvelin (44KDa) into the medium (Lin et al., 2005; Zhang et al., 2005).

Orthologs of the human hemojuvelin are found in mouse, rat and zebrafish. Sequence comparison shows that the human hemojuvelin is >85% identical to the mammalian orthologs and -45% identical to the fish ortholog (Papanikolaou et al., 2004). In the same study, mouse tissues were also analyzed using Western blot for the presence of *HJV* protein. The Western blot was positive in nearly all tissues in which *HJV* mRNA was detected by reverse transcription–PCR (Celec, 2005).

Although hemojuvelin's function is unknown, humans with HJV mutations and $Hfe2^{-/-}$ mice have low hepcidin levels, and siRNA inhibition of HJV decreases hepcidin expression in vitro, suggesting that hemojuvelin positively regulates hepcidin expression. However, the mechanism of the hemojuvelin action is still unclear. It is likely to involve interactions with other proteins, since hemojuvelin lacks a cytoplasmic tail for direct signaling to the cell interior, and other members of the RGM family (RGMa and RGMb) function as receptor ligands.

Recently, it was demonstrated that the RGM family members act as co-receptors to enhance bone morphogenetic protein (BMP) signaling (Babitt et al., 2005; Samad et al., 2005). BMPs are a protein subfamily of the transforming growth factor b (TGF-b) superfamily of ligands with key roles in regulating cell proliferation, differentiation, apoptosis, migration and pattering during development and in adult tissues (Zhao, 2003). All BMPs share a common signaling pathway, which involves interaction with complexes resulted from association of two type I and two type II serine/threonine kinase receptors (Shi and Massague, 2003). The binding of a BMPs ligand to the type I receptors induces an association with the type II receptors, leading to an active signaling complex. After the formation of this complex, the type II receptors phosphorylate the type I receptors, which at their turn will phosphorylate Smad proteins. The BMPs subfamily signals via Smad1, Smad5 and Smad8, whereas the TGF-b subfamily signals via Smad2 and Smad3. Phosphorylated Smad proteins associate with the common mediator Smad4 to form heteromeric complexes, which will transfer into nucleus to modulate gene expression.

Babitt et al demonstrated that HJV, a member of the bone morphogenetic protein (BMP) co-receptor family, also uses the BMP signaling pathway to up-regulate hepcidin expression (Babitt et al., 2006). As investigated in mice, it is proposed that

HJV acts as a co-receptor that binds to BMP ligands (for example BMP2) and BMP type 1 or BMP type 2 receptors on the cell surface. This complex (HJV–BMP2–BMP-receptor) then induces an intracellular BMP signaling cascade. This in turn activates the Smad4 signaling pathway to directly increase hepcidin gene expression (Babitt et al., 2006). Indeed, mice whose hepatocytes are deficient in Smad4 have reduced hepcidin expression and showed a phenotype of iron overload (Wang et al., 2005a). However, this pathway is not used by HFE or TfR2 (Truksa et al., 2006) and the mechanism of the action of these upstream regulators is unclear.

HJV is typically membrane-bound by a glycosylphosphatidylinositol (GPI) anchor, although a soluble (sHJV) form of it does exist (Lin et al., 2005). It is hypothesized that sHJV competes with the membrane-bound HJV for BMP receptor binding on the cell surface, resulting in sHJV inhibiting the signaling cascade that induces hepcidin expression (Lin et al., 2005). These studies were performed in vitro on primary human hepatocytes using recombinant sHJV at levels similar to those present physiologically in human sera (Lin et al., 2005). These preliminary findings require further investigation, as understanding how these molecules modulate each other might provide important insights into many iron-overload diseases.

In a recent study, it was demonstrated that iron accumulation in HEK 293 cells is dependent on HJV–neogenin interaction (Zhang et al., 2005). Neogenin, a transmembrane protein expressed by different tissues, functions as a receptor for a variety of ligands implicated in the regulation of neuronal survival and guidance of axon growth during development (Matsunaga et al., 2004; Rajagopalan et al., 2004), determination of cell polarity, or direction of migration in both neuroectodermal and mesodermal cells (Mawdsley et al., 2004). It was found that the expression pattern of HJV overlaps with that of neogenin in muscle and liver (Niederkofler et al., 2004). Zhang et al. proposed a model in which the HJV–neogenin interaction in the muscle cells probably triggers the activation of a signal pathway that regulates iron levels for incorporation into the heme of myoglobin, and in hepatocytes the HJV–neogenin complex up-regulates hepcidin mRNA through an unidentified signaling pathway.

1.3.2.2 Molecules Involved in Inflammation Regulation of Hepcidin Expression

Although functional HFE is required to maintain basal hepatic hepcidin mRNA levels, the weight of evidence indicates that HFE is not needed for hepcidin up-regulation in response to inflammation. Lee and colleagues saw increased

hepcidin expression in response to lipopolysaccharide (LPS)-induced inflammation in both *Hfe* knockout and wild-type mice. Interleukin-6 (IL-6) upregulated hepcidin in hepatocytes cultured from either *Hfe* knockout or wild-type mice. Frazer and colleagues reported similar findings and proposed that hepatic hepcidin expression is stimulated by a HFE-dependent mechanism in response to iron but may be overcome in acute phase inflammatory responses, possibly through a pathway involving Toll-like receptor 4 (TLR-4), which is important in the innate immune response. Hepcidin is up-regulated in the iron-deprived wild-type mice injected with LPS but not in the TLR-4–deficient mice. This is consistent with a TLR-4–dependent inflammatory pathway that overrides the iron-sensing pathway (Constante et al., 2006).

In the liver, TLR-4 is involved in the Kupffer cell activation. It may also be required for hepatic ischemia/reperfusion injury and hepatic injury following hemorrhagic shock, and may contribute to alcoholic liver disease. Regulation of hepatocyte hepcidin expression through the iron-sensing pathway appears independent of either Kupffer or peripheral myeloid cells (Lou et al., 2005; Montosi et al., 2005; Peyssonnaux et al., 2006). However hepcidin regulation through the inflammatory pathway in response to bacterial pathogens could involve paracrine effects through

Kupffer cells or might primarily act through TLR-4 expressed on peripheral myeloid cells (macrophages, neutrophils)(Peyssonnaux et al., 2006).

Signaling through TLR-4 activates nuclear factor κB (NF-κB), leading to IL-6 production, which induces the expression of the signal transducer and activator of transcription 3 (STAT3) molecule that in turn binds the hepcidin promoter (Verga Falzacappa et al., 2006). Hepcidin is down-regulated in alcoholic liver injury, suggesting that it may also be regulated through TLR-2 or TLR-3, which down-regulates signaling through TLR-4 to reduce hepatic injury in response to LPS (Jiang et al., 2005). Although most hepcidin studies used LPS, which is a model for Gram-negative bacterial infection, other stimuli trigger different Toll pathways.

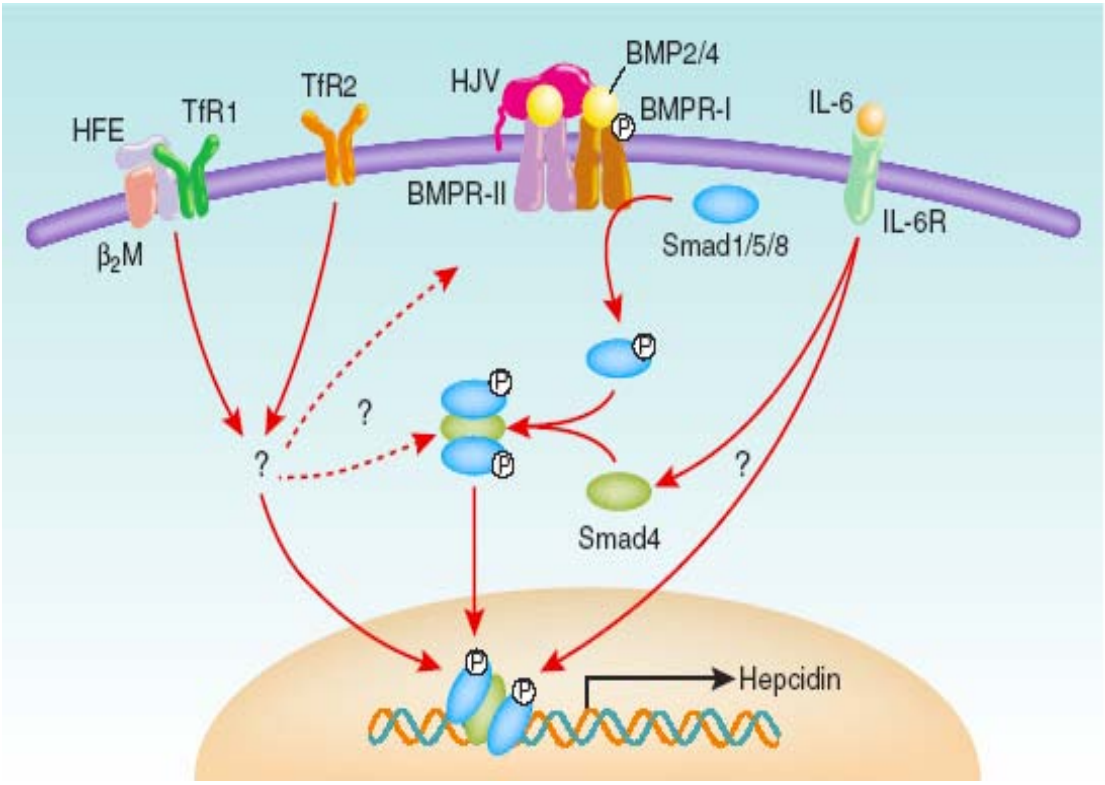


Figure 1-4. Hemojuvelin, a glycosylphosphatidylinositol (GPI)-linked membrane protein, acts as a BMP coreceptor and modulates hepcidin expression by stimulating BMP2 and BMP4 signaling. Upon binding by BMPs, BMP receptor type II phosphorylates type I receptors, and this complex, in turn, phosphorylates the receptor Smads 1, 5 and 8. These form heteromeric complexes with Smad4, and this complex translocates to the nucleus and alters the expression of target genes. Hepcidin can also be regulated by HFE (which forms a complex with TfR1 and β_2-microglobulin (β_2M)) and TfR2, but how these proteins exert their effects and whether HJV and/or the BMP signaling pathway are involved remains to be resolved. Inflammatory cytokines such as IL-6 can also stimulate hepcidin expression, and recent data suggest that Smad4 is involved in regulating this response (Anderson and Frazer, 2006).

1.4 BIOLOGICAL FUNCTIONS OF HEPCIDIN

Hepcidin is a β-defensin-like peptide hormone that functions both as a homeostatic regulator of iron metabolism and as a mediator of host defense and inflammation.

1.4.1 Hepcidin Antimicrobial Activity

During a comprehensive search for bioactive peptides, Park and Krause isolated a new antimicrobial peptide, hepcdin, from urine and blood. Like other antimicrobial peptides, hepcidin is rich in cystines and appears to be engaged in four intramolecular disulfide bonds. It displays spatial separation of its positively charged hydrophilic side chains from the hydrophobic ones, a characteristic of peptides that disrupts bacterial membranes (Ganz, 2003).

In vitro, human hepcidin exhibited antifungal activity against *Candida albicans, Aspergillus fumigatus,* and *Aspergillus niger* and antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis,* and group B *Streptococcus* at 10–30 mM concentrations (Park et al., 2001). As is the case with many other cationic peptides, the antimicrobial activity is favored by low ionic

strength media. Urinary hepcidin concentrations are typically in the 3–30 nM range (10–100 ng/ml) and can be at least tenfold higher during infections. It is thus unlikely that hepcidin can exert antimicrobial activity in urine.

1.4.2 Hepcidin Iron-Regulatory Activity

Of all the known micronutrients, iron has the longest and best described history. Iron is the second most abundant metal and the fourth most abundant element in the Earth's crust. Iron is involved in many metabolic processes, including storage and transport of oxygen, electron transport and oxidation-reduction reactions, as well as DNA of cells and organisms. So it is an essential element for nearly all living organisms (Aisen et al., 2001), particularly those in oxygen-rich environments. It has been suggested that reactions catalyzed by iron might have constituted a first step in the origin of life. All living cells possess an absolute requirement for iron.

1.4.2.1 Cell Iron Metabolism

The mechanisms of cellular iron homeostasis involved in cellular iron uptake, storage, transport and the regulation of intracellular iron balance are very important

because they are the basis of body iron metabolism.

1.4.2.1.1 Iron Uptake

Before being incorporated into the cellular heme and non-heme iron-containing proteins to exert its roles, iron must be carried across the cellular membranes firstly. There are two iron transport mechanisms involved: Tf-TfR dependent iron transport and Tf-TfR independent iron transport.

Tf and TfR Dependent Iron Uptake

Most cells obtain iron from transferrin (Tf) by receptor-mediated endocytosis. Tf-Fe uptake or Tf and transferrin receptor (TfR) mediated-endocytosis is considered to be the main route for cellular iron accumulation. Based on current information, this process can be divided into seven main steps (Li and Qian, 2002; Qian and Shen, 2001; Qian and Tang, 1995): 1) Binding: Tf-Fe binds to the extracellular portion of TfR on the cellular membrane, which is a simple physiological process independent of cell metabolism. 2) Internalization or endocytosis: Fe-Tf-TfR complexes are clustered together and localized in clathrin-coated pits, which eventually bud off to

form coated vesicles called endosomes or receptorsomes. 3) Acidification: intravesicular pH is lowered to about 5-6 by the activity of H^+ ATPase on the membrane of the endosomes. 4) Dissociation and reduction: intravesicular acidification induces the release of iron (Fe^{3+}) from Tf and its reduction to Fe^{2+} within the endosomes. 5) Translocation: iron (Fe^{2+}) is transported through the membrane of the endosomes to the cytoplasm. DMT1 has been recently proposed to be involved in this process, but the precise mechanism remains unclear. 6) Cytosolic transfer of iron into intracellular compounds: after translocation from endosome to cytoplasm, ATP or AMP transfers iron to the sites where it is needed for physiological activity, e.g. haem synthesis, electron transport in mitochondria etc., or to ferritin for storage. 7) Return of Tf-TfR complex to the plasma membrane: endosomes containing Tf-TfR return to the plasma membrane. The apotransferrin is replaced by new Tf-Fe molecules from extracellular fluid and the uptake process is repeated. Apotransferrin released form the receptor returns to the plasma or surrounding solution (Qian et al., 2002; Qian and Tang, 1995).

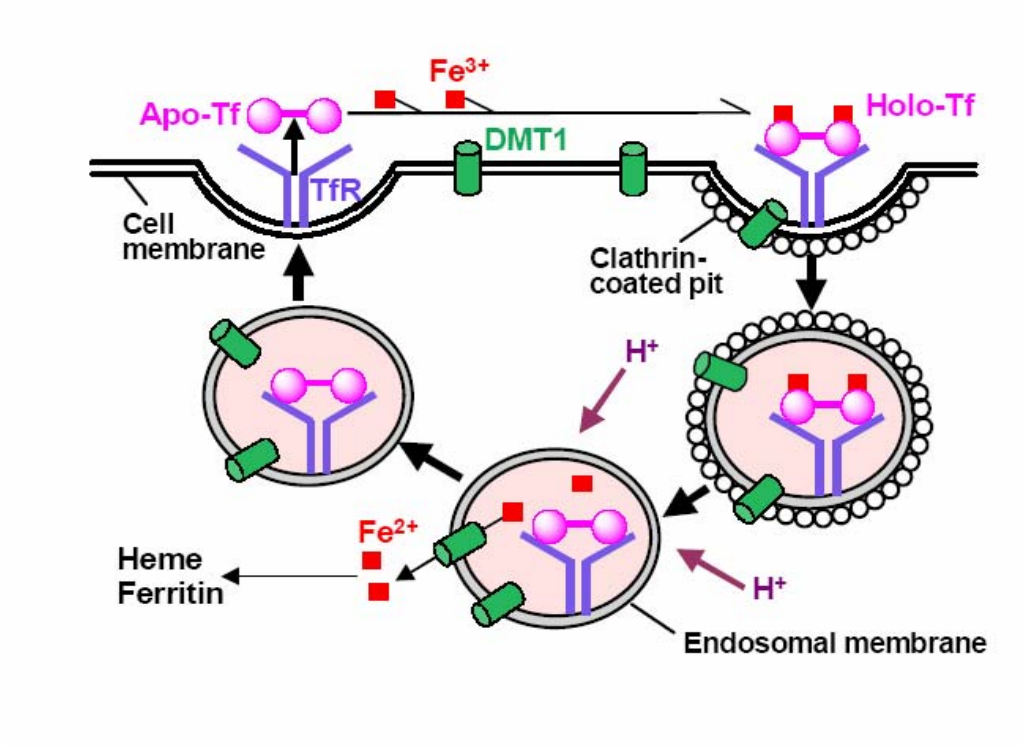


Figure 1-5. The pathway of cellular iron uptake from Tf via TfR mediated endocytosis (Qian and Tang, 1995).

Tf and TfR Independent Iron Uptake

Although Tf-dependent iron uptake probably predominates under normal circumstances, several lines of evidence indicate that there are distinct transport pathways for non-Tf bound iron. First, mice and humans lacking Tf develop massive iron overload in nonhematopoietic tissues such as the liver and pancreas (Trenor et al., 2000). Second, TfR1-deficient mice show normal embryonic organ development before they succumb to severe anemia in mid-gestation, suggesting a second iron uptake mechanism (Levy et al., 1999). Third, rapid clearance of plasma Fe is

observed in diseases (e.g., hereditary hemochromatosis) where the iron binding capacity of Tf is exceeded. Morgan and Qian's result show that reticulocytes can take up Fe^{2+} and this route is not associated with TfR but dependent on reduction of iron to the ferrous state and then across the membrane by an iron carrier (Qian and Morgan, 1992; Qian and Tang, 1995; Trinder et al., 1996).

Divalent metal transporter 1 (DMT1) is the first mammalian transmembrane iron transporter to be identified (Fleming et al., 1997; Gunshin et al., 1997). It is considered as a membrane iron carrier and mediates Tf and TfR independent ferrous iron transport through enterocytes (Fleming et al., 1998). Dietary iron in the lumen of the gut is predominantly in the ferric form, but it must first be reduced to the ferrous form before it can be utilized. Duodenal cytochrome b (Dcytb) is a strong candidate for this brush border ferrireductase (McKie et al., 2001). The membrane transporter DMT1 then facilitates the transport of ferrous iron across the brush border membrane. Little is known about the trafficking of iron through the cell, but it requires the intracellular protein hephaestin (Heph) and is likely to involve the ferroxidase activity of this molecule. Basolateral transfer to the body is thought to be mediated by the membrane iron exporter ferroportin 1 (FPN1) that is strongly expressed in the duodenum. Iron that enters the circulation is loaded onto Tf for transportation to the

body tissues. Molecules that have been strongly implicated in the regulation of iron absorption are the hemochromatosis protein HFE, TfR2 and the antimicrobial peptide hepcidin (Nicolas et al., 2001). They are most strongly expressed in the liver (Frazer et al., 2001; Kawabata et al., 1999; Pigeon et al., 2001).

There is another pathway, independent of TfR but not of Tf, available for iron uptake from Tf in the human melanoma cell or CHO cells lacking TfR but transfected with p97 (Jefferies et al., 1996; Kennard et al., 1995). This pathway may involve the transfer of iron from Tf or simple chelators to GPI-p97, and hence to the cell interior by an endocytic route that is less sensitive to the effect of weak bases but more accessible to hydrophilic chelators than the receptor-mediated pathway. The Tf independent iron uptake may be mediated by gelatinase-associated lipocalin (NGAL). Lipocalins are a large group of proteins that are related more in structure than in sequence and which bind small molecules. Lipocalins have been implicated in the transport of small organic molecules such as retinol, prostaglandins, fatty acids and oderants. NGAL binding iron is internalized into endosomes and, like Tf, is recycled. However, the detailed mechanism is not clear (Kaplan and Kushner, 2000). Besides, there maybe other routes which are involved in the Tf and/or TfR independent iron uptake.

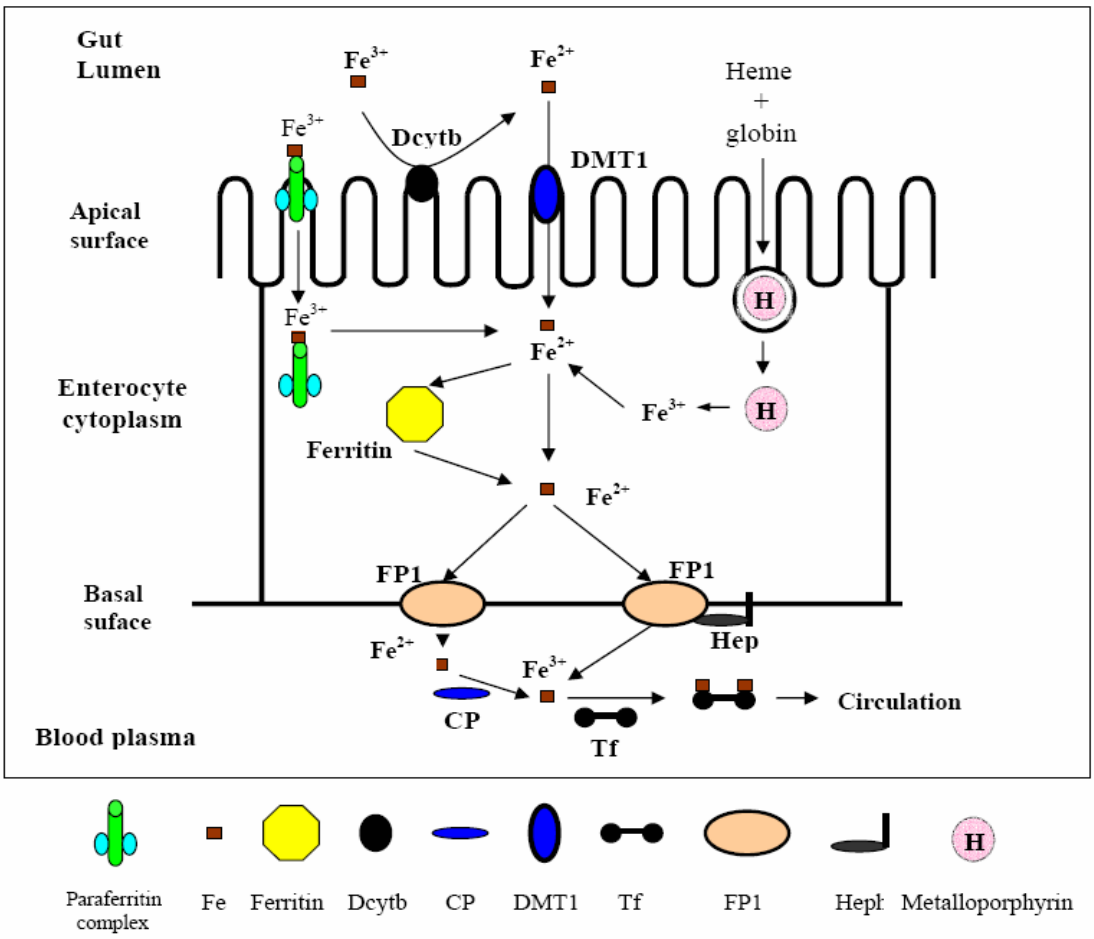


Figure 1-6. Pathways of heme and non-heme iron uptake and transport in the intestine. Fe^{3+} is reduced by Dcytb forming Fe^{2+}. Fe^{2+} in the intestinal lumen is transported across the apical surface by DMT1. Fe^{3+} and Fe^{2+} can also enter absorptive enterocytes via a pathway mediated by paraferritin complex, consisting of β-integrin, mobilferrin and flavin mono-oxygenase. Heme is processed in the gut lumen and enters the enterocytes as an intact metalloporphyrin (H). The mechanism of heme iron transport is not well defined, but is probably mediated by an endocytic pathway. Once inside the cytoplasm, heme is degraded by heme oxygenase to release inorganic iron. Once inside the cell, iron can either be stored in ferritin or transported

across the basolateral membrane by FPN1. The species of iron that is exported by FPN1 is not known, but the need for ferroxidase activity to load iron onto Tf indicates that it is probably Fe^{2+}. FPN1 and Heph may work together in the absorptive enterocyte. In other cell types, FPN1 may work with CP to load iron onto Tf. Abbrevs: DMT1, divalent metal transporter; CP, ceruloplasmin; Tf, transferrin; FP1 (FPN1), ferroportin 1; Heph, hephaestin; Dcytb, duodenal cytochrome b.

1.4.2.1.2 Iron Efflux

Intracellular iron balance is dependent not only upon the amount of iron taken up and sequestered but also upon the amount of iron released from cells. Duodenal enterocytes, macrophages, hepatocytes, placenta syncytiotrophoblasts and cells of the central nervous system (CNS) require mechanisms to release iron in a controlled fashion to ensure that the metal is available where it is needed. The only putative iron exporter identified to date is ferroportin 1 (also known as Ireg1, MTP) (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2001; McKie et al., 2000). Ferroportin 1 is located at the basolateral membrane of duodenal enterocytes, where it mediates iron export into the bloodstream, apparently in concert with hephaestin, a ferroxidase that is homologous to the abundant plasma protein ceruloplasmin (Vulpe

et al., 1999).

Iron export from nonintestinal cells requires ceruloplasmin (Cazzola et al., ; Harris et al., 1999). CP converts Fe^{2+} to Fe^{3+} that is loaded onto Tf for transport in the plasma. In the brain, the glycosylphosphatidylinositol (GPI)-anchored form of CP physically interacts with ferroportin1 to export iron from astrocytes (Jeong and David, 2003). Patients and mice with CP deficiency accumulate iron in macrophages, hepatocytes and cells of the CNS, resulting in iron-restricted erythropoiesis and neurodegeneration (Hentze et al., 2004).

1.4.2.2 System Iron Homeostasis

There are about 35 and 45 mg iron per kg body weight in adult women and men, respectively (Andrews, 1999a; Andrews, 1999b). More than half of the body's iron, about 60 – 70%, is found in hemoglobin in erythrocytes and their precursors. About 20–25 mg iron is required on a daily basis to support the hemoglobinization of new erythrocytes. Another 10% of essential body iron is present in the forms of myoglobins, cytochromes and iron-containing enzymes, amounting to no more than 4 – 8 mg of iron. In a healthy individual, the remaining 20 – 30% of surplus iron is

stored as ferritin and hemosiderins in hepatocytes and reticuloendothelial macrophages. Additionally, less than 1% iron (approximately 4 mg) binds to transferrin (Tf). This transport compartment, although small, is by far the most dynamic iron compartment in the body because it has the highest turnover with at least ten times daily.

The process of body iron mobilization is composed of five stages: absorption, transportation, utilization, storage and excretion. The duodenum and proximal jejunum are the principal sites for iron absorption. Iron handling by the mucosal (intestine) is divided into three distinct steps: uptake of iron into the mucosal cell from the lumen of the gut across the brush-border of the mucosal epithelial cell, transport of iron across the mucosal cell from the brush-border membrane to the other side of the cell, and its release from the mucosal cell to the transport protein of the plasma, Tf. Not all of the iron taken up from the lumen into the cells is transferred. A variable proportion can be sequestered within the mucosal cells and eventually be discarded into the gastrointestinal tract when the cells are exfoliated. This is principally determined by the body's requirements for iron (essentially the rate of erythropoiesis). It was suggested by Granich that the absorbed iron in excess of body requirements might be incorporated into mucosal cell ferritin where it would

function somehow as a ‘mucosal block’ against unnecessary assimilation of dietary iron. When iron passes into the plasma, it is oxidized to the ferric state and transported in combination with Tf to be stored, utilized or excreted. Although the amount of iron in plasma is small, which is about 0.05-0.18 mg/kg body weight, its turnover rate is rapid, averaging approximately 0.56 mg/kg body weight per day. Of this, a total of approximately 20-25 mg daily is used by the bone marrow for hemoglobin synthesis. A small amount is taken up by other cells of the body for the formation of myoglobin and cellular enzymes, while the remaining 10-15 mg iron is either transported for storage or excreted from the body.

When blood cells are destroyed, almost all the iron released from hemoglobin is re-used. Approximately 20-25 mg of iron released from the hemoglobin of red cells is re-used. Iron released from other cells that die is similarly conserved. A small but significant amount, therefore, is excreted. In men, the daily iron losses are about 1 mg (14 µg/kg). It is estimated that two-thirds of iron are lost from the gastrointestinal tract by the exfoliation of mucosal cells and the loss of red cells and one third by the exfoliation of cells from the skin and urinary tract. The human body lacks an effective means of iron excretion. This means that only very small amounts of dietary iron are absorbed. In view of the poor means of excretion available, the iron content

of the body is controlled almost entirely through the regulation of absorption. It is possible that a slight excessive mucosal iron absorption in man can already lead to parenchymal iron overload and cause hemochromatosis.

In addition, the human body also develops an efficient storage function and provides a strong backup when the body iron needs arise. It has been demonstrated that (1) the amount of iron stored in the tissues of a healthy adult is approximately 1 gm., (2) the liver and spleen are the chief storage sites, and (3) the stored iron is found intracellularly in a protein complex as ferritin and as hemosiderin. Both compounds are capable of being mobilized by the body for metabolism hemoglobin synthesis when the need arises.

Excessive tissue iron causes widespread organ damage. It is also possible that a rapid release of iron from macrophages could create local iron overload and cause localized tissue injury. The toxic effects of free iron are ascribed to its ability to catalyze the generation of reactive free radicals (Aisen et al., 2001). So it is important to tightly control the systemic iron homeostasis.

Systemic iron homeostasis, the control of iron balance throughout the body, requires

mechanisms for regulating iron entry into and mobilization from stores for meeting erythropoietic needs and for scavenging previously used iron. There is no efficient pathway for iron excretion; hence, intestinal absorption must be modulated to provide the appropriate amount of iron to keep stores replete and erythroid demands met. There must be effective communication between cells that consume iron (primarily erythroid precursors) and cells that acquire and store iron (duodenal enterocytes, hepatocytes, tissue macrophages). Transfer between tissues must be orchestrated to maintain homeostasis (Fig 1-7)(Hentze et al., 2004).

Since the sites of iron absorption, recycling, storage and utilization are distant from each other, it is reasonable to expect that iron-regulatory hormones must exist to account for the observed interactions between these compartments, and that inflammatory substances may also be involved in iron regulation. However, the molecular basis of these signals was elusive for many years, until a series of converging and often serendipitous discoveries of hepcidin provided the long-sought after opening (Ganz, 2003).

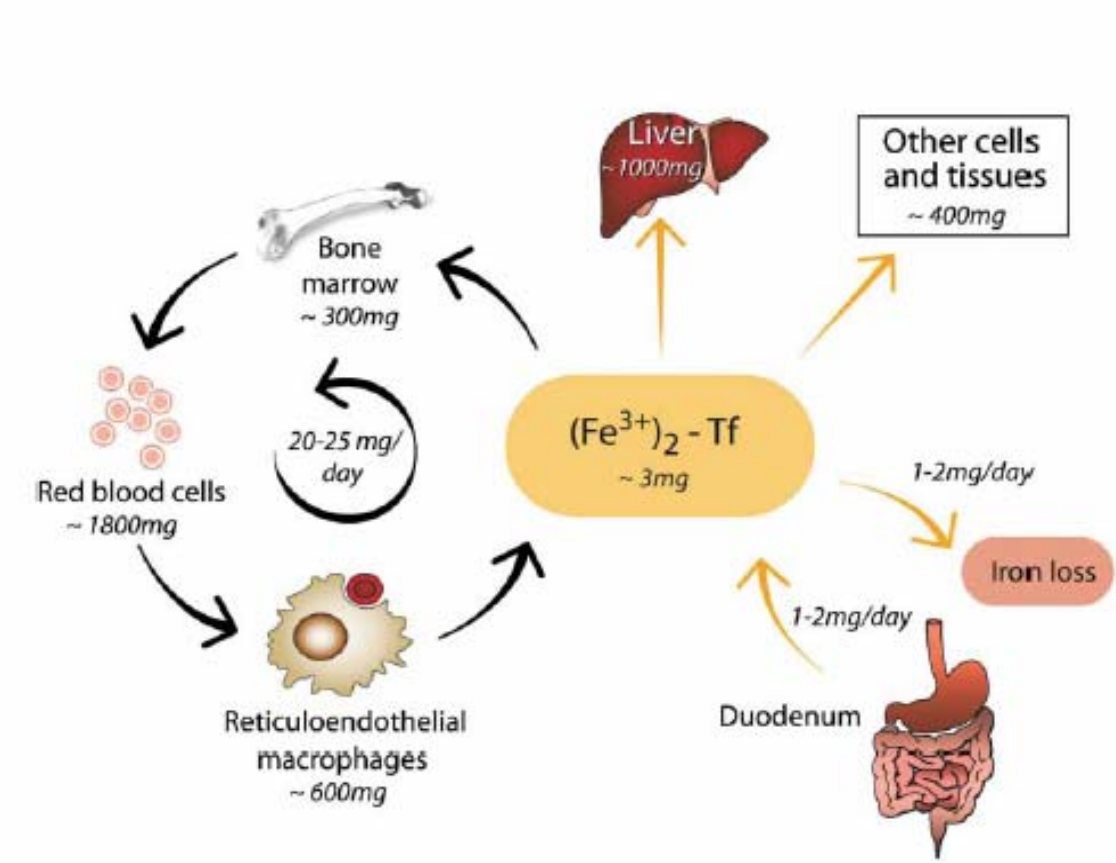


Figure 1-7. Systemic Iron Homeostasis

Major pathways of iron traffic between cells and tissues are depicted. Normal values for the iron content of different organs and tissues are stated, and the approximate daily fluxes of iron are also indicated. Note that these values are approximate and subject to significant person-to-person variations. Iron losses result from sloughing of skin and mucosal cells as well as blood loss. Importantly, there exists no regulated excretion pathway to control systemic iron homeostasis (Hentze et al., 2004).

1.4.2.3 Proteins Related to Iron Metabolism

1.4.2.3.1 Transferrin

The transferrin (Tf) protein is an 80 kDa glycoprotein which contains 679 amino acid residues and can bind 2 iron atoms (MacGillivray et al., 1998; Parkkinen et al., 2002). Tf is synthesized primarily in the liver and secreted into the serum (Morgan, 1983). It has been detected in various body fluids including plasma, bile, amniotic, cerebrospinal, lymph and breast milk (Qian et al., 2002). Plasma concentration of Tf is stable from birth, ranging from 2 g/L to 3 g/L, and the *in vivo* half-life of this protein is eight days (van Campenhout et al., 2003). Free iron can be toxic, promoting free radical formation via the Fenton and Haber–Weiss reactions, thus resulting in oxidative damage to tissues (van Campenhout et al., 2003). So all non-heme iron in the circulation is bound to Tf. Only about 30% of Tf binding sites are occupied, most of the protein is free of iron. Affinity of Tf for iron is pH dependent, in which iron is released from Tf as the pH is lowered below pH 6.5. In addition to iron, Tf might be involved in the transport of a number of metals, such as aluminum, manganese, copper and cadmium (Moos and Morgan, 2000). Neurons (Dwork et al., 1988; Mollgard et al., 1987) and certain types of astrocytes (Dwork et

al., 1988; Qian et al., 2000; Qian et al., 1999) also express Tf. Expression of Tf mRNA in rat oligodendrocytes is iron independent and changes with increasing age (Moos et al., 2001).

1.4.2.3.2 Transferrin Receptors (TfR)

Transferrin receptors (TfR) provide a controlled access of Tf to cells. Transferrin-bound iron (Tf-Fe) uptake or Tf and TfR mediated-endocytosis has been considered the main route for cellular iron accumulation (Qian et al., 2002). Two kinds of receptors have been described. The first and much more studied of these is now known as transferrin receptor 1 (TfR1), but before the discovery of transferrin receptor 2 (TfR2), it was simply designated the TfR. TfR2 is a newly described receptor, which is mainly expressed in the liver (Kawabata et al., 1999).

1.4.2.3.2.1 Molecular Characterization and Expression of TfR

TfR1 is a homodimer of disulfidebonded 760-residue subunits (Hu and Aisen, 1978; McClelland et al., 1984; Seligman et al., 1979). From the primary sequence, three regions can be identified within each subunit of the protein: a globular extracellular

portion (residues 90–760) where Tf binds, a hydrophobic intramembranous region (residues 62–89) and the remaining 61 residues lying within the cytoplasm. Human TfR1 bears one O-linked and three N-linked oligosaccharides. N-linked glycosylation is particularly important for proper folding and transport of the protein to the cell surface (Hayes et al., 1997). Palmitylation sites are also present within the intramembranous portion (Cys 62 and Cys 67) and presumably help anchor TfR1 within the cell membrane (Alvarez et al., 1990; Omary and Trowbridge, 1981) as well as assist endocytosis. An intracellular phosphorylation site (Ser 24) of uncertain function plays no role in endocytosis (Rothenberger et al., 1987).

The human TfR2 is predicted to be a type II transmembrane protein with an 80 amino-acid transmembrane domain, and an extracellular domain comprising residues 105-801. TfR2-α has a number of similarities to TfR1. These include the presence of the motif YQRV, a protease-associated domain (PA domain) and the presence of cell attachment sequences (RGD motifs). It is yet to be determined if the internalization signal is functional and whether the RGD sequences are the binding sites for Tf, as has been shown for TfR1 (Subramaniam et al., 2002).

Human TfR2 mRNA is highly expressed in liver and normal erythroid precursor

cells, erythroleukaemic cell lines (e.g. K562, OCI-M1) and bone marrow samples from several patients with erythroleukaemia and myeloid leukaemia. The affinity of TfR2 for transferrin was 25–30-fold less than that of TfR1 (Kawabata et al., 2000; West et al., 2000). The binding of diferric transferrin and apotransferrin to TfR2 were pH-dependent. Binding of apotransferrin was greater at a lower pH, while binding of diferric transferrin increased with pH (Kawabata et al., 2000). This is similar to TfR1 and is consistent with transferrin receptor mediated endocytosis. Also, like TfR1, the uptake of ^{55}Fe-transferrin increased with time, suggesting that TfR2 mediated the cellular uptake of transferrin-bound iron (Kawabata et al., 1999).

1.4.2.3.2.2 Regulation of TfR1 and TfR2 Gene Expression

Both transcriptional and post-transcriptional regulations play important roles in the control of TfR1 expression. Transcriptional regulation may play a more significant role in tissue- or stage-specific regulation. Earlier studies of nuclear run on and reporter gene assays demonstrated that at least a portion of the reciprocal regulation of the TfR1 mRNA levels by iron is mediated at the transcriptional level. Transcriptional regulation is also involved in serum/mitogenic stimulation of TfR1 expression, in the TfR1 induction during T and B lymphocyte activation, in the

enhanced TfR1 expression by SV40 viral infection, in the decrease of TfR1 during 54 terminal differentiation of myeloid and lymphoid leukemic cell lines, and also in the marked increase of TfR1 during erythroid differentiation (Ponka and Lok, 1999). A minimal region of about 100 bp upstream from the transcriptional start site was shown to drive both basal as well as serum/mitogenic stimulation of the promoter activity (Casey et al., 1988; Miskimins et al., 1986; Owen and Kuhn, 1987). This region contains putative regulatory elements similar to AP-1 and SP-1. Due to the five similar IRE motifs identified within the 2.7 kb 3'UTR of TfR mRNA, regulation is achieved via iron regulatory proteins (IRPs) and IREs. At low intracellular iron levels, both IRP1 and IRP2 bind to the stem loop IREs and protect the mRNA from degradation. At high intracellular iron concentrations, IRP1 binds iron, rendering it unable to bind mRNA. IRP2 is oxidized, ubiquitinated and degraded via proteasomes. The TfR1 mRNA with no IRP bound to it is rapidly degraded, resulting in low steady state levels of TfR1 mRNA. Consequently, less TfR1 is synthesized (Aisen et al., 2001).

The TfR2 transcript lacks IRE(s), so its expression is not sensitive to iron status (Fleming et al., 2000; Kawabata et al., 2000). TfR2 is normally expressed in the iron-overloaded liver of hemochromatosis (Fleming et al., 2000),

aceruloplasminemia (Yamamoto et al., 2002) and desferrioxamine (DFO) treated Chinese hamster ovary (CHO) cells (Tong et al., 2002). During embryonic development, TfR2 expression in the liver is up regulated. In contrast, TfR1 expression is the greatest in the embryonic liver and decreases to low levels in adults (Kawabata et al., 2001). So regulation of TfR2 may be via the cell cycle accommodating the needs of proliferating cells (Kawabata et al., 2000).

1.4.2.3.3 Divalent Metal Transporter 1

Divalent metal transporter 1 (DMT1) has four names: natural resistance associated macrophage protein 2 (Nramp2) (Vidal et al., 1995), Divalent cation transporter 1 (DCT1) (Gunshin et al., 1997), DMT1 (Andrews, 1999b) and solute carrier family 11, member 2 (SLC11A2) (http://www3.ncbi.nlm.nih.gov/omim). In this thesis we will use the DMT1.

In recent years, the study of DMT1 has advanced rapidly. There are at least two different splice forms, encoding alternative carboxy-termini and alternative 3'-UTR (Fleming et al., 1998; Lee et al., 1998). One form contains an IRE in its 3' untranslated region (3'-UTR), which is similar to IREs found in the 3' untranslated

region of the TfR1 mRNA. This suggests that DMT1 protein expression may be controlled post-translationally by intracellular iron concentration. Another form does not contain any recognizable IRE. In other words, the two mRNA isoforms differ in the 3' UTR: DMT1+IRE has an IRE (Iron Responsive Element) but DMT1-IRE lacks this feature. DMT1+IRE appears to serve as the apical iron transporters in the lumen of the gut; DMT1-IRE mainly facilitates the exit of iron from endosomes.

1.4.2.3.3.1 Characteristic of DMT1 Gene and Protein

The DMT1 gene is on the 12q13 of human chromosome (Vidal et al., 1995) and mouse chromosome 15 (Andrews and Levy, 1998). The human DMT1 consists of 17 exons spreading over more than 36 kb (Lee et al., 1998). The DMT1 genes of murine and rat have a similar structure. In detailed studies, Tchernitchko et al (Tchernitchko et al., 2002) characterized DMT1 mRNA isoforms containing the alternative 3' exons: one contained an iron responsive element (IRE) in the 3' untranslated region (+IRE), while the second lacked the IRE (-IRE), and the C-terminal 18 amino acids were replaced by a novel 25 amino acids segment. The IRE isoform was produced by the alternative splicing at a site within exon 16.

The DMT1 protein is highly hydrophobic, with 12 predicted transmembrane domains. Both amino- and carboxy-termini are predicted to be within the cytoplasm. There are four isoforms of rat DMT1. In the C-terminal there are two isoforms variants: The –IRE's, which is associated with the absence of an IRE in the 3'UTR of the mRNA and encodes a 561-amino-acid protein (Gunshin et al., 1997), and the +IRE mRNA, which is associated with its presence and encodes a 568-amino-acid protein (Fleming et al., 1998; Gunshin et al., 1997; Lee et al., 1998). More recently, two N-terminal variants were identified: One originates with a MV sequence as originally reported (Fleming et al., 1998; Gunshin et al., 1997) and one starts with 31 amino acid residues proximal to that sequence (Hubert and Hentze, 2002). The longer peptide species has a potential nuclear localization signal (NLS) motif within it. The N-terminal isoforms are due to an alternative exon to exon 1; investigators have dubbed the new sequence 1A (the old becoming an untranslated 1B and the next exon remaining as 2), so exon 1A predicts 29 additional residues for human DMT1 and 31 for rat DMT1. Antisera have been raised that recognize a ~90-116 kDa DMT1 protein which can be deglycosylated to a ~50-55 kDa protein (Gruenheid et al., 1999; Tabuchi et al., 2000). The latter mass is closer to the predicted molecular weight based on the amino acid composition of DMT1. However, other groups have generated specific antisera that recognize a ~65-66 kDa species; these antibodies

appear to block iron transport activity, suggesting that this antigen is a true transporter protein (Conrad et al., 2000; Roth et al., 2000; Tandy et al., 2000). It is possible that cell-type specific glycosylation patterns may account for such differences in the mass profile.

1.4.2.3.3.2 Expression and Regulation of DMT1 Gene

Northern-blot analysis revealed that DMT1 is prominently expressed in proximal intestine, followed by kidney, thymus and brain, and is faintly present in the testis, liver, colon, heart, spleen, skeletal muscle, lung, bone marrow, stomach and all tissues examined. In the kidney and thymus, two strong bands at ~ 3.5 kb and ~4.5 kb were detected, indicating that two isoforms mRNA exist in those tissues (Gunshin et al., 1997). In the brain, DMT1 mRNA is found in neurons, glial and ependymal cells (Burdo et al., 2001; Gunshin et al., 1997). A qualitative examination of sagittal sections indicates that most neurons express DMT1 mRNA at low levels. More prominent labeling is present in densely packed cell groups, such as the hippocampal pyramidal and granule cells, cerebellar granule cells, the preoptic nucleus and pyramidal cells of the piriform cortex, and in moderate amounts in the substantia nigra (Burdo et al., 2001; Wang et al., 2002).

Each DMT1 isoform exhibits a differential cell type-specific expression pattern and distinct subcellular localizations. Epithelial cell lines predominantly express DMT1A, whereas the blood cell lines express DMT1B. In HEp-2 cells, GFP-tagged DMT1A is localized in late endosomes and lysosomes, whereas GFP-tagged DMT1B is localized in early endosomes. In polarized MDCK cells, GFP-tagged DMT1A and DMT1B are localized in the apical plasma membrane and their respective specific endosomes. Disruption of the N-glycosylation sites in each of the DMT1 isoforms affects their polarized distribution into the apical plasma membrane but not their correct endosomal localization. These data indicate that the cell type-specific expression patterns and the distinct subcellular localizations of the two DMT1 isoforms may be involved in the different iron acquisition steps from the subcellular membranes in various cell types (Tabuchi et al., 2002).

DMT1 gene expression is regulated in response to iron status. Rats that were made iron deficient had markedly increased DMT1 mRNA in the intestinal epithelial cells and cell lines, and iron sufficient rats had markedly decreased levels (Frazer and Anderson, 2003; Gunshin et al., 1997; Han et al., 1999; Martini et al., 2002). The basis of this regulation has not yet been fully defined. The putative promoter of the human DMT1 gene contains several potential metal response elements, suggesting

that there may be transcriptional regulation in response to the metal levels (Lee et al., 1998). It is likely that there is also post-transcriptional regulation (Gunshin et al., 2001; Ke et al., 2003). The IRE sequence found in the 3' untranslated region of one of the two alternative splice forms of DMT1 mRNA binds iron regulatory protein *in vitro* and appears to respond to cellular iron levels. Under low iron conditions, IRPs bind to the IREs in the 3'UTR of DMT1 mRNA and protect the mRNA from degradation. Conversely, they lower mRNA stability when iron is abundant. It has been shown that IRP can bind to the 3'UTR stem-loop in DMT1+IRE mRNA *in vitro* (Guerrini et al., 1998; Wardrop and Richardson, 1999). Therefore, DMT1 expression is likely to be modulated by IRP-IRE at the post-transcriptional level. Other mechanisms may be involved in the regulation of DMT1 gene expression. Studies of the alternative 5' exon forms of DMT1 demonstrated that both the 1A/IRE+ and 1A/IRE - isoforms are sensitive to iron regulation in the duodenum, but not in the kidney (Hubert and Hentze, 2002). Thus both the 5' and 3' ends of the mRNA may hold important clues for iron response in different tissues. It has been demonstrated that the 5' regulatory region of human DMT1 contains a single interferon (IFN)-γ regulatory element, three potential SP1 binding sites, two potential Hif-1 binding sites, and five potential metal response elements, all of which may play some roles in the regulation of this molecule (Lee et al., 1998). The role of cytokines

in DMT1 regulation is largely unstudied except in human monocytic cell lines (Ludwiczek et al., 2003). In the THP-1 human monocytic cell line, DMT1 mRNA levels increased in a dose-dependent way in response to increasing concentrations of interferon-γ, lipopolysaccharide or TNF–α. Wardrop and Richardson showed that there was a seven-fold increase in the expression of the 2.3 kb DMT1 mRNA transcript when compared with the control, but little effect on the DMT1 3.1 kb transcript in RAW264.7 macrophage cell line after incubation with LPS/IFN-γ. They suggested that DMT1 mRNA expression can be influenced by factors other than iron levels in macrophages (Wardrop et al., 2002). However, the regulation of DMT1 gene expression is still not yet fully understood.

1.4.2.3.3.3 Functions of DMT1

Microcytic anemia (mk) mice and Belgrade (b) rats have naturally occurring mutations that result in identical missense substitutions in their DMT1 genes (Simovich et al., 2003; Trenor et al., 2000). Both animals have defects in the transport of iron from the gut lumen into the absorptive enterocyte and from plasma transferrin into erythroid precursors. This indicates that DMT1 is important for both of these iron transport steps. When expressed in Xenopus lavis oocytes, rat DMT1

exhibited moderately high apparent affinity for Fe^{2+}, Zn^{2+} and several other transition metal ions (Gunshin et al., 1997). DMT1-mediated Fe^{2+} transport was shown to be pH-dependent and coupled to proton symport. In duodenal iron uptake, Fe^{3+} made soluble by gastric acid is reduced to Fe^{2+} presumably by Dcytb (McKie et al., 2001) or a similar reductase on the apical surface and enters the brush border via DMT1. Because DMT1 may act as a proton symporter, one assumes that Fe^{2+} uptake is facilitated by the mildly acidic pH expected in the proximal duodenum. DMT1 is found on the apical surface of the enterocyte (Canonne-Hergaux et al., 1999; Trinder et al., 2000). This location is consistent with the finding (Knopfel et al., 2000) that divalent cation transport activity is associated with the brush-border membrane vesicles. Like this form of TfR-independent iron transport in the brush border, it is possible that the TfR-independent iron transport via DMT1 is present in other tissues.

The second role of DMT1 is mediating iron transport across the endosome. Tf-TfR mediated iron uptake is a main route in the cell (Qian and Wang, 1998). Iron is taken up in most cells by Tf mediated endocytosis. In this process, Fe is released from protein by a decrease in the intravesicular pH (Richardson and Ponka, 1997). Once Fe is released from Tf, it must cross the endosomal membrane, probably via a membrane-bound transporter that is recruited from the cell surface. Recent studies

have shown that the molecule involved in Fe transport from endosome is DMT1. Fleming et al studied erythroid precursor cells of anemic Begrade rat with mutation in DMT1. They found that these cells could take up iron into an endosomal compartment by receptor-mediated endocytosis of diferric Tf bound to TfR, but they were subsequently unable to export iron from the endosome into the cytoplasm. As a result, endosomal iron was returned to the cell surface and released and very little was retained for hemoglobin synthesis (Fleming et al., 1998). DMT1 can be expressed on the endosomal membrane and co-localizes with Tf to export iron from the endosome into the cytoplasm of the cell (Su et al., 1998; Tabuchi et al., 2000). In their study, DMT1 showed clear colocalization with FITC-Tf both at the plasma membrane and in recycling endosomes. The evidence strongly supports the role of DMT1 in transporting Fe^{2+} into the cytoplasm after acidification of the Tf-positive endosome.

1.4.2.3.4 Ceruloplasmin

Ceruloplasmin (CP, or the sky-blue protein) is a major protein that circulates in the plasma and functions as a copper transporter that is able to couple and transport 90–95% of serum copper (Giurgea et al., 2005)**.** As a copper-containing ferroxidase,

it is essential for normal iron homeostasis. Loss-of-function mutations in the CP gene will induce hereditary aceruloplasminemia. CP is expressed by astrocytes in the brain, cerebellum, retina and by the epithelial cells of the choroids plexus.

1.4.2.3.4.1 Characteristic of Ceruloplasmin Gene and Protein

The gene encoding human CP has been mapped on chromosome 3q23-q24 (Daimon et al., 1995) and in rats on chromosome 2 (Miura et al., 1993). It contains 20 exons with total length of about 65 kb (Koschinsky et al., 1987; Koschinsky et al., 1986). The nucleotide of CP mRNA is about 3321 bp (NM-000096). Rat CP nucleotide is 3700 bp (NM-012532). The derived amino acid sequence of rat CP is 93% homologous to the corresponding human sequence and contains a 19-amino acid leader peptide plus 1040 amino acids of mature protein.

CP is an α-2 plasmatic glycoprotein with a molecular weight of ~132 kDa. This protein has a single polypeptide chain with 1046 amino acids and belongs to the blue multinuclear copper oxidases family that includes ascorbate oxidase and laccase. The molecule is comprised of six plastocyanin domains arranged in a triangular array.

There are two forms of CP. One is the secreted form (Klomp and Gitlin, 1996;

Loeffler et al., 1996) as mentioned above. The other is the glycosylphosphatidylinositol (GPI)-anchored form of CP (Fortna et al., 1999; Patel and David, 1997; Salzer et al., 1998). The mature GPI-CP protein has 1065 amino acids compared with 1040 amino acids for secreted ceruloplasmin. The C-terminal 5 amino acids of secreted CP are replaced with 30 alternative amino acids. The alternative stretch of 30 amino acids is predominantly non-polar, as it is for all known GPI-anchored proteins, with a large fraction of hydrophobic residues. In addition, this stretch of amino acids contains, proximally, a potential site for GPI anchor addition. GPI anchors are added to a small amino acid (denoted the ω amino acid) upstream of the non-polar C-terminal signal peptide, with concomitant cleavage of the signal peptide (Udenfriend and Kodukula, 1995). Thus, it appears that this alternatively spliced CP with its alternative C-terminal tail satisfies all the known requirements for GPI anchor addition (Patel et al., 2000).

1.4.2.3.4.2 Expression and Regulation of Ceruloplasmin Gene

CP is synthesized in a variety of organs such as the testes, placenta, yolk sac (Aldred et al., 1987), heart (Linder and Moor, 1977), uterus, lung (Fleming and Gitlin, 1990), lymphocytes (Pan et al., 1996), mammary gland (Jaeger et al., 1991) and synovium

(Dixon et al., 1988). But the main synthesis tissue is the liver. The hepatocytes synthesize and secrete CP (secreted form) into the circulation and the concentration is about 350µg/ml serum (Lamb and Leake, 1994). Factors reported to cause changes in the synthesis of CP are copper, hormones and leucocytic endogenous mediator (Weiner and Cousins, 1983). In peripheral the factors of inflammation (Fleming et al., 1991; Gitlin, 1988), interferon, IFN-γ (Mazumder and Fox, 1999; Sampath et al., 2003), endotoxin (Yang et al., 1996) and ovarian cancers (Hough et al., 2001) can lead to increased CP gene expression. This supports ceruloplasmin's critical role in host defense against oxidative damage, infection and injury (Yang et al., 1996). IL-1beta can also increase CP mRNA expression and induce two CP peaks at 8 and 20 h, while IL-6 has little effect in HepG2 cells (Daffada and Young, 1999). Transcript-selective translational silencing by gamma interferon is directed by a novel structural element in the CP mRNA 3' untranslated region (Sampath et al., 2003). Serum CP activity was also significantly elevated by the early neonatal administration of T4. Furthermore, gestational hypothyroidism resulted in a significant decrease in CP activity after postnatal Day 3. These data suggest a role for thyroid hormone and possibly glucocorticoids in the normal developmental regulation of CP (Fitch et al., 1999). Thomas et al. showed that the CP RNA level is closely related to the estrous cycle; there is the maximum expression of CP at estrous

(Thomas et al., 1995). Insulin was also observed to function as a bi-directional, condition-dependent regulator of hepatic cell CP expression (Seshadri et al., 2002).

A number of factors can induce changes in CP gene expression. However, little is known about the cellular and molecular mechanism(s) involved. Mukhopadhyay et al. reported that iron chelators increase CP mRNA expression and protein synthesis in human hepatocarcinoma HepG2 cells. Furthermore, the increase in CP mRNA is due to the increased rate of transcription. They cloned the 5'-flanking region of the CP gene from human genomic library. A 4774-base pair segment of the CP promoter/ enhancer driving a luciferase reporter was transfected into HepG2 or Hep3B cells. Iron deficiency or hypoxia increased luciferase activity by 5 to10-fold compared with the untreated cells. Examination of the sequence showed three pairs of consensus hypoxia-responsive elements (HREs). Deletion and mutation analysis showed that a single HRE was necessary and sufficient for the gene activation. The involvement of hypoxia-inducible factor-1 (HIF-1) was shown by gel-shift and supershift experiments that showed HIF-1alpha and HIF-1beta binding to a radiolabeled oligonucleotide containing the CP promoter HRE. These results are consistent with *in vivo* findings that iron deficiency increases plasma CP and provides a molecular mechanism that may help understand these observations (Mukhopadhyay et al., 1998;

Mukhopadhyay et al., 2000).

1.4.2.3.4.3 Biological Function of Ceruloplasmin

There are at least four main functions attributed to CP, which include copper transport, iron metabolism, antioxidant defense and involvement in angiogenesis and coagulation. It has been shown that CP catalyzes the oxidation of Fe^{2+} to Fe^{3+}. Ryan et al stated that iron is the best substrate for CP (Ryan et al., 1992). As a substrate, Fe^{2+} has the lowest apparent Km and the highest Vmax of any of CP's multiple substrates. It catalyzes the oxidation of 4 atoms of Fe^{2+} with the concomitant production of water from molecular oxygen. This prevents the free radical formation that occurs during spontaneous ferrous oxidation (de Silva and Aust, 1992).

$$4Fe^{2+} + O_2 + 4H^+ \xrightarrow{CP} 4Fe^{3+} + 2H_2O$$

This ferroxidase activity is considered pivotal in mobilizing iron for transport via the protein transferrin (Saenko et al., 1994). When Tf is present, it binds the ferric product and thus protects it from subsequent reduction. The presence of both CP and Tf in the plasma normally provides considerable antioxidant protection by preventing iron-induced free radical formation (Logan, 1996; Saenko et al., 1994). This activity as ferroxidase is increased during inflammation, infections and other conditions.

These observations seem to suggest that there is a possibility that CP acts both as an antioxidant and an acute-phase reactant (Kang et al., 2001).

Another role of CP may be to facilitate the loading of iron into ferritin. Investigators have proposed that the mechanism by which iron is placed into ferritin is catalyzed by CP and found that ferritin plus CP is an effective 'antioxidant'. Human cells store iron in the ferric state in ferritin and haemosiderin. When the body needs iron, the ferric iron is reduced and moved to the outside of the cells. Because spontaneous ferrous oxidation cannot provide a sufficiently large supply of ferric iron for the binding to Tf and the subsequent distribution to the body, CP-catalyzed oxidation is still required (Logan, 1996).

Thus, by catalyzing the oxidation of Fe^{2+}, CP promotes the incorporation of iron into Tf and into ferritin. Its ferroxidase activity also eliminates free iron from the plasma, thereby protecting blood and membrane lipids from peroxidative damage. This is supported by its ability to protect phospholipids in vascular walls and erythrocyte membranes from peroxidation as well as DNA from scission. Thus, it acts as an antioxidant defense (Saenko et al., 1994). Moreover, it can partially protect rat heart from myocardial injury induced by oxygen free radicals (Chahine et al., 1991). Thus,

the ‘free iron’ concentrations in the plasma are related to the copper-binding properties and ferroxidase activities of CP. Because copper atoms in CP have no access to O_2 through diffusion, CP must protect cells from this progenitor of free radical reactions through other mechanisms. As mentioned above, CP is capable of increasing the rate of incorporation of iron into Tf and ferritin, effectively eliminating unbound iron in plasma as soon as it forms. This putative and protective role of the ferroxidase activity in the antioxidant action of CP that extends from membrane lipids to DNA has been confirmed by a number of laboratories (Saenko et al., 1994).

1.4.2.3.5 Hephaestin

Hephaestin was identified as a key component of the intestinal iron transport by a study of the sex-linked anemia (*sla*) mouse (Vulpe et al., 1999). The locus responsible for the *sla* phenotype was mapped to the central region of mouse chromosome X. Much later, Vulpe and his coworkers narrowed down the chromosomal region to 2 cM, identified the *sla* candidate gene and named it hephaestin (*Heph*, Hp) after the Greek metalworking god, Hephaestus (Vulpe et al., 1999).

1.4.2.3.5.1 Characteristic of Hephaestin Gene and Protein

The human hephaestin gene maps on 14.55 cR of DXS1194 in Xq11-q12 (lod score = 7.81), a region with homology by synteny to the *sla* region, and comprises 20 exons spanning approximately 100 Kb (Vulpe et al., 1999). The complete human *Heph* cDNA encodes a polypeptide of 1185 amino acids with a putative N-terminal signal peptide. The predicted molecular mass of the hHeph protein with the signal peptide is 130.4 kDa and without the putative signal peptide 127.8 kDa. However, these calculations do not take glycosylation into account. Anderson and co-workers, futher using a polyclonal antibody raised against a peptide in the C-terminus of murine Heph, detected a major polypeptide species of a molecular mass 155 kDa and a minor protein of 135 kDa, in both wild-type mouse tissues and transfected COS-7 cells. The disparity in molecular mass would indicate post-translational modification, most probably in the form of glycosylation.

Hephaestin is highly homologous to CP (50% identity, 68% similarity) and significantly, all the residues involved in copper binding and disulfide bond formation in CP are conserved in Heph (Vulpe et al., 1999). In contrast to its soluble serum homologue, hephaestin contains additional 86 amino acids at the C-terminus.

This segment includes a single predicted transmembrane domain and a short cytosolic tail, suggesting that hephaestin is a membrane-bound protein with a large ceruloplasminlike ectodomain. Comparative structural modeling of the hephaestin ectodomain (representing more than 92% of its sequence) was based on the known crystal structure of ceruloplasmin and revealed several important facts. Both proteins share the same beta fold, and the key structural features critical for folding and function of ceruloplasmin are conserved in hephaestin. All copper-binding sites of ceruloplasmin are also conserved in the putative structure of hephaestin. All cysteine residues essential for the formation of disulphide bridges are also present. In addition, the putative iron-binding site with a negatively charged aspartaterich tract in its vicinity is also conserved in hephaestin (Syed et al., 2002). These observations indicate that hephaestin has a ferroxidase activity similar to ceruloplasmin. CP has a ferroxidase activity that is likely to facilitate iron export from the reticuloendothelial system and various parenchymal cells to the plasma (Harris et al., 1999; Lee et al., 1968), and it is very likely that Heph plays a similar role in intestinal enterocytes.

In addition, Heph from both the intestinal cell line and enterocytes is capable of oxidizing paraphenylenediamine (PPD), like CP in plasma. These data combined with the *sla* phenotype, suggest that Heph is a multicopper oxidase that plays a

central role in whole body iron homeostasis due to its involvement in intestinal iron export at the basolateral membrane of duodenal enterocytes (Chen et al., 2003).

1.4.2.3.5.2 Regulation of Hephaestin Gene Expression

Because the Heph mRNA does not possess any IRE structure, hephaestin expression is not regulated via iron responsive elements and IRE-binding proteins, and the transcription is thought to be important for the regulation of Heph gene expression. Known factors influencing expression levels of hephaestin in the intestine include iron and copper levels. Studies in rodents have shown that the expression of Heph mRNA is increased to a small degree under iron deficient conditions and decreased with iron loading (Frazer et al., 2001; Sakakibara and Aoyama, 2002). Similarly, a decreased expression of hephaestin was observed in the duodenum of iron-overloaded patients with hereditary hemochromatosis (Stuart et al., 2003) and an increased expression of mRNA and protein in patients with iron deficiency (Zoller et al., 2003). However, no iron-dependent regulation of the Heph gene expression was observed in hereditary hemochromatosis (HH) patients (Rolfs et al., 2002). Recent studies showed that Heph and FPN1 expression responds to systemic rather than local signals of iron status (Chen et al., 2003; Frazer and Anderson, 2003). The

change of Heph protein response to iron status is larger than mRNA, so the post-translational modification of Heph may play a significant role (Anderson et al., 2002a). Taken together, however, the current evidence suggests that Heph is not highly regulated in response to iron requirements.

Hephaestin expression also responds to the concentration of copper. Increased hephaestin mRNA expression was observed in copper repleted $CaCO_2$ cells (Han and Wessling-Resnick, 2002). Copper seems to be critical not only for hephaestin synthesis and ferroxidase function but also for hephaestin stability. In copper-depleted cells hephaestin protein does not mature into its stabile 160 kDa form, but is tagged by ubiquitin and quickly degraded in the proteasome (Nittis and Gitlin, 2004).

1.4.2.3.5.3 Biological Function of Hephaestin

Heph expression is consistent with its predicted role. Expression studies with northern blots prepared from adult mouse tissues revealed a high Heph expression in the small intestine and colon (Fleet et al., 2003; Linder et al., 2003; Thomas and Oates, 2002), and low levels of expression in other tissues such as the brain, spleen,

lung and placenta (Frazer et al., 2001; Vulpe et al., 1999). This expression contrasts with that of CP, which is highly expressed in the liver and is expressed to a lesser extent in other tissues including the brain and lung, but is not expressed in the intestine. The high expression of Heph in the duodenum is consistent with the primary role it plays in intestinal iron transport, and this is a feature it shares with other important intestinal iron transport molecules including DMT1, FPN1 and Dcytb (Abboud and Haile, 2000; Donovan et al., 2000; Frazer and Anderson, 2003; Frazer et al., 2001; Gunshin et al., 1997; McKie et al., 2001; McKie et al., 2000).

The subcellular distribution of Heph is intriguing. Immunohistochemical studies have shown that the protein is located at intracellular and supranuclear site, instead of on the plasma membrane (Frazer et al., 2001; Simovich et al., 2002). This raises the possibility that Heph is involved in the intracellular trafficking of absorbed iron before the movement of iron across the basolateral membrane by FPN1.

On the basis of the above findings, Heph presumably plays a crucial role in the release of iron from the basolateral membrane of the enterocyte. However, Heph has only a single transmembrane domain, so it is also to be able to transport iron itself. It has been suggested that FPN1 is a candidate basolateral iron transporter with which

Heph may associate. The FPN1 is expressed at high levels on the basolateral membranes of intestinal epithelium which is expected to be involved in iron efflux. Hephaestin could be in direct contact with a membrane bound iron exporter (possibly Ireg1) transporting ferrous iron through the membrane, and hephaestin may oxidize the ferrous iron to enable its subsequent binding by transferrin. This scenario is supported by the observation that the yeast orthologue of hephaestin Fet3p forms a functional complex with an iron permease Ftr1p (Stearman et al., 1996). In addition, it has been shown that the recently identified GPI-anchored ceruloplasmin physically associates with FPN1 transporter in astrocytes (Jeong and David, 2003; McKie et al., 2000). It will be important to determine whether hephaestin is engaged in similar complexes in enterocytes.

1.4.2.3.6 Ferroportin1

Ferroportin1 is a newly discovered molecule that may play a role in iron export. This protein was first named as iron-regulated transporter 1 (IREG1) by McKie et al (McKie et al., 2000), who were the first to identify and clone it. Subsequently, the same molecule was also named ferroportin1 (FPN1) (Donovan et al., 2000) and metal transport protein 1 (MTP1) (Abboud and Haile, 2000). In this thesis, this

molecule will be referred to as FPN1. The gene mutation developed a hypochromic anaemia (Donovan et al., 2000), which suggests that FPN1 is involved in iron export. In addition, FPN1 expression in humans and mice has been shown to occur in areas that are critical for iron absorption, i.e. duodenal enterocytes and macrophages (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). The following is the current state of knowledge regarding the role of FPN1 as a putative iron exporter.

1.4.2.3.6.1 Characteristic of Ferroportin1 Gene and Protein

The human FPN1 gene encompasses 20 kb of DNA, comprises 8 exons and maps to 2q32. Mouse FPN1 cDNA encodes a protein 570 amino acids in length with a predicted mass of 62 kDa (McKie et al., 2000). Sequence data showed that ferroportin1 has 12 putative transmembrane domains (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). This protein is extremely well conserved in humans, mice and rats, showing >90% homology. Further analysis of the FPN1 protein by McKie et al (McKie et al., 2000) showed a putative NADP/adenine-binding site and basolateral localization signal present at its C-terminus. The NADP/adenine-binding sequence of FPN1 comprises an IFVCGP

motif that is also found in yeast ferric reductase and the neutrophil oxidoreductase gp91-phox (Segal and Abo, 1993). This suggests that FPN1 may have reductase activity involved in iron transportation. Both Fe^{2+} and Fe^{3+} are known to exist in complex equilibrium within cells and the conversion between these redox states may be important in terms of transportation and sequestration (Richardson and Ponka, 1997; St Pierre et al., 1992). Indeed, the export of Fe^{3+} from cells may involve the conversion of Fe^{3+} to Fe^{2+} by the reductase region of FPN1 and this should be further investigated. The last four amino acids (TSVV) of FPN1 comprise a T/S-X-V/L PDZ target motif that is readily recognised by postsynaptic density-95 (PSD-95)/discs large (Dlg)/zona occludens-1 (ZO-1) (PDZ) proteins. PDZ proteins are thought to be involved in the basolateral localization of proteins containing this target motif. Sequence analysis of FPN1 mRNA revealed the presence of an IRE in its 5' untranslated end. The 5' IRE sequence of FPN1 is well conserved in humans, mice and zebrafish. Similar 5' IREs occur in transcripts encoding ferritin, erythroid-5-aminolevulinate synthase and mitochondrial aconitase (Richardson and Ponka, 1997).

1.4.2.3.6.2 Expression and Regulation of FPN1

FPN1 was originally isolated from duodenal mucosa where it is expression is high. In other regions of the intestine expression of the FPN1 mRNA varies from moderate in the colon to virtually undetectable in the jejunum and ileum (Frazer et al., 2001; McKie et al., 2000). Another site of high expression of FPN1 is the macrophage, particularly those residing in the red pulp of the spleen and the Kupffer cells of the liver. These cells play a key role in recycling iron from the haemoglobin of effete red blood cells. Recent genetic evidence has highlighted the critical role that FPN1 plays in this process. FPN1 mRNA is also highly expressed in placenta where the protein is involved in the transfer of iron between the maternal and foetal circulations. In the placenta, FPN1 is expressed in the basolateral, but not the apical, membrane of the syncytiotrophoblast that separates the two circulations (Donovan et al., 2000). Expression at this site is consistent with a role in the transfer of iron to the foetus. Moderate FPN1 expression levels are found in kidney and lung (Donovan et al., 2000) and there is one report on its expression in brain where FPN1 protein was found by immunostaining in neurons and oligodendrocytes of the white matter (Burdo et al., 2001).

FPN1 expression is regulated at several different levels. FPN1 shows transcriptional regulation, as it is highly expressed in professional iron exporters such as duodenum,

macrophages and placenta. The 5'-untranslated region (UTR) of FPN1 contains a well-conserved IRE. The IRE is 100% conserved among mouse, human and rat indicating an important regulatory role. Band-shift experiments using cell extracts containing IRPs show that the FPN1 IRE is active and binds both IRP1 and IRP2 (McKie et al., 2000). It can be speculated that the presence of the IRE in the 5' UTR of FPN1 mRNA suggests that it is regulated by the IRPs in a similar way to that of ferritin mRNA. In this latter case, IRP1 binds to the 5'-IRE in the ferritin mRNA and prevents translation (Hentze and Kuhn, 1996).

However, in contrast to the IRP-IRE theory, it was reported that iron-deficiency induces FPN1 expression in duodenal enterocytes, while lower expression was shown in iron-replete mice (Abboud and Haile, 2000). These results suggest the presence of an IRE-independent pathway that controls the expression of FPN1 in enterocytes. Recently, Nemeth et al (Nemeth et al., 2004b) proposed that FPN1 was regulated post-translationally by hepcidin. Together, these results suggest a complex tissue-specific regulation of FPN1 that may be related to various functions of the molecule in different cell types.

1.4.2.3.6.3 Biological Function of Ferroportin 1

Functional studies of FPN1 demonstrated that it mediates iron efflux across membranes by a mechanism that requires an auxiliary ferroxidase activity (Donovan et al., 2000; McKie et al., 2000). Iron efflux studies were conducted to further evaluate the iron export function of FPN1. Donovan et al demonstrated iron efflux in the absence of ceruloplasmin but in the presence of transferrin (Donovan et al., 2000), whereas McKie et al showed that ceruloplasmin was necessary for efflux but transferrin was not (McKie et al., 2000). Although contradictory results were obtained from these studies, both authors were able to show that FPN1 may play a role in iron export in the *Xenopus* expression model. Recently, a glycosylphosphatidylinositol (GPI)-linked form of ceruloplasmin found to be associated with ferroportin1 and to facilitate the iron efflux from brain astrocytes (Jeong and David, 2003). There is speculation that similar complex forms in the enterocyte between the membrane-bound hephaestin and ferroportin1. Hephaestin may interact with the Fe chaperone to facilitate Fe transfer to FPN1. Once transported, Fe^{2+} may then be released into the circulation and oxidized by CP (Le and Richardson, 2002). Regardless of its precise export mechanism and the presumed role for ferroxidase activity, overexpression of ferroportin1 in tissue culture cells confirms its function in iron release because depletion of cytosolic iron can be shown by decreased levels of the iron storage protein ferritin (Abboud and Haile, 2000).

Disruption of ferroportin1 expression in zebrafish (Donovan et al., 2000) and mice (Donovan et al., 2005) results in embryonic lethality, but for both animals this effect appears to be due to an impaired maternal-fetal iron transfer. Donovan et al (Donovan et al., 2005) selectively inactivated its postnatal expression in the intestine. Once intestinal expression was suppressed, the mice developed severe iron-deficiency anemia and accumulated duodenal iron consistent with the critical role for the transporter in dietary iron assimilation. On the basis of these data, we concluded that ferroportin1 provides the major, if not the only, iron export pathway in the small intestine.

Recent discovery indicates that ferroportin1 functions as a receptor for hepcidin. Through an elegant series of labeling experiments, Nemeth et al showed that ferroportin1 was bound with hepcidin (Nemeth et al., 2004b). This finding implicates a homeostatic mechanism that allows systemic regulation of intestinal iron absorption directly in response to the body's iron demands. Donovan et al (Donovan et al., 2005) have further suggested that hepcidin may affect the body iron status by influencing its levels of "excretion". Reduced ferroportin1 levels due to increased circulating levels of hepcidin during iron overload would result in increased enterocyte iron, an effect observed in zebrafish and mice with impaired ferroportin1

function. These authors pointed out that in addition to the newly absorbed dietary iron, the amount of plasma iron entering enterocytes is also quite large given the surface area of the villous epithelium. Because enterocytes are continually sloughing off into the gut as they mature, any iron they contain due to reduced ferroportin-mediated efflux would also be lost from the body. Although the hypothesis that hepcidin regulates body iron excretion remains to be rigorously tested, this exciting new model certainly challenges the established view that mechanism for regulated iron loss does not exist.

1.4.2.4 Iron Metabolism in the Reticuloendothelial System

Of the typical 3 to 4 g of iron contained in the normal adult human, only about 0.03% is lost per day. To replace these basal losses and remain in iron balance, the body must absorb a roughly equivalent amount of iron from the diet. This relatively small daily exchange of iron between body and environment contrasts sharply with the comparatively large exchange of this metal between internal organs. For example, each day the bone marrow utilizes approximately 24 mg of iron to produce over 200 billion new erythrocytes. To meet the demand for heme production necessary for erythropoiesis, iron must be recycled from senescent red cells. This process is carried

out by macrophages of the reticuloendothelial system (RES).

1.4.2.4.1 Iron Acquisition by the RES

Macrophages of the RES acquire most of their iron by phagocytosing senescent red blood cells. With each red cell ingested, the macrophage accrues approximately one billion iron atoms (Kondo et al., 1988). After erythrophagocytosis, hydrolytic enzymes present in the phagolysosome degrade the red blood cell. Proteolytic digestion of hemoglobin liberates heme, which is assumed to cross the phagolysosomal membrane either by diffusion or by a specific transporter in order to reach heme oxygenase (HMOX). HMOX proteins are localized to the endoplasmic reticulum (ER), where they catabolize heme to produce biliverdin, carbon monoxide and Fe^{2+} (Maines, 1997). The iron thus liberated is then either released from the macrophage or stored.

Uptake of hemoglobin-haptoglobin via CD163 is another significant pathway of normal iron acquisition by the RES. From kinetic studies of hemoglobin turnover in humans, it has been calculated that 10% to 20% of normal erythrocyte destruction occurs intravascularly, resulting in the release of hemoglobin (Garby and Noyes,

1959). Recent studies have identified a hemoglobin scavenger receptor, CD163, expressed exclusively on monocytes and macrophages (Kristiansen et al., 2001). Found in the highest concentrations in the spleen and liver, CD163 scavenges hemoglobin by mediating endocytosis and subsequent degradation of the hemoglobin-haptoglobin complex (Kristiansen et al., 2001). The detection of hemopexin receptors on human monocytic cell lines (Alam and Smith, 1989; Taketani et al., 1990) also suggests that the RES is able to acquire heme from this pathway, but the amount taken up is probably not significant under normal circumstances.

Through endocytosis of the plasma iron-binding protein transferrin by TfR, iron is delivered to most tissues. The transferrin receptor is a dimmer of 90-kDa subunits that associates with a regulatory molecule called HFE (Feder et al., 1998; Parkkila et al., 1997). Isolated human monocytes express transferrin receptors (Bjorn-Rasmussen et al., 1985) and are able to take up iron from transferrin (Sizemore and Bassett, 1984). When cultured monocytes differentiate into macrophages, the expression of transferrin receptor increases greatly (Andreesen et al., 1984). Transferrin-binding activity has also been demonstrated in various macrophages from mice (Hamilton et al., 1984), rats (Kumazawa et al., 1986;

Nishisato and Aisen, 1982) and humans (Andreesen et al., 1984; Montosi et al., 2000; Testa et al., 1987).

1.4.2.4.2 Iron Storage in the RES

The main sites of body iron stores are the hepatic parenchyma and the RES, particularly the RE cells of the bone marrow, spleen and liver. The liver and the total bone marrow each contain approximately 100 to 300 mg of storage iron in healthy Western individuals (Gale et al., 1963). The concentrations of iron in the liver and bone marrow have been shown to correlate well over a wide range (up to 9000 μg/g tissue) (Gale et al., 1963). Iron in the RES most likely accumulates secondary to the catabolism of red cell heme. RE iron acquired via erythrophagocytosis that is not utilized or released is first destined for storage in ferritin, a cytosolic protein which is comprised of 24 subunits of two types, H and L. In RE cells, ferritin is comprised mainly of the L-subunit (Invernizzi *et al.*, 1990), the form most associated with iron storage (Levi et al., 1994). Cell culture studies using monocytes and macrophages document the formation of ferritin protein within hours after red cell ingestion (Bornman et al., 1999; Custer et al., 1982). Recent serial analyses of gene expression in human monocyte-derived macrophages highlight the importance of ferritin in the

RE cell (Hashimoto et al., 1999). The storage of iron from the uptake of hemoglobin appears to be influenced by genetic polymorphisms in haptoglobin. Of the three haptoglobin polymorphisms in humans (Langlois and Delanghe, 1996), the multimeric Hp2-2 phenotype has the highest functional affinity for the hemoglobin scavenger receptor, CD163 (Kristiansen et al., 2001). As the amount of iron in the cell increases, a larger percentage of it deposits in hemosiderin, an insoluble, aggregated form of partially digested ferritin. Diversion of excess iron into hemosiderin permits the storage of more iron per unit volume in the cell and in fact, the highest concentrations of hemosiderin in the body are found in the RES.

1.4.2.4.3 Iron Release by the RES

Normal adult human plasma contains about 3 to 4 mg iron, essentially all bound to transferrin. Most of the circulating iron is contributed by the RES through the release of iron from the catabolized senescent red cells. Cyclic fluctuations in the RE iron release appear to cause a pronounced circadian variation in the plasma iron concentrations (Fillet et al., 1974). Neither the mechanism nor the significance of this diurnal variation in iron output from the RES is known. *In vivo* ferrokinetic studies have characterized RE iron release using trace amounts of ^{59}Fe heat-damaged red

blood cells (^{59}FeHDRBCs). After injection into the circulation, ^{59}FeHDRBCs are rapidly scavenged and processed by the RES. Studies in dogs (Fillet et al., 1974) and humans (Fillet et al., 1989) show that iron given in this manner is released in two distinct phases: an early phase, in which two-thirds of the iron freed from hemoglobin is returned to the plasma within the first few hours, and a late phase, in which the remainder is released from the RE stores over days and weeks. A similar biphasic pattern of iron release after erythrophagocytosis has been observed in isolated human monocytes (Moura et al., 1998) and macrophages (Custer et al., 1982), cultured rat peritoneal macrophages (Saito et al., 1986) and Kupffer cells (Kondo et al., 1988). The efficient release of erythrocyte-derived iron appears to require heme catabolism by HMOX1, as mice lacking this enzyme develop iron-deficiency anemia (Poss and Tonegawa, 1997). Most of the iron released into the plasma is bound by transferrin. Studies of cultured macrophages confirm that iron is released as a low-molecular-weight species that readily binds to plasma transferrin (Haurani and O'Brien, 1972; Kondo et al., 1988; Moura et al., 1998; Rama et al., 1988).

Several lines of evidence indicate that FPN1 functions as an iron exporter in various cell types. The double immunofluorescence staining has confirmed the localization

of FPN1 to RE cells (Yang et al., 2002), which implicated that this protein plays an important role in iron recycling in the RES. Consistent with this possibility is the observation that loading the RES with iron dextran enhances FPN1 expression in mouse Kupffer cells (Abboud and Haile, 2000).

Normal iron release also does seem to require ceruloplasmin, a multicopper ferroxidase. Early studies showed that copper-deficient pigs developed iron-deficiency anemia despite having normal or elevated iron stores (Lee et al., 1968). The iron deficiency appeared to result from an inefficient release of iron from the RES because serum iron concentrations did not increase significantly after intravenous administration of damaged erythrocytes.

Marrow iron requirements appear to be an important factor in the physiological regulation of iron release from the RES. When body (marrow) requirement increases, as in iron deficiency or venesection, iron release increases (Beamish et al., 1971; Lipschitz et al., 1971; Noyes et al., 1960). Within hours of being given ^{59}FeHDRBCs, iron deficient individuals released 100% of the iron, whereas normal subjects had a mean release of 64% (Fillet et al., 1989). Conversely, decreased marrow requirements resulting from either hypertransfusion (Finch et al., 1982) or bone

marrow aplasia (Fillet et al., 1989) are associated with decreased iron release. How a distant stimulus from the bone marrow regulates RE iron release is not understood. Recently, Pietrangelo has proposed that the extent of transferrin saturation relays information about the iron status of the bone marrow to the RES. Another model with a signaling role of transferrin saturation, in combination with levels of soluble transferrin receptor in plasma, has been suggested by Townsend and Drakesmith.

1.4.2.5 Heart Iron Metabolism

1.4.2.5.1 Iron Uptake and Release in Heart Cells

Like most cells, iron uptake of cardiomycytes is mediated through the internalization of the transferrin-iron complex bound to high-affinity membrane receptors (de Silva et al., 1996). A second mechanism of iron uptake occurs through a transferrin-independent process. The mechanisms of non–transferrin-bound iron (NTBI) uptake by heart cells are unclear. However, it has been suggested that, in addition to the L-type Ca^{2+} channels and voltage-independent uptake system, divalent metal transporter 1 (DMT1) might play a key role in NTBI uptake by cardiac myocytes (Tsushima et al., 1999). This NTBI transport process is considered

to have a minor role in iron uptake under normal physiological conditions but becomes the primary uptake mechanism when serum iron is severely elevated (e.g. primary and secondary hemochromatosis). Under these conditions, iron saturation of transferrin and reductions in the number of transferrin receptors occur, which result in excessive transferrin-independent iron uptake via an unknown transporter pathway (de Silva et al., 1996). Of importance to our studies, it has been shown that a critical step in NTBI uptake is the reduction of ferric iron (Fe^{3+}) to the ferrous state (Fe^{2+}) by a membrane-associated ferrireductase.

Intracellular iron balance depends on the amount of iron taken up as well as on the amount of iron released by cell (Qian and Shen, 2001; Qian and Wang, 1998). Our earlier studies have provided direct evidence for the existence of the expression of FPN1 and Heph, CP and TfR mRNAs and proteins in the rat heart by Northern blotting, Western blotting and immunohistochemistry. CP is an abundant plasma protein mainly synthesized in hepatocytes，which played an important role in iron efflux to promote iron incorporation into Tf (Osaki, 1966). Recent gene mapping studies have identified a CP homologue, Heph, which is expressed predominantly in the small intestine (Vulpe et al., 1999). Heph facilitates the transport of iron from enterocyte to plasma, but it is not a membrane transporter. Ferroportin1 was a

recently identified duodenal iron export molecule (Donovan et al., 2000). The basolateral membrane of the duodenum is the primary iron regulatory site; FPN1 and Heph may work together in iron transport from the enterocytes into the circulation. In other cell types, FPN1 may work with CP to load iron onto Tf and enhance iron release from the cell (Fleming and Sly, 2001a).

1.4.2.5.2 Regulation the Iron Metabolism of Heart Cells

Under physiological conditions, cellular iron levels are precisely regulated. FPN1, TfR and DMT1+IRE and many other mRNAs encoding proteins involved in iron uptake and storage contain IRE within their 3' or 5'UTR, which is a binding site for iron-regulatory protein (IRP). An IRE/IRP-dependent iron-regulatory pathway for the FPN1 gene appears to be present in the rat heart and lung cells (Yang et al., 2002). However, studies have demonstrated that iron status can regulate the expression of a number of mRNAs without IRE. It has been reported that desferrioxamine (DFO), an iron chelator, can decrease TGF (transforming growth factor) 1 mRNA and its activity, collagen type I mRNA and collagen synthesis, and biglycan mRNA in cardiac myocytes and non-myocytic fibroblasts (Nieto et al., 2000; Parkes et al., 2000). These imply that there are other mechanisms involved in the regulation of

mRNAs expression and protein synthesis by iron in addition to the IRE–IRP mechanism, because these mRNAs, like the DMT1-IRE mRNA, do not contain any IRE. Therefore, it is highly likely that these mechanisms that are involved in the regulation of the above mRNAs (non-IRE) might also have roles in the regulation of DMT1-IRE mRNA expression and translation in the heart. It is also possible that these mechanisms could affect the translation of DMT1+IRE mRNA. Currently, we know very little about the non-IRE–IRP mechanisms involved in the regulation of iron transporters in the heart. The full understanding of these basic issues is critical for elucidating the possible role of these proteins in heart iron homeostasis.

1.4.2.5.3 Iron-Heart Disease Connection

Heart cells require iron for many aspects of their physiology. On the other hand, iron is an effective catalyst in free radical reaction and excess iron in the heart can be potentially harmful via the generation of reactive oxygen species. Studies show that iron-mediated injury might play an important role in the development of a number of cardiovascular diseases. These include heart ischemia-reperfusion injury (Berenshtein et al., 1997; Chen et al., 2002), hemochromatosis (Pereira et al., 2001; Sullivan and Zacharski, 2001), bata-thalassemia (Aessopos et al., 2001), heart failure

(Ide et al., 2001), atherosclerosis (de Valk and Marx, 1999; Haidari et al., 2001), acute myocardial infarction (Klipstein-Grobusch et al., 1999; Tuomainen et al., 1998) and coronary heart disease (Tzonou et al., 1998). Iron may also be involved in the development of cardiotoxicity induced by doxorubicin, an anthracycline antineoplastic agent, via iron-induced oxygen free radicals (Minotti et al., 1999). Clinical investigation demonstrates that iron chelation therapy can significantly alleviate heart reperfusion injury, and the prognosis of thalassemia patients and endothelial function of patients with coronary artery diseases (Duffy et al., 2001; Horwitz and Rosenthal, 1999; Horwitz et al., 1998; Taher et al., 2001). At present, it is not known how iron increases to pathological level in the heart. The uptake of NTBI (non-transferrin-bound iron) by heart cells might be a key cause in heart injury because the toxicity of NTBI is much higher than that of Tf-Fe as judged by its ability to promote hydroxyl radical formation. However, decreased iron release by the heart cell is also another important mechanism. Therefore, the study of NTBI uptake and iron release by the heart cells is critical (Qian and Wang, 1998). It will provide important insights into the cause of excessive accumulation of heart iron as well as the pathogenesis of iron-induced heart diseases.

1.4.2.6 Brain Iron Metabolism

Brain iron research began in the second half of the 19th century when Perls (Andrews, 1999a) described the histochemical visualization of ‘‘iron oxide’’ in tissues by the exposure of sections to a mixture of potassium ferrocyanide (1%) and hydrochloric acid (1%). However, it is still not known why iron levels are abnormally high in some regions of the brain in NDs. In recent years, much information has been accumulated. Several studies support the theory that high concentrations of iron in the brain are the primary cause of neuronal death in some neurodegenerative disorders (Qian and Shen, 2001). The disrupted expression of iron metabolism proteins, which is induced by genetic or non-genetic factors, is the cause of the increased brain iron.

1.4.2.6.1 The Role of Iron in the Brain

Iron is important for the normal functions of neurons. It has been argued that iron is likely to be especially critical in the brain because neurons are dependent upon the iron-inducing oxidative aerobic metabolism (Connor and Benkovic, 1992). Iron is an essential component of cytochromes a, b, and c oxidase, iron–sulfur complexes of the

oxidative chain (ATP production), a cofactor for tyrosine hydroxylase and tryptophan hydroxylase, ribonucleotide reductase (the rate-limiting step for DNA synthesis), succinate dehydrogenase and aconitase. At a more organizational level, iron is important in the function and synthesis of neurotransmitters. It is involved in the function and synthesis of dopamine, serotonin, catecholamines, possibly γ-aminobutyric acid (GABA) and myelin formation (Beard et al., 1993). It is the key component of the heme in cytochrome proteins, permitting mitochondrial electron transfer during cellular respiration. Moreover, iron is involved in the synthesis and degradation of fatty acids and cholesterol and is likely to have an important role in both myelinogenesis and myelin maintenance (Connor and Benkovic, 1992). Thus, iron has a central role at many levels in the CNS, and is accordingly a vital element for its normal funciton. A number of physiological functions listed in Table 1-1 have been attributed to brain iron.

Table 1-1. Biological functions of brain iron

1. Catalytic role in enzymatic processes

A. Tricaboxylic cycle enzymes : Succinate dehydrogenase [a], Aconitase [a]

B. Oxidative phosphorylation enzymes, e.g., cytochrome oxidase C [a]

C. Amino acid and neurotransmitter metabolism enzymes

Phenylalanine hydroxylase [a], Monoamine oxidase (MAO) [a]

Aldehyde oxidase, Aminobutyric acid transaminase, Glutamate dehydrogenase

2. Effect on D2 receptor function

3. Effect of other neurotransmitters:

γ-Aminobuturic acid, Serotonin, Opiate-peptides

4. Role in peroxidation, oxidation, and hydroxylation reactions

5. Other possible functions (not established)

Role in protein synthesis

Role in maintenance of blood-brain barrier

[a] Brain enzyme levels remain unchanged in iron deficiency.

1.4.2.6.2 Iron Distribution in the Brain

Iron is abundant in the brain and has a distinct regional and cellular pattern of distribution. Hallgren and Sourander in 1958 found that the iron content in globus pallidus, red nucleus, putamen and substantia nigra are higher than the iron levels in the liver, the main site of iron metabolism in the body (Gerlach et al., 1994). Indeed, iron is present in the basal ganglia at a concentration equal to that of the liver (Hu and Connor, 1996). Biochemical and histochemical analysis (Connor and Benkovic, 1992; Dwork et al., 1988; Morris et al., 1992) indicate that the white matter is a major site of iron concentration throughout the brain. Recent MRI mapping of iron distribution in the brain of children and adolescents also shows that the highest concentrations of iron occur in the globus pallidus, caudate nucleus, putamen and substantia nigra while the cortex and cerebellum contain substantially lower concentrations.

In the cellular iron distribution, it has been found that iron is distributed to different cell types in the brain in a heterogeneous fashion. Iron is found in many cell types of the CNS, including neurons, oligodendroglia, microglia and astrocytes. The predominant cell type containing iron in the brain is oligodendrocyte (Dwork et al.,

1988; Gerber and Connor, 1989; Hill and Switzer, 1984), which is consistent with the important role of oligodendrocytes in brain iron metabolism. Neuronal iron staining is characteristically different from that seen in oligodendrocytes. Neurons, particularly pyramidal neurons in the cerebral cortex and hippocampus, have small punctatum of iron reaction in their somata that increases in density with age in rats (Benkovic and Connor, 1993). One additional cell type staining prominently for iron is the tanycyte that lines the third ventricle. The epithelial cells of the choroids plexus within the ventricles also stain intensely for iron. These cells may be involved in iron transport between the brain and the cerebrospinal fluid.

1.4.2.6.3 Iron Transport Across the Blood-Brain Barrier (BBB)

There is a specialilzed barrier system that excludes plasma proteins, metals and polar substances from gaining free access to the brain interior. As a number of transition metals are essential to normal brain function, the brain ensures their transport from the circulation by specialized mechanisms of transport. However, to date, the mechanisms of iron transport across the BBB have not been completely clarified. Accumulated evidence suggests that the Tf/TfR pathway may be the major route of iron transport across the luminal membrane of the capillary endothelium (Malecki et

al., 1999; Moos and Morgan, 2000), and that iron, possibly in the form of Fe^{2+}, crosses the abluminal membrane and enters the interstitial fluid (IF), although the molecular events of this process are unknown (Fig 1-8). It has been shown that the uptake of Tf-Fe by TfR-mediated endocytosis from the blood into the cerebral endothelial cells is no different in nature from the uptake into other cell types (Bradbury, 1997). This process includes several steps: binding, endocytosis, acidification, dissociation and translocation of iron across the endosomal membrane, probably by a DMT1-mediated process (Fleming et al., 1998; Gunshin et al., 1997). Most of the Tf will return to the luminal membrane with TfR, while the iron crosses the abluminal membrane by an undetermined mechanism. Recent studies show that FPN1/Heph and/or Heph-independent [perhaps is FPN1-CP (GPI-anchored CP or CSF CP)] iron-export systems might play a key role in Fe^{2+} transport across the basal membrane of enterocytes in the gut (Donovan et al., 2000; Kaplan and Kushner, 2000; Vulpe et al., 1999). It is unknown at present if these two systems have the same role in Fe^{2+} transport across the abluminal membrane of the BBB as in enterocytes. The expression of Heph in the BBB has not been clarified and is worth investigating. Another proposed mechanism for Fe^{2+} transport across abluminal membrane involves astrocytes. The astrocytes probably have the ability to take up Fe^{2+} from endothelial cells through their end feet processes on the capillary

endothelia (Malecki et al., 1999; Oshiro et al., 2000). In addition to the Tf/TfR pathway, it has been suggested that the LfR/Lf and GPI-anchored p97/secreted p97 pathways might play roles in iron transport across the BBB (Faucheux et al., 1995; Malecki et al., 1999; Qian and Shen, 2001). It is also possible that a small amount of iron might cross the BBB in the form of intact Tf-Fe complex by receptor-mediated transcytosis (Moos and Morgan, 1998).

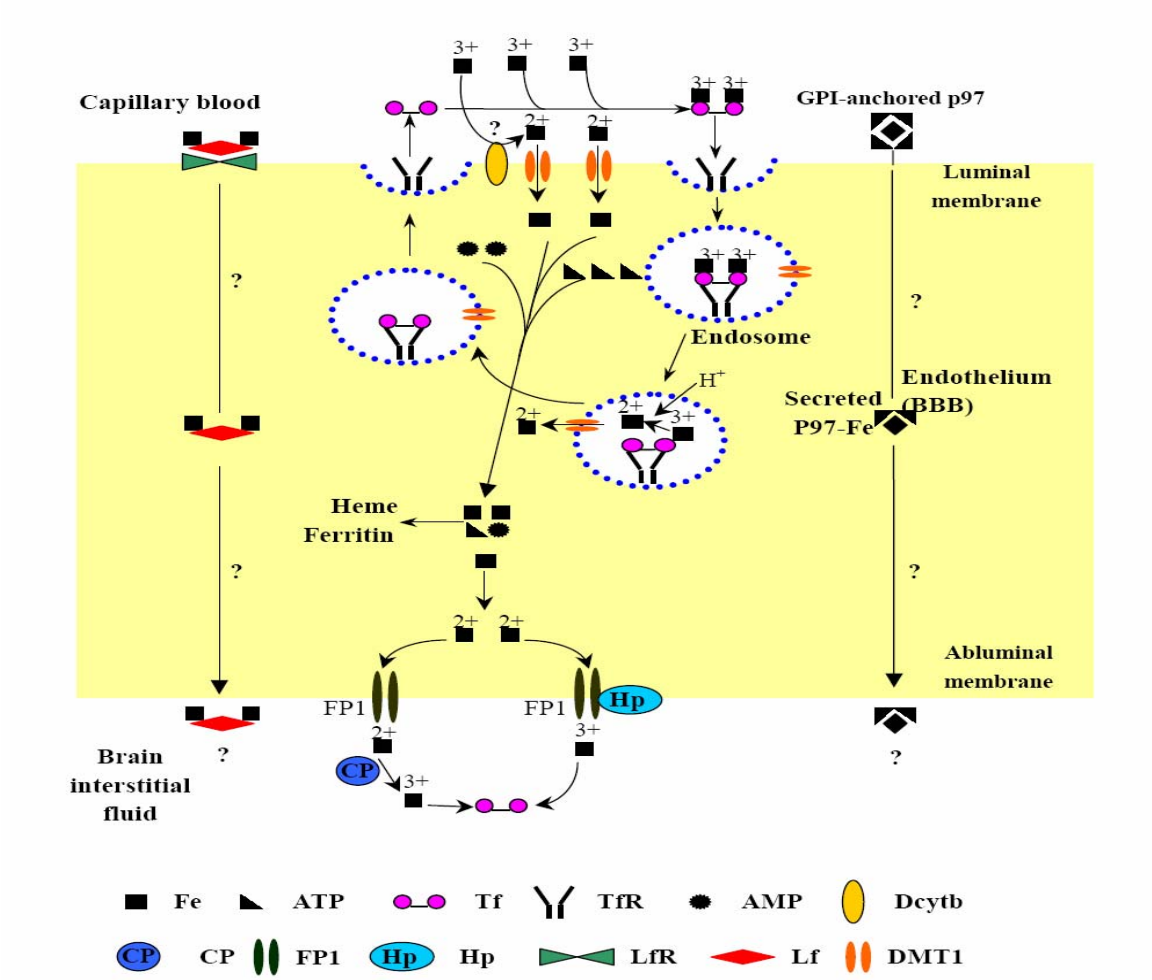


Figure 1-8. Proposed scheme for iron transport across the BBB. The Tf-TfR pathway might be the major route of iron transport across the luminal membrane of the BBB. Tf-Fe uptake by endothelial cells is similar in nature its uptake into other cell types. DMT1 might play a role in the translocation of iron from endosome to cytosol. Iron (Fe^{2+}) probably crosses the abluminal membrane via FPN1-Heph and /or Heph independent [perhaps is FPN1-CP (GPI-CP or CSF CP)] export systems. LfR-Lf and GPI-anchored p97-secreted-p97 pathways might also be involved in iron transport across the BBB; however, further study is needed. Abbreviations: BBB, brain-blood barrier; Tf, transferrin; TfR, transferrin receptor; CP, ceruloplasmin; DMT1, divalent metal transporter; FPN1, ferroportin 1; Heph, hephaestin; Dcytb, duodenal cytochrome b; Lf, lactoferrin; LfR, lactoferrin receptor; NTBI, non-transferrin-bound iron; Tf-Fe, transferrin-bound iron.

1.4.2.6.4 Iron Uptake and Release in Brain Cells (Fig 1-9)

The Tf-TfR mediated endocytosis is the main pathway of iron uptake by most of the extraneural cells. In the brain, TfR is found to be widely distributed on both the membrane of glial cells and neurons, and the mechanism of Tf-TfR mediated pathway was first identified by Swaiman and Machen in the cultured mammalian cortical neurons and glial cells (Swaiman and Machen, 1985). It has been demonstrated that iron uptake in cortical neuronal and glial cell cultures could be inhibited by the lysosomotropic agents ammonium chloride and methylamine, which revealed the possibility that iron transport to brain cells is Tf-mediated just like in other tissues. Qian et al. also detected a Tf-bound iron uptake pathway in the cultured cerebellar granule cells (Qian and Wang, 1998).

Based on the accumulated information, however, it is highly likely that Tf-dependent iron uptake is not the only route in brain cell, nor is TfR expression the only factor determining iron uptake by brain cells. The following evidence supports this possibility: 1) Photomicrograph study shows amazingly that there is little overlap in the distribution of TfR and iron. Except for the interpeduncular nucleus, no other iron-rich area of the brain has abundance of TfRs, and the areas with dense TfRs

have little or no stainable iron. 2) Some iron transporters were newly found in the brain: Two putative iron transporters, DMT1 and SFT (stimulator of Fe Transport) have been identified in some neurons and postnatal astrocytes. CP, which has been postulated as the critical ferroxidase for over 30 years, was found in the astrocyte and its involvement of iron uptake and release in brain cells was suggested. LfR is found to increase within the neurons of the pathological brain areas in PD, where neuronal loss is the greatest and iron accumulation is the heaviest. P97, first identified on melanoma cells, is also found in a subset of reactive microglia associated with senile plaques in AD brain, where there are high levels of iron and the iron storage protein ferritin.

The Tf-independent iron uptake, perhaps via LfR or GPI-anchored p97-mediated processes, will take up the Lf-Fe and secreted p97-Fe (Malecki et al., 1999). NTBI will be acquired by neuronal cells or other brain cells, probably via DMT1 or trivalent cation-specific transporter (TCT)-mediated mechanisms (Fig 1-9) (Attieh et al., 1999). However, the mechanisms are still unknown.

In addition, little is known about the molecules which are involved in iron release from the brain cells. The results of clinical and CP gene knockout mice support CP

involvement in iron release (Gitlin, 1998; Harris et al., 1999). However, our previous study (Qian et al., 2001a) and the Fox group results (Mukhopadhyay et al., 1998; Attieh et al., 1999) exhibited the role of CP in iron uptake. Recent evidence also shows that the FPN1 is expressed in rat brain (Jiang et al., 2002). The expression of Heph gene in the brain is worth studying. Whether the Heph-FPN1 export pathway (like in duodenum) plays a role in the brain is still a matter of debate (Ke and Ming Qian, 2003). So, elucidating the regulation and expression of iron metabolism-related proteins and brain iron metabolism is a worthwhile and realistic goal.

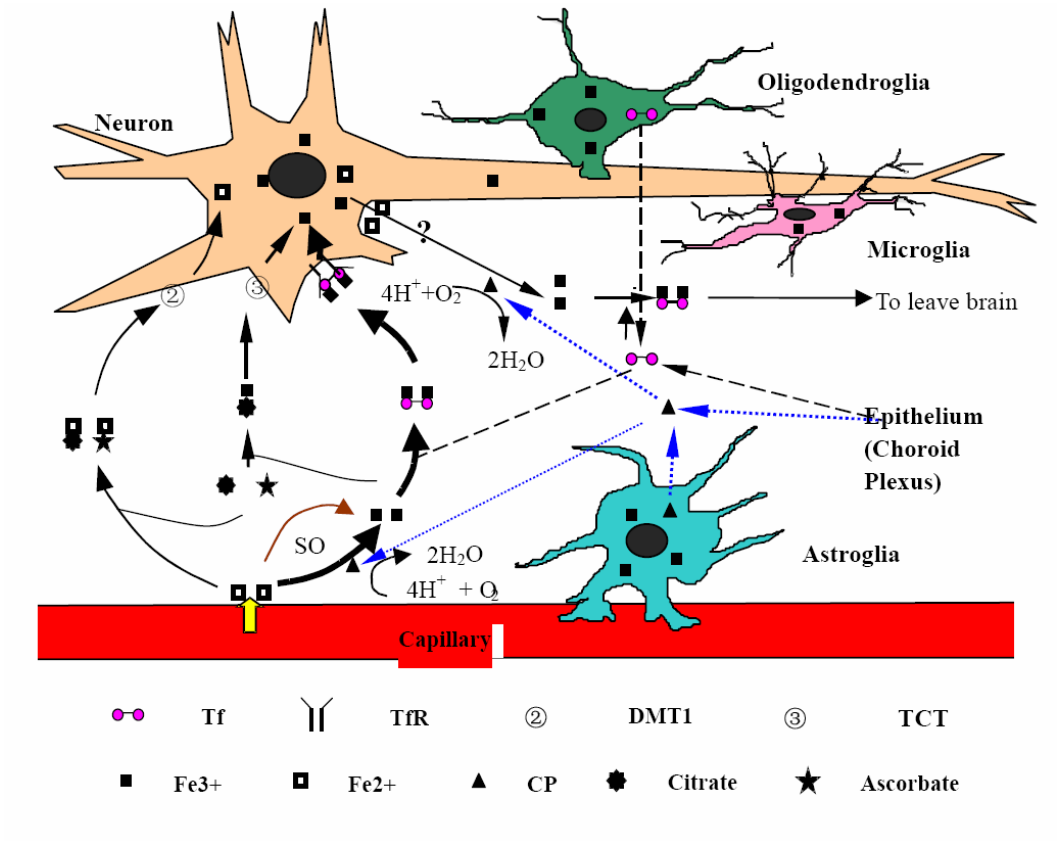


Figure 1-9. The suggested role of ceruloplasmin in iron uptake by neuronal cells in the brain. CP may play a role in both in iron efflux from and in iron influx into the brain cells via its ferroxidase activity. It is also possible that the involvement of CP in iron uptake is more important physiologically than its role in iron efflux. CP may be necessary for Fe^{2+} to be oxidized to Fe^{3+} after it crosses the abluminal membrane of the BBB. A small amount of Fe^{2+} might be oxidized to Fe^{3+} via spontaneous oxidization (SO) activity. The Fe^{3+} can than bind to transport carrier Tf in the cerebrospinal fluid and interstitial fluid that are secreted from the oligodendrocytes and choroids plexus epithelial cells and be acquired by neurons (or microglia and other relevant brain cells) via TfR-mediated endocytosis. NTBI (including

citrate-Fe^{3+} or Fe^{2+}, ascorbate-Fe^{2+}, and albumin-Fe^{2+}or Fe^{3+}) will be acquired by neuronal cells (or other brain cells) probably via DMT1 or TCT-mediated mechanisms. CP may be also involved in iron egress from neuronal cells to extracellular fluid. It has been hypothesized that iron, probably in the form of Fe^{2+}, is presented to the surface of the brain cell and then oxidized to Fe^{3+} by CP and combined with Tf in extracellular fluid for eventual excretion. The relevant mechanisms have not been determined. Abbreviations: Tf, transferrin; TfR, transferrin receptor; CP, ceruloplasmin; DMT1, divalent metal transporter; TCT, trivalent cation-specific transporter; NTBI, Non-Tf-bound iron (Qian and Shen, 2001).

1.4.2.7 The Role of Hepcidin in Regulation the Iron Homeostasis

1.4.2.7.1 Evidence for the Role of Hepcidin in Regulation the Iron Homeostasis

The critical role of hepcidin as a negative regulator of iron absorption in the small intestine was first revealed in the USF2 knockout mouse, which developed hemochromatosis with progressive iron deposition in liver and pancreas (Nicolas et al., 2001). During a search for the causes of iron overload in the mouse, the animal

was found to lack hepcidin mRNA. USF2 is a gene located in a position adjacent to the hepcidin genes, and it encodes a transcription factor not involved in iron metabolism. In the USF2 knockout mouse, the disruption of this gene created an inadvertent effect on the nearby genes, including the hepcidin genes. Another USF2 knockout mouse, generated by a different knockout construct, expressed hepcidins normally and had normal iron metabolism, which showed that the loss of USF2 by itself was not the cause of hemochromatosis (Nicolas et al., 2002a). Final confirmation of the essential role of hepcidin in humans came from two families with juvenile hemochromatosis whose affected members were found to be homozygous for disruptive mutations in the hepcidin gene (Roetto et al., 2003).

In mice, only Hepc1 is responsible for the regulation of iron homeostasis. Mice overexpressing Hepc2 have normal iron metabolism. Initial studies showed that $Usf2^{-/-}$ mice failed to express both hepcidin genes and developed hemochromatosis with iron deposition in the liver and pancreas and sparing of the macrophage-rich spleen (Nicolas et al., 2001). Recently, it was demonstrated that hepcidin 1 is a negative regulator of iron absorption and iron release from macrophages. $Hepc1^{-/-}$ mice presented massive multivisceral iron overload, with sparing of the spleen and hepatic macrophages. Mice overexpressing hepcidin-1 under the control of a

liver-specific promoter were born with lethal iron deficiency anemia, and could be rescued with parenteral but not oral iron (Nicolas et al., 2002a). These experiments indicated that before birth, hepcidin inhibits iron transport from the maternal blood across the placenta to the fetal circulation, and after birth, it inhibits iron absorption in the small intestine. Moreover it appears that the inhibitory activity of transgenic hepcidin is not overridden by other mechanisms, even during severe anemia. Patients with large hepatic adenomas, hypoferremia and severe iron-refractory anemia whose tumors overexpressed hepcidin mRNA demonstrated an analogous effect of hepcidin excess in humans (Weinstein et al., 2002).

1.4.2.7.2 Hepcidin Regulates the Iron Metabolism

After been identified as a regulator, hepcidin was proposed to interact with receptors on the crypt cells of the duodenum and dictate the amount of iron absorbed by these cells once they have matured and migrated to the villus(Nicolas et al., 2001). Recently an inverse correlation was found between hepcidin expression and the expression of duodenal iron transporters and iron absorption in rats (Frazer et al., 2002). During the switch from high to low iron diet in rats, iron absorption increased, accompanied by a rise in duodenal expression of ferric reductase (Dcytb) and

duodenal iron transporters, DMT1 and FPN1. These changes correlated with decreases in hepatic hepcidin expression and transferrin saturation. At no time was a 2- to 3-day lag period evident between the decreased hepcidin expression and the increased expression of iron transporters, suggesting that hepcidin acts directly on mature villus enterocytes instead of crypt cells, which may take 2–3 days to mature and migrate to the villus. Frazer et al (Frazer et al., 2004) investigated the delay in the iron absorptive response following stimulated erythropoiesis by using phenylhydrazine induced haemolysis. They found that this delayed increase in iron absorption following stimulated erythropoiesis was attributable to a lag in the hepcidin response rather than in the crypt programming. This was consistent with a direct effect of the hepcidin pathway on the mature villus enterocytes expression of iron transport molecules which is altered simultaneously in all mature enterocytes, and not progressively as newly differentiated cells emerge from the crypts.

A recent study has indicated that hepcidin inhibits cellular iron export through binding directly to the iron exporter ferroportin1 and inducing its internalization and degradation in HEK-293 cells (Nemeth et al., 2004b). Serial deletion of the N-terminal amino acids of intact hepcidin causes progressive loss of bioactivity of the peptide, and with an almost complete loss when all five N-residues are deleted

(Nemeth E, 2006). Human urine contains two predominant hepcidin forms each comprised of 20 and 25 amino acids each, which differ only by the N-terminal truncation (Hunter et al., 2002). The N-terminal peptides alone do not internalize ferroportin1, nor modified hepcidin molecules when the C-terminus is deleted or the disulphide pattern is altered by replacing pairs of cysteines with alanines (Nemeth et al., 2006). These data indicate that the N-terminus within the intact molecules is responsible for the activity of this peptide with respect to ferroportin1 internalization and degradation.

A pharmacodynamic study of the effects of radio-labelled hepcidin injection in mice showed that a single 50 ug dose resulted in an 80% drop in serum iron within 1 h and it did not return to normal until 96 h (Rivera et al., 2005b). This time course is consistent with the blockage of recycled iron from macrophages and previous reports of the rapid hepcidin response to IL-6 administration (Nemeth et al., 2004a). The rapid disappearance of plasma iron was followed by a delayed recovery, possibly due to the slow resynthesis of membrane FPN1. Tissue concentrations revealed that hepcidin preferentially accumulates in the proximal duodenum and spleen, reflecting the high expression of FPN1 in these areas.

Laftah et al reported that direct injection of synthetic hepcidin peptide into mice inhibited iron uptake in isolated duodenal segments (Laftah et al., 2004). In contrast to the conclusions of other investigators, these authors suggested that hepcidin predominantly acted to diminish iron transport across the apical membrane, with little or no effect on basolateral iron transfer. New data presented demonstrate that hepcidin specifically decreases iron uptake across the apical surface of the $CaCO_2$ epithelial layer, which is consistent with previous findings (Laftah et al., 2004). At the molecular level these changes in iron transport are explained by a reduction in DMT1 (McKie et al., 2001) transporter expression following hepcidin treatment.

1.4.2.7.3 Hepcicin and RES Iron Metabolism

Although RES plays an important role in iron recycling, the precise regulation mechanisms involved is still unclear. Now studies on the hepcidin gave us some clues about the molecular aspects of RE iron handling. Nicolas et al (Nicolas et al., 2001) proposed that hepcidin may serve as an iron-status signaling molecule between tissues involved in iron mobilization. An iron-loaded liver would secrete increased amounts of hepcidin into the plasma, which maybe in turn signal the RES to downregulate iron release. Evidence showing that in the transgenic mouse, iron was

sequestrated in macrophages. However, in the absence of hepcidin, RE iron release is expected to continue unabated as liver iron accumulates. Recently Knutson et al treated J774 cells with hepcidin, and found that hepcidin could significantly decrease the release of ^{59}Fe from macrophages after the phagocytosis of ^{59}Fe-labeled red cells. These studies revealed that macrophages are a target for the hepcidin action (Knutson et al., 2003). However, there is little known about the effect of hepcidin on RES iron transport.

1.4.2.7.4 Hepcidin and Heart Iron Metabolism

Although it is unknown how iron increases to a pathological level in the heart under abnormal circumstances, recent studies of the hepcidin level in the hereditary haemochromatosis (HH) and beta-thalassemia give some clues about the mechanism of iron homeostasis of the heart. Mutations in the hepcidin gene have been shown to result in juvenile haemochromatosis (JH), which is a more severe form of iron loading disorder than HH. A second gene, hemojuvelin (HJV), has recently been identified as being involved in the same pathway as hepcidin. HJV mutation also results in JH. In JH, symptomatic organ involvement occurs as early as the second decade of life. The cardiomyopathy, arrhythmias and heart failure are far more

frequent in JH than in the adult-onset form. Heart failure and/or arrhythmias are the most frequent causes of death (De Gobbi et al., 2002; Vas et al., 2005). Using of Deferoxamine (DFO) and Deferiprone (DFP) could successfully treat the case of heart failure in the setting of unrecognized JH.

The relationship between the hepcidin, the HJV and the severe heart failure in JH indicate that the heart is perhaps another target of hepcidin. But at present, there is no data about the effect of hepcidin on heart iron hemeostasis.

1.4.2.7.5 Hepcidin and Brain Iron Metabolism

Iron is an essential cofactor for many proteins that are involved in the normal function of neuronal tissues. However, iron accumulation in the brain can contribute to disorders of the CNS (Zecca et al., 2004). There are several reports of neurologic dysfunction in hemochromatosis patients (e.g., cognitive decline, gait differences, cerebellar ataxia, and extrapyramidal dysfunction)(Costello et al., 2004). Moreover, Dekker et al. showed an increased prevalence of mutations in the hemochromatosis gene in Parkinson's disease patients (Dekker et al., 2003). Recent magnetic resonance imaging (MRI) findings suggest that there is excess iron deposition in the

basal ganglia of patients with hereditary hemochromatosis (Berg et al., 2000). Furthermore, the associations of hemochromatosis gene mutations with amyotrophic lateral sclerosis have been described (Goodall et al., 2005; Wang et al., 2004), indicating that imbalances of iron in the brain contributes to the neurodegenerative processes.

Several studies have been undertaken to localize proteins that are involved in the regulation of iron homeostasis in the CNS. These include the transferrin receptor (Moos, 1996), iron regulator protein (Leibold et al., 2001) and ferritin (Moos, 1996). Hepcidin is a protein that seems to act as an important iron-regulator protein in the peripheral organs (Nicolas et al., 2002a). It is conceivable that it may operate as a regulator of iron metabolism in the CNS as well. Besides liver, hepcidin is also detectable in the brain and spinal cord (Pigeon et al., 2001). Recently Zechel et al (Zechel et al., 2006) have used RT-PCR, in situ hybridization and immunohistochemistry to investigate the cellular distribution of hepcidin mRNA and protein in the brain, spinal cord and dorsal root ganglia. Their results show a wide-spread distribution of hepcidin in different brain areas, including the olfactory bulb, cortex, hippocampus, amygdala, thalamus, hypothalamus, mesencephalon, cerebellum, pons, spinal cord, as well as in dorsal root ganglia of the peripheral

nervous system. Hepcidin immunoreactivity is not restricted to neurons, but can be detected in both neurons and GFAP positive glia cells.

Due to the hepcidin widespread distribution within the CNS, it seems that it plays an important role in the CNS. However the molecular mechanisms of the hepcidin activity, the mechanism of its regulation, and the linkage between hepcidin and other iron transport proteins in the CNS are still unknown.

1.5 OBJECTIVES

This thesis will investigate some important aspects of hepcidin in regulating iron homeostasis and iron transport proteins in macrophages, cardiomyocytes and brain cells.

1.5.1 Part 1

Although these studies proposed the possibility that hepcidin affect the RES iron metabolism and can regulate iron release from the cancer cell line (J774), there is still an absence of evidence about the responsibility of the normal macrophages to

the hepcidin and little is known about the mechanism involved in hepcidin treatment.

Therefore, this study aims to:

(1) Determine the effect of hepcidin on the iron uptake and release in the peritoneal macrophages in vitro, and the role of hepcidin in the protein expression of DMT1, TfR1, FPN1 and CP in macrophages.

(2) In order to compare the differences of the hepcidin regulation in vivo and in vitro, an animal model inducing the hepcidin expression by LPS was used to examine the iron uptake and release of the peritoneal macrophages, and the changes of iron transporter proteins (DMT1, TfR1, FPN1, CP) on these cells. On the other hand, the correlation of hepcidin levels and serum iron concentrations and other blood parameters were examined.

These two sets of results will elucidate the role of hepcidin in the normal macrophages iron metabolism, explain how hepcidin regulates cell iron uptake or release, and increase our understanding of how iron transporters protein expression is regulated by hepcidin in macrophages.

1.5.2 Part 2

To determine whether the regulation of hepcidin is organ- or tissue-specific, the effect of hepcidin on iron uptake and release in the heart cells was also investigated. Studies show that iron-mediated injury may play an important role in the development of a number of cardiovascular diseases. In the part 2, we also analyze the hepcidin regulation of the DMT1, TfR1, FPN1, CP and Heph protein expression in the cardiomyocytes and propose a possible role of hepcidin in heart iron homeostasis.

1.5.3 Part 3

In order to confirm whether hepcidin has the same role in CNS like out of brain, we further investigate the effect of hepcidin on iron transport of C6 glioma cells. In this part, we firstly detect the expression of hepcidin mRNA in different brain regions, including the cortex, hippocampus, striatum and substantia nigra of male SD rats treated with lipopolysaccharide (LPS). In addition, the effect of hepcidin on the iron uptake and release and on the protein expression of the DMT1-IRE, TfR1, FPN1, CP and Heph in the C6 glioma cells are also investigated.

CHAPTER 2

MATERIALS, APPARATUS AND METHODS

2.1 MATERIALS

2.1.1 Reagents and Analysis Kits

Acrylamide/bis-acrylamide 30% solution, electrophoresis grade	Bio-Rad Technology Ltd., Hercules, CA, USA
Agarose standard low-*Mr*	Bio-Rad Technology Ltd., Hercules, CA, USA
Ammonium persulphate	Bio-Rad Technology Ltd., Hercules, CA, USA
α-naphthyl acetate	Sigma Chemical Co. St. Louis, MO, USA
Apo-transferrin (Rat)	Sigma Chemical Co. St. Louis, MO, USA
Aprotinin	Sigma Chemical Co. St. Louis, MO, USA
Bathophenanthroline disulfonic (BP)	Sigma Chemical Co. St. Louis, MO, USA
Bromophenol blue	Bio-Rad Technology Ltd., Hercules, CA, USA
Chloroform	Sigma Chemical Co. St. Louis, MO, USA

DC protein assay kit	Bio-Rad Technology Ltd., Hercules, CA, USA
Diethyl ether	Lab-Scan Ltd., Stillorgan / Co Dublin, Ireland
Dimethyl sulfoxide	Sigma Chemical Co. St. Louis, MO, USA
DMEM medium	Invitrogen, Carlsbad, CA, USA
ECL Western blotting analysis system (RPN2132)	Amersham Pharmacia biotech, Piscataway, NJ, USA
EDTA	Sigma Chemical Co. St. Louis, MO, USA
Ethidum bromide	Bio-Rad Technology Ltd., Hercules, CA, USA
Ethanol (100 %, 96 %)	Lab-Scan Ltd., Stillorgan / Co Dublin, Ireland
$FeCl_3$	Sigma Chemical Co. St. Louis, MO, USA
Fast blue BB salt	Sigma Chemical Co. St. Louis, MO, USA
$FeSO_4$	Sigma Chemical Co. St. Louis, MO, USA
$^{55}FeCl_3$	Perkinelmer Inc., Wellesley, MA,USA
Fetal bovine serum	Invitrogen, Carlsbad, CA, USA
Formaldehyde	Sigma Chemical Co. St. Louis, MO, USA
Glycerol	Bio-Rad Technology Ltd., Hercules, CA, USA
Glycine	Bio-Rad Technology Ltd., Hercules, CA, USA

Hepcidin	Peptides International, Inc., Louisville, Kentucky, USA
HEPES	Sigma Chemical Co. St. Louis, MO, USA
iQ SYBR Green Surmix	Bio-Rad Technology Ltd., Hercules, CA, USA
Iron and total iron binding capacity kit	Stanbio laboratory Co., Boerne, USA
Isopropanol	Sigma Chemical Co. St. Louis, MO, USA
Leupeptin	Sigma Chemical Co. St. Louis, MO, USA
Lipopolysaccaride	Sigma Chemical Co. St. Louis, MO, USA
$MgSO_4$	Sigma Chemical Co. St. Louis, MO, USA
2-mercaptoethanol	Bio-Rad Technology Ltd., Hercules, CA, USA
Methanol	Lab-Scan Ltd., Stillorgan / Co Dublin, Ireland
Hydrochloride acid	Lab-Scan Ltd., Stillorgan / Co Dublin, Ireland
Nitrilotriacetic acid (NTA)	Sigma Chemical Co. St. Louis, MO, USA
Nonidet P-40	Sigma Chemical Co. St. Louis, MO, USA
Penicillin-streptomycin	Invitrogen, Carlsbad, CA, USA
Pipes	Sigma Chemical Co. St. Louis, MO, USA
PMSF	Sigma Chemical Co. St. Louis, MO, USA
Prestained protein marker	Bio-Rad Technology Ltd., Hercules, CA, USA
RPMI 1640 medium	Invitrogen, Carlsbad, CA, USA

RT-for-PCR Kit, Cat# A3500	Promega corporation., Madison, WI, USA
Scintillation cocktail	Beckman Coulter, Inc., Fullerton, CA, USA
Sodium Acetate	Sigma Chemical Co. St. Louis, MO, USA
Sodium chloride	Sigma Chemical Co. St. Louis, MO, USA
Sodium deoxycholate	Sigma Chemical Co. St. Louis, MO, USA
SDS	Bio-Rad Technology Ltd., Hercules, CA, USA
Sodium orthovanadate	Sigma Chemical Co. St. Louis, MO, USA
Sucrose	Sigma Chemical Co. St. Louis, MO, USA
TEMED	Bio-Rad Technology Ltd., Hercules, CA, USA
Tris	Bio-Rad Technology Ltd., Hercules, CA, USA
Trizol® Reagent	Invitrogen, Carlsbad, CA, USA
Trypsin	Invitrogen, Carlsbad, CA, USA
Tween 20	Sigma Chemical Co. St. Louis, MO, USA
Mouse anti-Mouse Ceruloplasmin purified antibody	BD Transduction Laboratories., Rockville, MD, USA
Mouse anti-rat CD71 monoclonal antibody	BD Transduction Laboratories., Rockville, MD, USA
Rabbit anti-Mouse Hephaestin antibody	Alpha Diagnostic International,Inc., San Antonio, TX, USA
Rabbit anti-Rat DMT1+IRE antibody	Alpha Diagnostic International,Inc., San Antonio, TX, USA

Rabbit anti-Rat DMT1-IRE antibody	Alpha Diagnostic International,Inc., San Antonio, TX, USA
Rabbit anti-mouse FPN1 antibody	Alpha Diagnostic International,Inc., San Antonio, TX, USA
Mouse anti-rat β-actin monoclonal antibody	Sigma Chemical Co. St. Louis, MO, USA

2.1.2 Apparatus

Autoclave (model HA-30)	Hirayama manufacturing, Kasukabe-shi, Saitama, Japan
Jouan Centrifuge	DJB labcare Ltd., Buckinghamshire, England
Dri-bath	Barnstead/Thermolyne Ltd., Dubuque, Iowa, USA
GeneAmp PCR System 9700	Perkinelmer Inc., Wellesley, MA,USA
Incubator (model TC2323)	Shel LAB Inc., Cornelius OR USA
Leica microscope (Model DRIMB)	Leica Inc., Bensheim, Germany
LS 5600 scintillation counter	Beckman Coulter, Inc., Fullerton, CA, USA
Lumi-imager	Roche Molecular Biochemicals Ltd., Basel, Switzerland
Luminar flow cabinet (model Nu-425-400E)	Nuair Inc., Windsor , Nova Scotia, Canada
MBA spectrometer	Perkinelmer Inc., Wellesley, MA,USA

Microcentrifuge	Eppendorf Inc., Hamburg, Germany
Micro-hematocrit centrifuge and reader	Hawksley. Co., London, England
Minigel apparatus	Bio-Rad Technology Ltd., Hercules, CA, USA
Mini-protein II Electrophoresis Cell	Bio-Rad Technology Ltd., Hercules, CA, USA
Orbital incubater SI50	KOFA Enterprise Limited., HK
pH meter (Model 701 digital)	Orion research Inc., Beverly MA, USA
Power supply	Bio-Rad Technology Ltd., Hercules, CA, Reciprocal shaking bath USA
Reciprocal shaking bath	Precision Scientific Ltd., Ontario, NY, USA
Rotor–stator homogenizer	IKA-Labortechnik., Staufen, Germany
Smart cycler	Cepheid Corporate., Sunnyvale, CA,USA
Sonicator, Branson Sonifier 250	Branson Inc., Ann Arbor, Michigan, USA

2.1.3 Animal

In this project, rats were used to acquire the peritoneal macrophages and investigate the role of LPS *in vivo*. The use of animals was approved by the Department of Health of Hong Kong and Animal Ethics Committee of The Hong Kong Polytechnic University. The rats were supplied by the Centralised Animal Facilities of The Hong

Kong Polytechnic University. To eliminate the existence of any sex-related differences in the response of iron metabolism, only male Sprague-Dawley rats were used in all of the experiments. All the animals were housed in pairs in stainless steel rust-free cages at 21±2^{o}C. The rooms were in a light-dark cycle of 12 hours of light (7:00 to 19:00) and 12 hours of darkness (from 19:00 to 7:00).

Male Sprague–Dawley rats, specific pathogen-free and weighing 300–350 g (about 8weeks) were used. Before experiment, the rats were fasted for 24 h but given free access to water. They were killed by using diethyl ether anesthesia.

2.1.3.1 Animal Sacrifice and Sample Collection

For real-time RT-PCR and Western blot analysis, animals were anesthetized with diethyl ether. After taking the peritoneal macrophages and perfusion with ice-cold phosphate-buffered saline (Milli-Q water prepared and DEPC treated, pH 7.4) through the left ventricle, the brain was rapidly removed and immediately dissected into four brain regions: cortex, hippocampus (Hippo), striatum and substantia nigra (SNigra) according to modified methods published by Focht et al. (Focht et al., 1997). The liver were also rapidly removed, aliquots were rinsed with cold saline to remove

blood, and used directly. The remaining tissues were wrapped with aluminum, and immediately frozen below –70°C for storage after treatment with fluid nitrogen.

For the haematology analysis at the end of the study, the rats were anesthetized with diethyl ether. After the thorax of the animals were opened, blood samples were collected from the heart into heparinized syringes, and aliquots were taken immediately for hemoglobin (Hb) concentration and hematocrit (Hct) determination. The remaining blood was cooled to 4°C and centrifuged at 3,000 g for 10 min at 4°C, and serum was removed and frozen at -20°C before analysis for iron or total iron-binding capacity (TIBC).

2.2 GENERAL METHODS

2.2.1 Cell Culture

2.2.1.1 Cell Culture Medium, Solutions and Reagents

(1) Milli-Q water

All the solutions for cell culture and medium were made with Milli-Q water.

(2) Cell culture medium (Invitrogen, CA, USA)

Dulbecco's Modified Eagle Medium (DMEM), powder (Catalogue Number: 12100-038); RPMI 1640, powder (Catalogue Number: 31800-014); Fetal Bovine Sera (FBS), qualified, heat inactivated.

(3) Cell culture solution

Trypsin (2.5%, 1×) (Invitrogen, CA, USA)

Trypan blue solution (Fluka, Buchs, Switzerland)

2.2.1.2 Primary rat Peritoneal Macrophages (RPM) Culture

(1) Preparation of buffers and medium:

Phosphate-Buffered Saline (PBS) (10×)：80g NaCl + 29g Na_2HPO_4 •H_2O + 2g KCl + 2g KH_2PO_4+ 1000ml ddH_2O sterilized by autoclaved and stored at 4°C.

Medium A: 10.4 g RPMI 1640 powder+1000ml Milli-Q water,

Medium B: Medium A supplemented with 1% (v/v) FBS and 2.5% of penicillin/streptomycin solution (v/v)

Medium C: Medium A supplemented with 10% (v/v) FBS and 2.5% of penicillin/streptomycin solution (v/v)

All the mediums were adjusted to pH 7.6, sterilized by filtering and stored at 4°C.

(2) Cell separation and culture

The cells were prepared from the male SD rat using established methods with minor modification (Huang et al., 2003).

(A) Pre-cold medium and containers for 30min, and sterilized materials under UV light for about 15min.

(B) Anaethetize male SD rat with diethyl ether for 2-4min. Clean the rat by 70% ethanol, and fix position prior to sacrifice.

(C) Take off the skin of the abdomen and expose the abdominal wall. Wash the abdominal wall with 70% ethanol.

(D) Pierce a syringe with a small volume of air and 25ml medium B into the abdomen (Avoid damaging the internal organs). Inject a total of 50ml of ice-cold

medium B.

(E) Following a gentle massage, the macrophages resident on the abdomen released into the abdominal fluid. Use another syringe to take the supernatant out and place it into a collecting tube.

(F) Collect cells by centrifugation at 4°C for 10 min at 1100 rpm, discard the supernant and resuspend the pellet in medium C.

(G) Count the number of cells by using a cell-counting chamber and determine the cell density with 0.2% trypan blue in PBS. Plate the cells in 35mm culture dishes or 6-well culture plates and the average density should be 4 x 10^6 cells/well for Western blot and 1 x 10^6 cells/well for isotope experiments.

(H) After incubation at 37°C in 5% CO_2 atmosphere for 3h, remove the nonadherent cells by gentle washing with cold PBS. Incubate the plated cells with medium C in 5% CO_2 incubator at 37^{o}C for 24h, then change the medium to remove the dead cells and incubate until the day of the use.

(I)Determine the cell types by optical microscopy and α-Naphthyl Acetate Esterase Staining.

(3) Identification of macrophage by α-Naphthyl acetate esterase staining (Rademakers et al., 1989) (Yam et al., 1971)

(A) Principle

Cellular esterases are ubiquitous, apparently representing a series of different enzymes acting upon select substrates. Under defined reaction conditions, it may be possible to determine hemopoietic cell types by using specific esterase substrates. The described methods provide hematologists and hematopathologists means of distinguishing granulocytes from monocytes. The non-specific esterase is one kind of esterases which is detected primarily in monocytes, macrophages and histocyes, while it is normally absent in granulocytes. Lymphocytes may occasionally exhibit enzyme activity. It is demonstrated by exposing the sample to α-naphthyl acetate (NAE) which is substrate. To perform the test, blood and bone marrow films or tissue touch preparations are incubated with α-naphthyl acetate (NAE) in the presence of freshly formed diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds. These couple with the diazonium salt to form highly colored deposits at sites of enzyme activity.

(B) Preparation of the solution

Fix solution:

10% formaldehyde solution

Stocking solution:

A: $Na_2HPO_4 \cdot 12H_2O$ 2.388g dissolved with DDH_2O to 100ml

B: KH_2PO_4 0.908g dissolved with DDH_2O to 100ml

C: A solution 87ml+B solution 13ml, adjust the pH to 7.6

Working solution:

stocking solution C	40ml
10g/L α-naphthyl acetate	0.8ml
Fast blue BB salt	40mg

Stir and filter

(C) Procedure

1. Cells were seeded (1×10^5) on the glass coverslips (Sigma, USA) contained within the wells of 6-well culture plates and maintained as described in the culture of macrophages.
2. After 24 hours, the growth medium was removed and fixed with 10% formaldehyde solution.
3. Washed the cells in DDH_2O for five minutes.

4. Dipped them into working solution and incubated at 37^{o}C for 60 minutes.
5. Rinsed with DDH_2O for about 3 minutes.
6. Counterstain 2 minutes in Hematoxylin solution, and rinse in tap water and air dry.
7. Evaluate microscopically.

2.2.1.3 Trypan Blue Staining of Cell

(1) Cell were placed in complete medium without serum and diluted to an approximate concentration of 1×10^5 to 2×10^5 cells per ml. 0.5 ml of this cell suspension was placed in a screw cap test tube, to which was added 0.1 ml of 0.4% Trypan Blue Stain.

(2) The solution was mixed thoroughly and allowed to stand 5 min at 15-30 ^{o}C.

(3) A hemocytometer was filled with cell solution for cell counting. Non-viable cells were observed with the stain, and viable cells were not stained.

2.2.1.4 Cryopreservation of Cells

(1) The cultured cells were detached from the substrate using dissociation agents.

This was done as gently as possible to minimize damage to the cells.

(2) The detached cells were placed in a complete growth medium and established a viable amount of cells.

(3) The re-suspended cells was centrifuged at ~200 ×g for 5 min and the supernatant was withdrawn down to the smallest volume without disturbing the cells.

(4) The cell pellet was resuspended in freezing cold medium to a concentration of 5 × 10^6 to 1 × 10^7 cells/ml.

(5) Aliquot of the cells solution was placed into cryogenic storage vials. The vials were put on ice or in a 4 ^oC refrigerator, and freezing began within 5 min.

(6) The cells were slowly frozen at a rate of 1 ^oC /min. This could be done by programmable coolers or by placing the vials inside an insulated box placed in a -70 ^oC to -90 ^oC freezer, then transferred for storage in liquid nitrogen.

2.2.1.5 Thawing of Cryopreserved Cells

Cryopreserved cells are fragile and require gentle handling. The cells were thawed quickly and placed directly into complete growth medium. If the cells were particularly sensitive to cryopreservation (DMSO or glycerol), they were centrifuged to remove cryopreservative and then placed into the growth medium.

The following were the suggested procedures for thawing cryopreserved cells.

(1) Remove cells from storage and thaw quickly in a 37 ^{o}C water bath.

(2) Place 1 or 2 ml of frozen cells in ~25 ml of complete growth medium. Mix very gently.

(3) Centrifuge cells at ~80 × g for 2 to 3 min.

(4) Discard supernatant.

(5) Gently resuspend cells in the complete growth medium and perform a viable cell count.

(6) Culture the cells. Cell density should be at least 3 x 10^5 cells/ml.

Direct Plating Method:

(a) The cells from storage are thawed quickly in a 37 ^{o}C water bath.

(b) The thawed cells are directly placed in a flask with the complete growth medium. Add 1 ml of frozen cells to 10 to 20 ml of the complete medium to make a suspension cell density of at least 3 × 10^5 cells/ml.

(c) The cells are cultured for 12 to 24 hours, and replace the medium with the fresh complete growth medium to remove cryopreservatives.

2.2.1.6 C6 Glioma Cell and H9C2 Cell Culture

Both C6 glioma cell line and H9C2 cell line were obtained from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's modified Eagle's medium (DMEM/Glutamax; Life Technologies), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and 100U/ml of sodium penicillin G and 100 µg/ml of streptomycin sulfate. The medium was changed every 3 days. The subculture was prepared by removing the medium, adding 1-3 ml of fresh 0.25% trypsin or 2mM EDTA solution (for protein extraction) for several minutes, and then removing the trypsin solution. The culture was allowed to stand at room temperature for 10 to 15 minutes. Fresh medium was added, aspirated and dispensed until the cells were detached. Then the cells were transferred to a 15 ml centrifuge tube containing 3-5 ml of fresh medium and centrifuged at 1000 rpm for 5 minutes at 4^{o}C. The supernatant was discarded and the pellet triturated in 2 ml of fresh medium. The cell number was determined by trypan blue exclusion under the microscope, and the required number of cells was placed into the flasks (for maintenance) or in 6-well plates and 96-well plates. All the apparatus and mediums used for cell culture were sterilized before use. For certain immunocytochemistry experiments and the iron transport assay, the cells were replanted in serum-free DMEM or HBSS solution.

2.2.2 Methods of Molecular Biology

2.2.2.1 RNA Preparation

Total RNA was isolated from brain tissue or other tissues using Trizol® Reagent for real-time RT-PCR according to the manufacturer's instructions. Genomic DNA potentially present in RNA processing was removed by incubating the RNA with RNase free DNase I. The relative purity of the isolated RNA was assessed spectrophotometrically and the ratio of A260 nm to A280 nm exceeded 1.8 for all preparations or 28S RNA bands = twice the amounts of the 18S RNA.

2.2.2.2 cDNA Synthesis

1. Quickly thaw each tube in the RT-for-PCR Kit and place them on ice. Carry out all dilutions and additions on ice.
2. Spin each tube briefly in a tabletop microcentrifuge and return them to ice.
3. Place 1µg total RNA in a sterile 0.5-ml microcentrifuge tube and incubate at 70°C for 10 minutes. Spin briefly in a microcentrifuge, then place on ice.
4. Prepare 20µl reaction by adding the following reagents in the order listed:

Component Amount

$MgCl_2$, 25mM*	4µl
Reverse Transcription 10X Buffer	2µl
dNTP Mixture, 10mM	2µl
Recombinant RNasin® Ribonuclease Inhibitor	0.5µl
AMV Reverse Transcriptase (High Conc.)	15u
Oligo$(dT)_{15}$ Primer	0.5µg
total RNA	1µg
Nuclease-Free Water to a final volume of	20µl**

*The suggested magnesium concentration may be optimized for any given sequence to achieve better yields.

**Final concentration of reaction components: 5mM $MgCl_2$, 1X Reverse Transcription Buffer (10mM Tris-HCl [pH 9.0 at 25°C], 50mM KCl, 0.1% Triton® X-100), 1mM each dNTP, 1u/µl Recombinant RNasin® Ribonuclease Inhibitor, 15u/µg AMV Reverse Transcriptase (High Conc.), 0.5µg Oligo$(dT)_{15}$ Primer per microgram RNA.

Note: A master reagent mix would be prepared when more than one RNA sample would be used for RT-PCR. This would help ensure tube to tube consistency in the cDNA synthesis reaction. Make sure that an extra master mix is made so that there

would be sufficient master mix for all tubes.

5. Mix the contents of the tube by pipeting up and down.
6. Incubate the reaction at 42°C for 60 minutes.
7. Heat the sample at 95°C for 5 minutes, then incubate at 0–5°C for 5 minutes. This will inactivate the AMV Reverse Transcriptase and prevent it from binding to the cDNA. Then spin down the contents of the tube.
8. Store the first-strand cDNA at –20°C until use.

2.2.2.3 Real-time PCR

(1) Principle:

There are currently four competing techniques available that detect amplified product with about the same sensitivity (Wittwer et al., 1997). They use fluorescent dyes and combine the processes of amplification and detection of an RNA target to permit the monitoring of PCR reactions in real-time during the PCR. Their high sensitivity eliminates the need for a second-round amplification, and decreases opportunities for generating false-positive results (Morris et al., 1996). The simplest method uses fluorescent dyes that bind specifically to double-stranded DNA (dsDNA). The other

three rely on the hybridisation of fluorescence-labelled probes to the correct amplicon. SYBRGreen I is a DNA-binding dye that incorporates into dsDNA. It has an undetectable fluorescence when it is in its free form, but once bound to the dsDNA it starts to emit fluorescence. When monitored in realtime, this results in an increase in the fluorescence signal that can be observed during the polymerization step, and that falls off when the DNA is denatured. Consequently, fluorescence measurements at the end of the elongation step of every PCR cycle are performed to monitor the increasing amount of amplified DNA. Additional specificity and RT-PCR product verification can be temperature to generate a melting curve of the amplicon (Ririe et al., 1997). This is done by slowly increasing the temperature above the *T*m of the amplicon and measuring the fluorescence. As the *T*m of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product. A characteristic melting peak at the melting temperature (*T*m) of the amplicon will distinguish it from amplification artefacts that melt at lower temperatures in broader peaks.

(2) The sequences of primers:

Gene	Primar 5'→3'	Position	Size(bp)	Accession

hepcidin	Forward	acagaaggcaagatggcact	**13-32**	201	NM_053469
	Reverse	gaagttggtgtctcgcttcc	**194-213**		
β-actin	Forward	gtcgtaccactggcattgtg	**518-537**	181	NM_031144
	Reverse	ctctcagctgtggtggtgaa	**679-698**		

Note: To ensure real-time PCR was performed within the linear range of amplification, different template volums were selected. The anneal temperature was 59°C.

(3) Procedure:

A. Quickly thaw the iQ SYBR Green Supermix and primers and place them on ice. Carry out all dilutions and additions on ice.

B. Spin each tube briefly in a tabletop microcentrifuge and return them to ice.

C. Prepare a 25μl reaction according to the manufacturer's protocol.

D. The initial denaturation (95°C for 5 min) is followed by 40 cycles. Each cycle consists of 95°C for 15 seconds, 59°C for 15 seconds and 72°C for 30 seconds.

(4) Calculation:

Absolute quantification should be performed in situations where it is necessary to determine the absolute transcript copy number. In some situations, it may be unnecessary to determine the absolute transcript copy number and reporting the relative change in gene expression will suffice. In this thesis, the comparative C_T method is used for relative quantification.

In this method arithmetic formulas are used to calculate relative expression levels, compared with a calibrator, which can be for instance a control (non-treated) sample. The amount of target, normalized to an endogenous housekeeping gene (β-actin) and relative to the calibrator, is then given by $\triangle\triangle C_T$, where $\triangle\triangle C_T = \triangle C_T$(sample) — $\triangle C_T$(calibrator), and $\triangle C_T$ is the C_T of the target gene subtracted from the C_T of the housekeeping gene. For the untreated control sample, $\triangle\triangle C_T$ equals zero and 2^0 equals one, so that the fold change in gene expression relative to the untreated control equals one, by definition. For the treated samples, evaluation of $\triangle\triangle C_T$ indicates the fold change in gene expression relative to the untreated control.

2.2.2.4 Protein Preparation and Determination

(1) Protein Preparation

Tissue samples were homogenized in RIPA buffer containing 0.15mM NaCl, 10mM $NaPO_4$, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 1μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1mM sodium orthovanadate and 1% Triton X-100, and sonicated. The cell culture samples were washed with ice-cold PBS, lysed with Tris buffer containing 1% Triton X-100, 0.1% SDS, 1 mM PMSF and protease inhibitors (pepstatin 1μg/ml, aprotinin 1μg/ml, leupeptin 1μg/ml), and then supersonic using Soniprep 150 (MSE Scientific Instruments, England) for 3×10s. After centrifugation of homogenate at 10,000 × g for 30 min at 4°C, the supernatant was collected as crude cytosolic fraction.

(2) Protein Determination (*DC* Protein Assay kit, Bio-Rad, USA)

(A) Preparation of working reagent

Add 20 μl of reagent S to each ml of reagent A that will be needed for the run. (This working reagent A' is stable for one week even though a precipitate will form after one day. If precipitate forms, warm the solution and vortex).

(B) Five dilutions (0.2, 0.4, 0.6, 0.8, 1μg/μl) of a protein standard were prepared which is representative of the protein solution to be tested.

A standard curve should be prepared each time the assay is performed. For the

best results, the standards should always be prepared in the same buffer as the sample.

(C) Pipet 100 µl of the standards and samples into clean, dry test tubes.

(D) Add 500 µl of reagent A' or A (see note from step 1) into each test tube and vortex.

(E) Add 4.0 ml reagent B into each test tube and vortex immediately.

(F) After 15 minutes, absorbances can be read at 750 nm. The absorbances will be stable for at least 1 hour.

The protein concentration was adjusted to 2-4mg/ml and the fractions (containing 30 µg protein) in aliquots were stored at –70°C.

2.2.2.5 Western Blot

(1) Principle

Western- or immuno-blotting is a commonly employed technique for the detection of protein antigens in complex mixtures. It is highly sensitive to detect as little as picograms of protein with antibodies of known specificity. Samples are first separated by SDS-polyacrylamide gel electrophoresis. The separated proteins are

then transferred to a membrane. These membranes are incubated with an antibody specific for the protein of interest that binds to the protein band immobilized on the membrane. The antibody is then visualized with a detection system that is usually based on a secondary protein binding to Ig chains, which are linked to chemiluminescent substrate reaction.

(2) Procedure:

(A) Polyacrylamide gel electrophoresis and transfer

1) Polyacrylamide gel electrophoresis

A total of thirty micrograms protein were diluted in 2× sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol) and heated for 5 min at 95°C before SDS-PAGE on a 10% gel for FPN1, DMT1+IRE, DMT1-IRE, TfR and 7.5% gel for CP, Heph. Electrophoresis was conducted by running the stacking gel at 100V for about 15-20 min and running the separating gel at 200V for about 40-50 min.

2) Transferring gel

(a) The gel was disassembled from the plates carefully, the stacking gel was removed and the position marked by notching one corner.

(b) The gel was washed in the transfer buffer for 15 min.

(c) The PVDF membrane and the filter papers were cut to fit the dimension of the gel size.

(d) The filter paper and fiber pads were soaked in transfer buffer for at least 15min.

(e) The cassette was assembled as follows: white (+), fiber pads, filter papers, membrane, gel, filter papers, fiber pads, gray (-). Avoid the bubble by rolling a glass rod.

(f) The pre-chilled transfer buffer and frozen Bio-Ice cooling Unit were placed in the electrophoresis tank.

(g) The transfer unit was allowed to stand at 4°C overnight for 12-16h with 30 voltages and gentle agitation.

(h) After transferring, the blot was put on a clean filter paper, and stained with commassie blue to check the quality of transfer.

(i) The blots were used immediately or stored.

3) Immunoblotting (ECL western blotting analysis system, RPN 2132)

The membrane was blocked with 5% blocking milk (Bio-Rad, USA) in TBS containing 0.1% Tween 20 for 2h at RT or overnight at 4°C. The membrane was rinsed in three changes of TBS-T, incubated once for 15 min and twice for 5 min in fresh washing buffer, and then incubated with primary antibody for 2h at RT or

overnight at 4°C. The concentration of primary antibodies was maintained according to the instruction of the products. After three washes in washing buffer, the membrane was incubated for 2 hours in horseradish peroxidase-conjugated anti-rabbit or anti-mouse second antibody (1:5000, Amersham Biosciences, England) and developed using enhanced chemiluminescence (ECL Western blotting analysis system kit, Amersham Biosciences, England). The blot was detected by Lumi-imager F1 workstation (Roche Molecular Biochemical). The intensity of the specific bands was determined by densitometry with the use of LumiAnalyst 3.1 software (Roche Molecular Biochemical). To ensure even loading of the samples, the same membrane was probed with mouse anti-rat β-actin monoclonal antibody (Sigma-Aldrich, MO) at 1:5000 dilutions.

2.2.3 Methods of Biochemistry and Chemistry

2.2.3.1 Measurement of Hemoglobin (Hb) Concentration

(1) Principle:

Blood Hb concentration was measured colorimetrically by the cyanmethaemoglobin

method using Drabkin's reagent (Procedure No.525A, Sigma Chemical, St. Louis, MO,USA). Hb is a complex molecule composed of an iron-containing heme and the protein globin. The concentration of Hb in solution, i.e. when released from the red cells by haemolysis, can be estimated in various ways such as the measurement of its color, the amount of oxygen or carbon monoxide with which it can combine, or by determination of its iron content. The last technique, known as the cyanmethaemogkobin method, is used routinely, because it is simple, rapid and the coloured end product of the reaction is stable.

In this method, potassium ferricyanide (an oxidizing agent) converts the Fe^{2+} iron of haemoglobin to the Fe^{3+} state-methaemoglobin. This is then combined with cyanide (CN-) to form cyanmethaemoglobin. This is a stable coloured compound (i.e. unaffected by O_2 and CO_2 concentrations) and its concentration in solution can be estimated by its absorbance at 540nm in a colorimeter/spectrophotometer.

(2) Procedure:

Drabkin's solution

Sodium bicarbonate (1g) + Potassium ferricyanide (198mg) + Potassium cyanide

(52mg) + Distilled water (1000ml)

(A) 2.5 ml of Drabkin's solution was pipetted into each test tube and sample tube.

(B) 10 ml of standard human Hb (6g/dl, 12g/dl and 18g/dl) and whole blood was added into the test and sample tubes respectively.

(C) The tubes were covered with parafilm and mixed by swirling.

(D) The mixtures were left to stand at room temperature for at least 10 min.

(E) The absorbance of the test sample and the human Hb standard at wavelength of 540 nm were recorded against Drabkin's solution.

(F) The standard curve was plotted and the concentration of each unknown was determined according to the following equation.

(G) Hb conc. (g/100ml)=(A540 sample/A540 standard)×conc. Standard (g/100ml)

2.2.3.2 Measurement of Hematocrit (Hct)

Hematocrit is determined by centrifugation of blood collected into heparinized microcapillary tubes.

(1) Materials:

(A) Microhematocrit capillary tube

(B) Plasticine

(C) Microhematocrit centrifuge (Hawksley, England)

(D) Microhematocrit Reader

(2) Procedure:

(A) The microhematocrit capillary tube was filled with blood to 75%~90% of the tube length.

(B) The outside of the tube was wiped with parafilm and the opposite side was sealed by pushing it into the plasticine.

(C) The tube was placed in a Hawksley microhematocrit centrifuge with the sealed end towards the outside, and centrifuged at 12000rpm for 5min.

(D) After centrifugation, the Hct was read using the Microhematocrit Reader by placing the base of the red cell column on the '0' line, then moving the silver line on the adjuster until it reached the level of red cell/white cell-platelet interface.

(E) The result was expressed as a percentage.

2.2.3.3 Serum Iron, UIBC, TIBC Measurement

(1) Principle:

Serum iron and unsaturated iron binding capacity (UIBC) levels are determined by published methods (Persijn et al., 1971). The Tf-bound iron dissociates to form ferrous ions at an acidic pH and in the presence of reducing agents. The ferrous iron reacts with ferrozine to develop a magenta colored complex with maximum absorption at 560 nm. The difference in the color intensity at this wavelength, before and after the addition of ferrozine, is proportional to the serum iron concentration. At an alkaline pH (8.1), ferrous iron added to plasma binds specifically with Tf at unsaturated iron-binding sites. A separate technique for serum unsaturated iron-binding capacity (UIBC) involves addition of a known excess of ferrous ions, which saturate the available transferrin iron-binding sites. Excess (unbound) iron is then quantitated as described above, with UIBC being the difference in iron concentration between that added and that determined in the remaining excess. The serum total iron-binding capacity (TIBC) equals the total iron plus the UIBC.

(2) Materials:

Iron and total iron binding capacity kit (Stanbio laboratory Co., Boerne, USA).

(3) Procedure:

(A) 1 ml iron HA buffer reagent was added to the cuvettes labeled test, standard and blank for the measurement of serum total iron (A).

(B) In UIBC measurement (B), iron HA buffer was replaced by Tris Buffer.

(C) 0.1 ml iron-free water, iron standard and serum were respectively added to cuvettes and mixed.

(D) Incubate them for 1 minute at room temperature.

(E) The absorbance at 560 nm (A) of test sample and standard vs. blank was recorded as the INITIAL A or INITIAL B.

(F) 20 µL iron color reagent was added in each cuvette, mixed thoroughly and placed in water bath at 37^{o}C for 5 minutes.

(G) The absorbance at 560 nm of test sample and standard vs blank was recorded as the FINAL A or FINAL B.

Calculations:

A (B) test = FINAL A (B) test - INITIAL A (B) test

A (B) standard = FINAL A (B) standard – INITIAL (B) Standard

Serum Total iron (μg/dl) = Atest/Astandard × 500.

Serum UIBC (μg/dl) = 500 – (Btest/Bstandard × 500)

Serum TIBC (μg/dl) = Serum Total iron + Serum UIBC

2.2.4 Methods of Radiation

2.2.4.1 Non-transferrin Bound Iron (NTBI) Uptake Assay

(1) Preparation of buffers

(A) 0.27M Sucrose solution：9.242g sucrose + 100ml DDH_2O, buffered to pH 6.5 by 4mM Pipes.

(B) Cell lysis buffer： 1g SDS + 100ml DDH_2O

(C) Phosphate-Buffered Saline (PBS) (10×)：

80g NaCl + 33.6g $Na_2HPO_4 \cdot 12H_2O$+ 2g KCl + 2g KH_2PO_4+ 1000ml DDH_2O

(D) Radioactive solution: $^{55}FeCl_3$(Perkinelmer) dissolved with $FeSO_4$, the molar ratio is 1:10, then add 50 times beta-mercaptoethanol and 0.27M sucrose solution.

(2) Procedure

(A) Remove the incubation medium and wash the cells three times by cold PBS.

(B) Add 1ml 0.27 M sucrose solution and 32ul radioactive solution to each well.

(C) Incubate the cells at $37^{o}C$, 5% CO_2 for 30 minutes.

(D) Stop the reaction by placing the plates on ice and wash the cells by ice-cold PBS for 5 minutes for three times.

(E) After washing, lyse the cells by 500ul lysis buffer and shaked for 10min with the maximum shaking rate.

(F) Detach the cells by the scraper and transferred them into EP tubes and votex them.

(G) A 50ul aliquot was subjected to detect the protein concentration.

(H) Separate the cytosol from the stromal-mitochondrial membrane (membranes) fraction by centrifugation at 10,000 g for 20 minutes at $4^{o}C$ using a Jouan centrifuge (DJB labcare Ltd., England).

(I) Separate the cytosol from the membranes, and dissolve the pellet with 450 ul 1% SDS. Add both fractions into 3ml scintillation solution to count the cpm.

(J) Add 450ul 1% SDS to the blank group into 3ml scintillation solution to count the cpm.

2.2.4.2 Transferrin Bound Iron Uptake Assay

(1) Preparation of buffers

(A) Dialysis buffer：0.15 M NaCl.

(B) $NaHCO_3$ buffer：10mM $NaHCO_3$, 250mM Tris-HCl.

(C) Saturation $NaHCO_3$ solution

(D) 3.2mM NTA

(E) Acid solution：0.2N acetate，0.5M NaCl，1mM $FeCl_3$

(2) Preparation of the ^{55}Fe-Tf

(A) Take the $^{55}FeCl_3$ solution into 200ul NTA (3.2mM) solution. The molar ratio is $^{55}FeCl_3$: NTA=1:10

(B) Keep the solution in room temperature for 2 hours.

(C) Add the saturation $NaHCO_3$ solution to neutralize the reaction.

(D) Dissolve the rat apo-transferrin 2.6mg d in the 2ml 10mM $NaHCO_3$ buffer. The molar ratio is $^{55}FeCl_3$: Tf=2:1

(E) React at room temperature for 3 hours.

(F) Dialysis and removed the unbound-^{55}Fe by stirring in 250ml dialysis buffer for 7 times at 4^{o}C, and each time for 3 hours.

(G) Detect the Tf protein concentration (A280nm) and counted the radioactivity, then calculated the ratio.

(3) Procedure

(A) Remove the incubation medium and wash the cells three times with cold PBS.

(B) Add 1ml culture medium without FBS (but with 0.1% BSA) and incubat at 37^{o}C, 5% CO_2 for 1 hour.

(C) Wash cells with warm PBS for one time.

(D) Add 1ml culture medium without FBS (but with 0.1% BSA) to each well.

(E) Add the ^{55}Fe-Tf into each well to the final concentration of 10ug/ml, and incubate for 60 minutes at 37^{o}C.

(F) Cool the plates on the ice and discard the medium.

(G) Add 1ml ice-cold PBS to terminate the uptake of iron by cells. Wash the cells with cold PBS for two times.

(H) Wash the cells with 1ml acid washing buffer to remove the nonspecific attachment of the ^{55}Fe-Tf.

(I) Discard the buffer and wash the cells for three times by cold PBS.

(J) Add 500ul cell lysis buffer and keep on ice for 10 minutes.

(K) Detach the cells by the scraper and transferred them into EP tubes and votex them.

(L) A 50ul aliquot is subjected to detect the protein concentration.

(M) SeparateThe cytosol from the stromal-mitochondrial membrane (membranes) fraction by centrifugation at 10,000 g for 20 minutes at 4^{o}C using a Jouan centrifuge (DJB labcare Ltd., England).

(N) Separate the cytosol from the membranes, and dissolve the pellet with 450ul 1% SDS. Add both fractions into 3ml scintillation solution to count the cpm.

2.2.4.3 Iron Release Assay

(1) Preparation of buffers

(A) 0.27M Sucrose solution：9.242g sucrose + 100ml DDH_2O, buffered to pH 6.5 by 4mM Pipes.

(B) Cell lysis buffer： 1g SDS + 100ml DDH_2O

(C) Phosphate-Buffered Saline (PBS) (10×)：

80g NaCl + 33.6g $Na_2HPO_4 \cdot 12H_2O$+ 2g KCl + 2g KH_2PO_4+ 1000ml DDH_2O

(D) Radioactive solution: $^{55}FeCl_3$(Perkinelmer) dissolve with $FeSO_4$, the molar ratio is 1:10, then add 50 times beta-mercaptoethanol and 0.27M sucrose solution.

(2) Procedure

(A) Remove the incubation medium and wash the cells three times by cold PBS.

(B) Add 1ml 0.27 M sucrose solution and 32ul radioactive solution to each well. Incubate them at 37^oC and 5% CO_2 for 30 minutes.

(C) After this incubation, place the cell culture plates on a tray of ice, aspirate the medium, and wash the cell monolayer three times with ice-cold PBS.

(D) Incubate the cells at 37 ^oC for different times with or without hepcidin.

(E) After this incubation, transfer the overlying supernatant (efflux medium) to counting tubes.

(F) Remove the cells from the wells after adding 500ul of cell lysis buffer and use a plastic spatula to detach them.

(G) Votex them and a 50ul aliquot is subjected to detect the protein concentration.

(H) Separate the cytosol from the stromal-mitochondrial membrane (membranes) fraction by centrifugation at 10,000 g for 20 minutes at 4^oC using a Jouan

centrifuge (DJB labcare Ltd., England).

(I) Subsequently separate the cytosol from the membranes and dissolve the pellet with 450ul 1% SDS. Add both fractions into 3ml scintillation solution to count the cpm.

(J) Iron activity in the supernatant or cell fractions was expressed as percentage of the total iron present in the system.

CHAPTER 3

EFFECT OF HEPCIDIN ON THE IRON TRANSPORT PROTEINS EXPRESSION AND IRON UPTAKE AND RELEASE IN THE PERITONEAL MACROPHAGES

3.1 ABSTRACT

Hepcidin is a recently discovered peptide which is made in liver, distributed into plasma and excreted from urine. This peptide functions as an iron homeostatic regulator and presumably acts by regulating the expression or activity of one or more iron transporters on the cell membrane of the intestine, macrophages and liver. In this study, we investigated the effect of this regulatory peptide on iron metabolism of macrophages. First, we investigated the iron uptake related proteins DMT1, TfR1 and the role of hepcidin in iron uptake in macrophages. DMT1 and TfR1 protein expression were up-regulated by hepcidin; the effect was concentration-dependent with the optimal concentration of hepcidin at 700nM. In addition, after hepcidin treatment, both Fe(II) and Tf-Fe uptake were increased. In contrast, hepcidin

dramatically reduced the FPN1 expression on the macrophages. At the same time, the ^{55}Fe efflux from macrophages was decreased after hepcidin treatment. These data suggested that the iron accumulation in macrophages induced by hepcidin maybe due to a decrease of iron release from cells and an increase of iron uptake of cells from the environment.

KEY WORDS

Hepcidin, Macrophages, DMT1, Ferroportin1, Transferrin receptor, Ceruloplasmin, Iron uptake, Iron release

3.2 INTRODUCTION

One of the most distinguishing features of iron metabolism is the degree to which body iron is conserved. With a relatively small daily exchange of iron between the body and the environment, this metal exchanges largely among the internal organs. The reticuloendothelial system (RES) is responsible for this process. The RES, which is mainly comprised of monocytes and tissue macrophages, plays two major roles in iron metabolism: it recycles iron from senescent red blood cells and it serves as a

large storage depot for excess iron.

Macrophages of the RES acquire most iron by ingesting senescent red blood cells. RE iron acquired via erythrophagocytosis that is not utilized or released was firstly stored in ferritin. Cell culture studies using monocytes and macrophages documented the formation of ferritin protein within hours after the red cell ingestion (Bornman et al., 1999; Custer et al., 1982). On the other hand, RES also releases the stored iron into circulation to meet the need of bone marrow. A biphasic pattern of iron release after erythrophagocytosis has been observed in isolated human monocytes (Moura et al., 1998), macrophages (Custer et al., 1982) and cultured rat peritoneal macrophages (Saito et al., 1986).

Recently, our understanding of iron metabolism has been greatly advanced by the identification and characterization of transmembrane iron transport proteins such as natural resistance macrophage-associated protein 1 (Nramp1), divalent metal ion transporter 1 (DMT1) and ferroportin 1 (FPN1). The main function of Nramp1 appears to be the transporter of iron and other metals into and/or out of phagosome (Goswami et al., 2001). DMT1, the apical membrane iron transporter of the duodenum (Gunshin et al., 1997), also localizes to the recycling endosomes where it

transports iron from transferrin into the cytosol (Fleming et al., 1998; Tabuchi et al., 2000). Like Nramp1, DMT1 has been shown to become recruited to the phagolysosome in J774 cells (Gruenheid et al., 1999), suggesting that this protein plays a role in the intracellular iron handling in the macrophage as well. FPN1 is the first cellular iron exporter to be identified (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). The observation that FPN1 is particularly abundant in the RE macrophages of liver, spleen and bone marrow (Yang et al., 2002) supports the hypothesis that this protein may also function to export iron out of macrophages.

Although iron recycling by the RES represents the largest pathway of iron efflux in the body, the precise regulatory mechanisms involved remain elusive. Recently, studies characterizing the function and the regulation of hepcidin have rapidly expanded our knowledge about the molecular aspects of RE iron handling. Hepcidin is a small 25-amino-acid peptide, which is mainly synthesized by the liver, secreted into the bloodstream and excreted through the kidneys. Hepatic hepcidin expression is decreased in iron deficiency (Frazer et al., 2002; Nicolas et al., 2002b) and during stimulated erythropoiesis (Nicolas et al., 2002b); while it is increased in iron overload (Ahmad et al., 2002; Mazur et al., 2003; Pigeon et al., 2001) and during inflammation (Nemeth et al., 2003).

The mature bioactive 25-amino-acid form was proposed to act as the principal iron-regulatory hormone to maintain iron homeostasis (Pigeon et al., 2001). Recently, several groups investigated the mechanism behind hepcidin's influence on intestinal iron absorption at the in vivo and molecular level. An inverse correlation between hepcidin expression and the expression of duodenal iron transporters and iron absorption in rats was found. During the switch from high- to low-iron diet in rats, iron absorption increased, which was accompanied by an increase in duodenal expression of ferric reductase (Dcytb) and the duodenal iron transporters, DMT1 and FPN1. These iron transporters were also abundantly expressed in RE cells, and characterization of their functions is starting to reveal how the RES handles iron at the molecular level.

It is clear that hepcidin could regulate the intestinal iron transport through acting on the tranporters, but there is still little known about the responsibility of the normal macrophages to the hepcidin and there is an absence of evidence about the iron transporters expression after hepcidin treatment. The aim of the present study was to directly examine the role of hepcidin on the iron transport of the macrophages and its related mechanism.

3.3 MATERIAL AND METHODS

3.3.1 Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA. Isotope $^{55}FeCl_3$ was purchased from Perkinelmer Company, Wellesley, MA, USA. The scintillation cocktail and tubes were purchased from Beckman Coulter Company, Fullerton, CA, USA. Tris and prestained protein marker were purchased from Bio-Rad Laboratories, Hercules, CA, USA. ECL Western blotting analysis system was from Amersham Biosciences, England. The antibodies against DMT1+IRE, DMT1-IRE and FPN1 were purchased from Alpha Diagnostic International Company, San Antonio, TX, USA. The purified antibody against CP and mouse anti-rat CD71 monoclonal antibody were purchased from BD Transduction Laboratories, BD Biosciences Pharmingen, USA. The monoclonal antibody against β-actin was from Sigma Chemical Company, St. Louis, MO, USA.

3.3.2 Animals and Samples Collection

Male Sprague-Dawley (SD) rats were supplied by the Animal House of The Hong

Kong Polytechnic University. All the animals were housed in pairs in stainless steel cages at 21 ± 2^{o}C and provided free access to food and water. Rooms were in a cycle of 12 hours of light (7:00 to 19:00) and 12 hours of darkness (from 19:00 to 7:00). All of the animals were fed the Laboratory Rodent Diet (PMI, Brentwood, MO. Catalog# 5001) ad libitum. The shavings and plenty of distilled water were supplied at all times.

The 300–350g (about 8 weeks old) male Sprague–Dawley rats, specific pathogen-free were used. Before experiment, the rats were fasted for 24 hours but given free access to water. They were killed by using diethyl ether anesthesia and the peritoneal macrophages were taken by lavaging the rat peritoneum.

3.3.3 Methods

3.3.3.1 Primary Rat Peritoneal Macrophages (RPM) Culture

Primary rat peritoneal macrophage cell culture was established from the 8-week-old SD rats as previous reported (Cohn and Wiener, 1963; Tsuyuki et al., 2002). Briefly, 50 ml of ice-cold, sterile RMPI 1640 supplemented with 1% non-heat inactivated

fetal bovine serum, 100U/ml penicillin and 100µg/ml streptomycin, were injected intraperitoneally. Following a gentle massage, the abdominal fluid and cells were withdrawn. Cells were collected by centrifugation at 4°C for 10min at 1100 rpm and resuspended in RPMI-1640 containing 10% FBS and 100U/ml penicillin and 100µg/ml streptomycin. The cells were seeded at 4 x 10^6 cells/well for western blot and 1 x 10^6 cells/well for isotope experiments in 35mm culture dishes or 6-well culture plates. After incubation at 37°C in 5% CO_2 atmosphere for 3 hours, the non-adherent cells were removed by gently washing with cold PBS. The plated cells were incubated with RPMI-1640 containing 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin in a 5% CO_2 incubator at 37°C for 24 hours, then the medium was changed to remove the dead cells and the cells were incubated until the day of use. The dishes containing RPM monolayers were then used for various assays after incubating with 70nM and 700nM hepcidin for 8, 16 and 24 hours.

3.3.3.2 Identification of Macrophage by α-Naphthyl Acetate Esterase Stain (Rademakers et al., 1989) (Yam et al., 1971)

Cultured macrophages were identified by the cytochemistry of α-naphthyl acetate esterase method. Cells were seeded (1×10^5) on the glass coverslips contained within

the wells of the 6-well culture plates and maintained as described in the culture of macrophages. After 24 hours incubation, the growth medium was removed and the cells were fixed with 10% formaldehyde. Then the cells were washed with water, and dipped into working buffer to incubate for 60min at 37^{o}C. After washed again, the cells were counterstained for 2 minutes in Hematoxylin solution and rinsed. Then the coverslips were air dried, and the cell types were determined by optical microscopy. As shown in Fig 3-1, more than 95% of the cells were macrophages.

3.3.3.3 Trypan Blue Staining of Cells

The cells were placed in a complete medium without serum and diluted to an approximate concentration of 1×10^5 to 2×10^5 cells per ml. 0.5 ml of this cell suspension was then placed in a screw cap test tube, to which was added 0.1 ml of 0.4% Trypan Blue Stain. The solution was mixed thoroughly and allowed to stand for 5 min at 15-30^{o}C. A hemocytometer was filled with the cell solution for cell counting. Non-viable cells were observed with the stain and viable cells were not stained.

3.3.3.4 Western Blot Analysis

The cells were washed and homogenized in RIPA buffer containing 1% Triton X-100, 0.1% SDS, 1 mM PMSF and protease inhibitors (pepstatin 1µg/ml, aprotinin 1µg/ml, leupeptin 1µg/ml), and then sonicated using Soniprep 150 (MSE Scientific Instruments, England) for 3×10 seconds. After centrifugation at 10,000×g for 15 min at 4 ^{o}C, the supernatant was collected. Protein content was determined using the *DC* Protein Assay kit (Bio-Rad, Hercules, CA). A total of thirty micrograms protein were diluted in 2× sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol) and heated for 5 min at 95 ^{o}C before SDS-PAGE on 10% or 7.5% gel and subsequently transferred to a PVDF membrane (Bio-Rad, CA). After transfer, the membrane was blocked with 5% blocking milk (Bio-Rad, USA) in TBS containing 0.1% Tween 20 for 2 hours at RT or overnight at 4 ^{o}C. The membrane was rinsed in three changes of TBS-T, incubated once for 15 min and twice for 5 min in fresh washing buffer, and incubated with primary antibody for 2 hours at RT or overnight at 4 ^{o}C. The concentration of the rabbit anti-rat DMT1+IRE, DMT1-IRE, FPN1 primary polyclonal antibody (Alpha Diagnostic, USA) was 1:5000, and the purified mouse anti-CP and TfR1 IgG1 (BD Transduction Laboratories, USA) was 1:1000. After three washes in the washing

buffer, the membrane was incubated for 2 hours in horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibody (1:5000, Amersham Biosciences, England) and developed using enhanced chemiluminescence (ECL western blotting analysis system kit, Amersham Biosciences, England). The blot was detected using a Lumi-imager F1 workstation (Roche Molecular Biochemical). The intensity of the specific bands was determined by densitometry with the use of LumiAnalyst 3.1 software (Roche Molecular Biochemical). To ensure even loading of the samples, the same membrane was probed with mouse anti-rat β-actin monoclonal antibody (Sigma-Aldrich, MO) at a 1:5000 dilution.

3.3.3.5 Non-transferrin Bound Iron (NTBI) Uptake Assay

The methods for the preparation of the radiolabelled $^{55}Fe(II)$ solution and measurement of the Fe(II) uptake were as previously described (Qian and Morgan, 1992). The $^{55}Fe(II)$ ($^{55}FeCl_3$, Perkin-Elmer Life Sciences Company, Wellesley City, MA, USA) solution was prepared by mixing $^{55}FeCl_3$ and $^{56}FeSO_4$ in a molar ratio of 1:10, then adding 50-fold molar 2-mercaptoethanol and 0.27M sucrose to give a final concentration of 62.5uM. The incubation medium was the 0.27 M sucrose-4mM pipes, pH 6.5. Following incubation with 700nM hepcidin for 8, 16 and 24 hours

respectively, the cells were washed with cold PBS to avoid contamination with iron. Then the cells were incubated with 1ml 0.27M sucrose containing ^{55}Fe(II) to investigate the uptake of non-transferrin bound iron into the cells. At each time point, the ^{55}Fe(II) solution was added in an amount required to give the desired final concentrations and incubated for varying periods of time. After the cells were lysed for 10min at room temperature with 500ul cell lysis buffer, the cells were scraped off and transferred into Eppendorf tubes. A 50ul aliquot was subjected to detect the protein concentration. The cytosol was separated from the stromal-mitochondrial membrane (membranes) fraction by centrifugation at 10,000 g for 20 minutes at 4^{o}C using a Jouan centrifuge (DJB labcare Ltd., England). The cytosol was subsequently separated from the membranes and the pellet was dissolved with 1% SDS. All fractions was added 3ml scintillation solution to count the cpm. The blank group was added 1% SDS into 3ml scintillation solution.

3.3.3.6 Iron Release Assay

The ^{55}Fe(II) solution was prepared as described in the non-transferrin bound iron uptake assay. After washing three times with PBS, the macrophages (about 1 × 10^{6}cells/well) were incubated with ^{55}Fe(II) solution (2uM) for 30 minutes at 37^{o}C

and washed three times with PBS at 4^{o}C. The cells were then incubated at 37^{o}C for 2 hours, 8 hours, 16 hours and 24 hours with or without 700nM hepcidin (Peptides International, Inc. Louisville, Kentucky, USA) and with 0.1% BSA. After that, the medium was collected and measured following centrifugation. The cells were detached by 500ul lysis buffer. A 50ul aliquot was subjected to detect the protein concentration. The cytosol was separated from the stromal-mitochondrial membrane (membranes) fraction by centrifugation at 10,000 g for 20 minutes at 4^{o}C. The cytosol was subsequently separated from the membranes and the pellet was dissolved with 450ul 1% SDS. All fractions were added into 3ml scintillation solution to count the cpm. The sum of the radioactivity in the medium and in the cell (cytosol and pellet) was named as the total cellular radioactivity. The relative percentage of total radioactivity in the medium and in the cell was calculated.

3.3.3.7 Transferrin Binding Iron (Tf-Fe) Uptake Assay

Loading of rat apo-Tf with ^{55}Fe

The reaction was done as previously described (Roy et al., 1999). $^{55}FeCl_3$ (Perkin-Elmer Life Sciences Company, Wellesley City, MA, USA) was complexed

to nitrilotriacetic acid in 1:10 ratio (Fe:NTA). Then the ^{55}Fe-NTA was incubated with Tf in 2:1 ratio for 3 hours in a carbonate buffer (10 mM $NaHCO_3$, 250mM Tris-HCl). The ^{55}Fe-transferrin was separated from the free ^{55}Fe by stirring in 250ml dialysis buffer at 4^{o}C for 7 times and each time lasted for 3 hours.

^{55}Fe-Tf Uptake Protocol

1×10^6cells were seeded in 6-well culture dishes containing culture medium with or without (controls) 700nM hepcidin for 8, 16 and 24 hours. Before labelling, the cells were starved in 1ml culture medium without FBS (but with 0.1% BSA) for 1 hour, and followed by the addition of 10ug/ml ^{55}Fe-Tf. After incubated for 60 minutes at 37^{o}C, the cells were washed with cold PBS two times. The external bound Tf was stripped with an acid buffer (0.2 N acetic acid, 500 mM NaCl, 1 mM $FeCl_3$). The cells were solubilized in 500ul solubilization detergent same with described above. A 50ul aliquot was subjected to detect the protein concentration. The cytosol was separated from the stromal-mitochondrial membrane (membranes) fraction by centrifugation at 10,000 g for 20 minutes at 4^{o}C. The cytosol was subsequently separated from the membranes and the pellet was dissolved with 450ul 1% SDS. Both fractions were added into 3ml scintillation solution to count the cpm.

3.3.4 Statistical Analysis

Statistical analyses were performed using SPSS software for Windows (version 10.0). Data were presented as mean±SD. The difference between the means was determined by One-Way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. Differences with $P<0.05$ were considered significant.

3.4 RESULT

3.4.1 Effect of Hepcidin on DMT1 Protein Expression

There are two isoforms of DMT1 protein expressed in cells. Both forms are proteins with 12 putative membrane-spanning domains that are presumed to be located at the cell membrane and/or within the endosomal fraction of the cell. To determine whether hepcidin affects these proteins expression, the peritoneal macrophages grown in 35mm culture dishes were treated with 70nM to 700nM hepcidin for 8, 16 and 24 hours. Western blot analysis was quantified by densitometry and total protein loading was corrected using β-actin expression. Major bands were observed with these antibodies, with a molecular weight (*M*r) of ～56 kDa for DMT1+IRE

(Fig3-2A), ～60 kDa DMT1-IRE (Fig3-3A) and ～45 kDa for β-actin, which was in good agreement with the expected molecular weights based on published papers. The result demonstrated that there was no significant change in the expression of the DMT1+IRE protein. Similarly, hepcidin had no effect on the expression of the housekeeper protein, β-actin. Although there was little change of the DMT1+IRE protein, there were seemingly some changes ($P<0.05$) in the expression of the DMT1–IRE protein in the macrophage cells. As shown by the western blot, following 70nM and 700nM hepcidin treatment for 8 hours, the DMT1-IRE protein expression was slightly decreased. An expansion of the study showed that when raised the hepcidin incubation time to 16 hours, there was an increase of DMT1-IRE protein expression, especially when the concentration of hepcidin reached 700nM, the DMT1-IRE protein on the macrophage cells recovered and rose above the pre-hepcidin levels. To continue the incubation to 24 hours, there was no significant change in the DMT1-IRE protein with low concentration hepcidin treatment (70nM), but the DMT1-IRE protein level reached to 137% of the control after the 700nM hepcidin treatment ($P<0.01$)(Fig 3-3 B and C).

3.4.2 The Non-transferrin Bound Iron (NTBI) Uptake of Macrophages

Before performing the Fe(II) uptake in macrophages, we first established conditions for studying the Fe(II) uptake.

3.4.2.1 Effect of Iron Concentration on the NTBI Uptake

The concentration of the Fe(II) used varied from 0.05 to 5uM and the incubation time at 37^{o}C was 30 minutes. As shown in Fig3-4, when macrophages were incubated with an increasing concentration of Fe(II), the rate of uptake of Fe(II) into the cytosolic and stromal fractions of macrophages showed evidence of saturation at an iron concentration of 2.5 uM, but there was a little increase when further increased the Fe(II) concentration. This was evidence of both a saturable or specific process and a non-saturable or non-specific process.

3.4.2.2 Time Course of Fe(II) Uptake

The time of Fe(II) uptake by macrophages was optimal when incubation was performed under the standard condition (isotonic sucrose solution, pH 6.4-6.5, 2uM Fe(II)). The samples were taken out to measure the radioactivity within the cells (cysotol) and in the membrane (stroma) at certain minutes of intervals. The result

showed that Fe(II) was acquired by the cells in a linear manner ($y=0.0008x+0.241, R^2=0.9554$) throughout the 60-minute incubation period (Fig3-5). The iron incorporation into stroma was approximately 50% of that into the cytosol at each time point. Since iron uptake is linear within 30 minutes, the NTBI uptake experiments were performed by taking a single 30-minute time course and incubated with 2uM Fe(II).

3.4.2.3 Effect of Hepcidin on the Fe(II) Iron Uptake of Macrophages

Since preincubation of hepcidin changed the DMT1 protein level in macrophages, which was related to the Fe(II) uptake, we then studied whether the effects of hepcidin on DMT1 expression may have implications on the NTBI uptake of the macrophages. Purified macrophages were preincubated with or without (controls) 700nM hepcidin for 8, 16 and 24 hours respectively. At the desired time point, the $^{55}Fe(II)$ solution was added to the incubation medium to give the final concentration of 2uM and it was then incubated at 37^oC for 30 minutes. As it was evident from Fig3-6, there were no significant differences between the untreated and the hepcidin-treated 8 hours group in both cytosolic and stomal fractions. When the hepcidin incubation time was increased, the treatment of cells with hepcidin

significantly increased the uptake of radiolabeled Fe(II) by macrophages from the culture medium, and the separation of the cytosolic and the stromal fractions indicated that this was mainly due to the increase of the cytosolic iron uptake. When incubation was further prolonged to 24 hours, the Fe(II) uptake into cytosol was increased to about 125% of the control ($P<0.01$), which was paralleled to the increase of the DMT1 protein expression.

3.4.3 Effect of Hepcidin on Transferrin Receptor 1 (TfR1) Protein Expression

Iron is delivered to most cells via endocytosis of transferrin bound to their cell surface receptors, whereas most of the iron recycled by the RES after erythrophagocytosis through the same way. The iron-binding capacity of transferrin and the level of transferrin receptor on the cells affect the iron uptake of macrophages. The transferrin receptor 1 is a dimer of 90kDa subunits (Parkkila et al., 1997). The expression level of transferrin receptor 1 was detected using a special antibody, and the western blot result showed that after incubation with 70nM hepcidin for 8 and 16 hours, there were no significant differences in the expression of the transferrin receptor 1 protein between the control and the treated groups. But

when the incubation time was prolonged to 24 hours and the concentration of hepcidin increased to 700nM treatment for 16 and 24 hours brought a significant increase of the transferrin receptor 1 protein expression ($P<0.05$) (Fig 3-7).

3.4.4 Effect of Hepcidin on the Tf-Fe Iron Uptake of Macrophages

In parallel, we also studied the effect of hepcidin on the Tf mediated iron uptake in macrophages. These cells were incubated with or without (controls) 700nM hepcidin for 8, 16, 24 hours before the addition of ^{55}Fe-Tf (10ug/ml) into the incubation medium and incubated for 60 minutes at 37^{o}C. The intracellular radioactivity (cytosol) and the membrane-pellet (stoma) were measured respectively as described above. The result demonstrated that pre-treatment of cells with hepcidin for 16 hours and 24 hours resulted in an increase of cellular Tf-Fe uptake. The difference between the Tf-Fe uptake of each experimental and control group was statistically significant ($P<0.05$). At the 16 hours, the iron uptake of the control was 36.38±6.32 pmol/ug protein, while in the hepcidin treated group, the Tf-Fe uptake increased to 42.25±7.04 pmol/ug protein (116% of control). After the 24-hour incubation, the iron uptake of the control was 37.4±4.64 pmol/ug protein，while in the hepcidin treated group, the Tf-Fe uptake increased to 48.8±6.72 pmol/ug protein (130% of control).

However, no significant difference was found between the control group and the experimental group with the hepcidin treatment for 8 hours.

3.4.5 Effect of Hepcidin on Ferroportin1 (FPN1) Protein Expression

The most important role of RES is to serve as a large depot for iron storage after the senescence of red blood cells and to release the stored iron into plasma to meet the need of the bone marrow. Normal iron release does seem to require FPN1, an iron export protein. The expression profile of FPN1 in macrophages (Yang et al., 2002) suggests that the protein plays an important role in iron recycling in the RES. Hepcidin, a negative iron regulator, can reduce iron release from RES. To investigate the mechanism, we firstly detected the FPN1 protein expression on the macrophages. By western blot, we detected a single expected band with an approximate relative *M*r of ～62 KDa. Analysis of the bands showed that hepcidin could induce a decrease in ferroportin1 protein expression at both concentrations (70nM and 700nM). Before hepcidin treatment, there was a high basal level of FPN1 expression in the macrophages. After 8h incubation, the expression of FPN1 protein decreased markedly; with the level of FPN1 dropping to as low as 30% of the control at the hepcidin concentration of 700nM. But following 16 hours incubation, the protein

expression increased. By the treatment of 70nM hepcidin for 24 hours, the FPN1 had recovered to the pre-hepcidin levels, but the level of FPN1 of the 700nM hepcidin treating group was still lower than that of the control (Fig3-9). A more detailed investigation about the FPN1 protein expression with a high concentration of hepcidin (700nM) treatment was carried out. We found that the FPN1 protein began to decrease even after the hepcidin treatment for 1 hour, and it continued to decrease in the following 2, 4 and 6 hours. After incubation for 8 hours, the level FPN1 protein reached the lowest, and afterwards, there was an increase of this protein expression. By 24 hours, the FPN1 had recovered to about 80% compared with the control (Fig 3-10).

3.4.6 Effect of Hepcidin on Ceruloplasmin Protein Expression

Iron release from RES also required CP. Direct evidence of the role of CP in RES iron release was provided by a study of CP $^{(-/-)}$ mice (Harris et al., 1999). In our studies, in order to investigate whether the expression of CP was influenced by hepcidin, we used the western blot to analyse the CP protein level. We detected one expected band with a relative molecular weight (*Mr*) of ～150 KDa. Analysis the band showed that the CP protein expression did not change after treatment with

70nM hepcidin for 8, 16 and 24 hours, or with 700nM hepcidin for 8 hours and 16 hours. Some increase in the CP expression was observed only with 700nM hepcidin incubation for 24 hours (Fig 3-11).

3.4.7 Effect of Hepcidin on the Iron Release of Macrophages

Since FPN1 was the only way for iron to come out of cells, we next examined whether the hepcidin-induced FPN1 decrease would affect the iron release from the primary cultured macrophages. In order to further investigate the role of hepcidin in the iron release of macrophages, the cells were loaded with iron and incubated with or without 700nM hepcidin for 2, 8, 16 and 24 hours. After incubation with hepcidin for 2 hours and 8 hours, the iron release from the cells into the medium were 94% and 86% of the control respectively ($P<0.05$). These were consistent with the changes of the FPN1 protein expression. Investigation about the iron distribution also indicated that there was an increase of iron retention in the cysotol at the same time. When the incubation time increased to 16 hours and 24 hours, the iron continued to release into the medium, but there were no significant differences between the hepcidin treated group and the control group ($P>0.05$).

3.5 DISCUSSION

The RES, which is also known as the “mononuclear phagocyte system”, is composed of monocytes, macrophages and their precursor cells. Monocytes arise from progenitor cells in the bone marrow and are released into the blood. After migration to different tissues, they differentiate into macrophages with characteristic morphologic and functional qualities. It is too laborious to extract pure macrophages from tissues, and cultivated cell populations show different degrees of activation and differentiation (Olynyk and Clarke, 1998). The splenocyte cells, which are enriched for macrophages, are in reality consist of a variety of cell types. Peripheral blood monocytes can be obtained relatively easily but it has to be done either before or after differentiation to macrophages. More readily available source of macrophages is from the peritoneum. This kind of cell had been used as macrophage cell model to study the iron metabolism of RES in many papers (Kuriyama-Matsumura et al., 1998). So in our experiment, we separated the peritoneal macrophages according to the methods reported by Cohn (Cohn and Wiener, 1963; Tsuyuki et al., 2002). The α-naphthyl acetate esterase staining indicated that more than 95% cells were macrophages.

The prevailing serum hepcidin concentration is not exactly clear. Extrapolations from urinary measurements suggested that the circulating hepcidin was within the nanomolar range (Park et al., 2001). However, Dallalio's study indicated that the serum levels could be much higher, in the mid- to high-micromolar range (Dallalio et al., 2003). In 2005, Rivera et al estimated that hepcidin exerts its hypoferremic activity at blood concentrations of the 0.1 to 1 uM range, based on the blood levels of hepcidin after the intraperitoneal administration of 50 ug hepcidin (Rivera et al., 2005b). This concentration was also consistent with the concentration of hepcidin proposed by Nemeth (Nemeth et al., 2004b). The concentration of human synthetic 25 amino acids hepcidin used in our study (70nM, 700nM) sat between these 2 extremes.

The antimicrobial peptide hepcidin has emerged as a major hormonal regulator of systemic iron homeostasis. There are overwhelming genetic data which suggest that hepcidin negatively regulated dietary iron absorption in the duodenum and the recycling of iron from senescent red blood cells via reticuloendothelial macrophages (Fleming, 2005; Ganz, 2004). Another noteworthy finding is that hepcidin appears to affect iron storage in the RES (Nicolas et al., 2001). Some evidences showing that lipopolysacchride also enhances hepatic hepcidin mRNA expression raises the

possibility that the impaired RE iron release of ACD is mediated through changing the plasma hepcidin levels. However, in the absence of hepcidin, RE iron release is expected to continue unabated as liver iron accumulates. The negative regulatory effect of hepcidin *in vitro* is consistent with the observations that constitutive hepcidin expression prevents iron loading in $Hfe^{-/-}$ mice (Nicolas et al., 2003), a murine model of HFE-associated hemochromatosis, and that $Usf2^{-/-}$ mice lacking hepcidin gene expression develop severe tissue iron overload (Nicolas et al., 2001). In our project, we investigated the expression of these iron transport related proteins following hepcidin treatment and the related changes in iron uptake and release in macrophages.

Iron can be taken up by macrophages in several forms via different pathways. These include transferrin receptor-mediated uptake of transferrin bound iron, CD163 receptor-mediated uptake of hemoglobin-hepatoglobin complexes and phagocytosis-mediated uptake of erythrocytes, large iron complexes and iron-containing pollution particles. The presence of the transmembrane iron importer DMT1 in peritoneal macrophages suggested that DMT1-mediated transport could be another iron uptake pathway of these cells. In our experiments, we first detected the DMT1 protein level and NTBI uptake after hepcidin treatment. Our data presented here showed that

hepcidin had little effect on the expression of the DMT1+IRE, but had a significant regulation of the expression of DMT1-IRE protein as well as NTBI uptake. Similar studies by Laftah et al (Laftah et al., 2004) also demonstrated that hepcidin specifically decreased iron uptake across the apical surface of the $CaCO_2$ epithelial layer by depressing the DMT1 protein expression.

As we known, iron is delivered to most tissues via endocytosis of transferrin bound to its cell surface receptor. Isolated human monocytes express transferrin receptors (Bjorn-Rasmussen et al., 1985) and are able to take up iron from transferrin (Sizemore and Bassett, 1984). When cultured monocytes differentiate into macrophages, the expression of transferrin receptor increased greatly (Andreesen et al., 1984). Transferrin-binding activity has also been demonstrated in various macrophages from mice (Hamilton et al., 1984), rats (Nishisato and Aisen, 1982) to humans (Andreesen et al., 1984; Montosi et al., 2000; Testa et al., 1987). TfR1 plays an important role in the iron uptake of the RES. Unlike DMT1, we found that a low concentration of hepcidin had little effect on the TfR1 protein expression, but if the concentration was increased to 700nM, the TfR1 protein began to increase at 8 hours, and reached the maximal level after the hepcidin treatment for 24 hours. There was a significant increase which was about 40% higher than that of the control. To

determine whether this increase affected the iron uptake of the macrophages, we detected the Tf-Fe uptake after the hepcidin incubation. The data indicated that hepcidin could increase the transferrin binding iron uptake in a time-dependent manner, which was consistent with the changes of TfR1 protein. To our knowledge, this was the first report about the effect of hepcidin on TfR1.

In normal adult humans, about 80% of the circulating iron is en route between the RES and the bone marrow. A small amount of plasma iron is contributed by the hepatic iron stores and by the absorption of dietary iron from the duodenum, but most circulating iron is contributed by the RES through the release of iron from the catabolism of senescent red cells. FPN1 is a newly discovered transmembrane iron export protein. It is expressed on the surface of the absorptive intestinal enterocytes, macrophages, hepatocytes and placental cells, all of which release iron into plasma (Abboud and Haile, 2000; Donovan et al., 2000). A recent double immunofluorescence staining using antibodies to FPN1 and F4/80, a macrophage-specific cell surface antigen, has confirmed the localization of FPN1 to RE cells (Yang et al., 2002). Contrasted to enterocytes, FPN1 had been shown in the intracellular vesicles in macrophages. New observations of Delaby indicated that ferroportin1 is expressed in the vesicular compartments that can reach the plasma

membrane of the primary culture of the bone marrow-derived macrophages (Delaby et al., 2005; Rivera et al., 2005b). The expression profile of FPN1 in macrophages suggests that the protein plays an important role in iron recycling in the RES. Consistent with this possibility is the observation that loading the RES with iron dextran enhances FPN1 expression in the mouse Kupffer cell (Abboud and Haile, 2000). Nonetheless, recent clinical reports continue to support the link between FPN1 and RE iron metabolism. Patients with FPN1 mutations exhibit an autosomal dominant form of hemochromatosis (Montosi et al., 2000; Njajou et al., 2001).

In this study hepcidin was used to examine its effects on FPN1 expression. Similar to the report by Nemeth *et al*, our study revealed that treatment with hepcidin rapidly induced a dramatic reduction in the FPN1 protein levels. The suppression of FPN1 expression might result from an increase of degradation or impaired synthesis. Nemeth et al gave the explanation of the mechanisms involved in these findings, which demonstrated that FPN1 expression is post-translationally regulated by hepcidin (Nemeth et al., 2004b). In epithelial cells expressing GFP-ferroportin fusion protein, hepcidin was shown to decrease ferroportin1 expression through a direct protein-protein interaction leading to the internalization and degradation of this protein in lysosomes (Nemeth et al., 2004b). The post-translational regulation of

ferroportin1 by hepcidin may thus complete a homeostatic loop: Iron regulates the secretion of hepcidin, which in turn controls the concentration of ferroportin1 on the cell surface. Importantly, our result showed that the loss of FPN1 was associated with a decrease in ^{55}Fe release after the loading of $^{55}Fe(II)$. Similar results were also found in the macrophage cell line J774 (Knutson et al., 2005), in which hepcidin induced a rapid drop in ferroportin1 protein and significantly reduced the efflux of ^{59}Fe after erythrophagocytosis. In other cells, such as the THP1 monocytes (Andriopoulos and Pantopoulos, 2006) and the bone marrow derived macrophages (Delaby et al., 2005), hepcidin displayed the same effect.

Normal iron release does seem to require ceruloplasmin, a multicopper ferroxidase. CP appears to mobilize iron from storage sites by catalyzing the oxidation of ferrous iron to the ferric form, which can be incorporated into apotransferrin (Osaki et al., 1971). Direct evidence of the role of CP in RES iron release is provided by a study of the ceruloplaminemic ($CP^{-/-}$) mice (Harris et al., 1999). As in copper-deficient animals, the serum iron concentration of the $CP^{-/-}$ mice does not change significantly after the administration of damaged red cells, but increases after the administration of ceruloplasmin but not apoceruplasmin. The observation that the Kupffer cells of the $CP^{-/-}$ mice display markedly increased iron levels is also consistent with a role for

ceruloplasmin in RE iron release (Harris et al., 1999). Interestingly, in our studies, the CP protein was not significantly affected by hepcidin until the 700nM hepcidin treatment for 24 hours. The possible reason for the changes of CP was not consistent with the response of FPN1 and the involved mechanism was still not known.

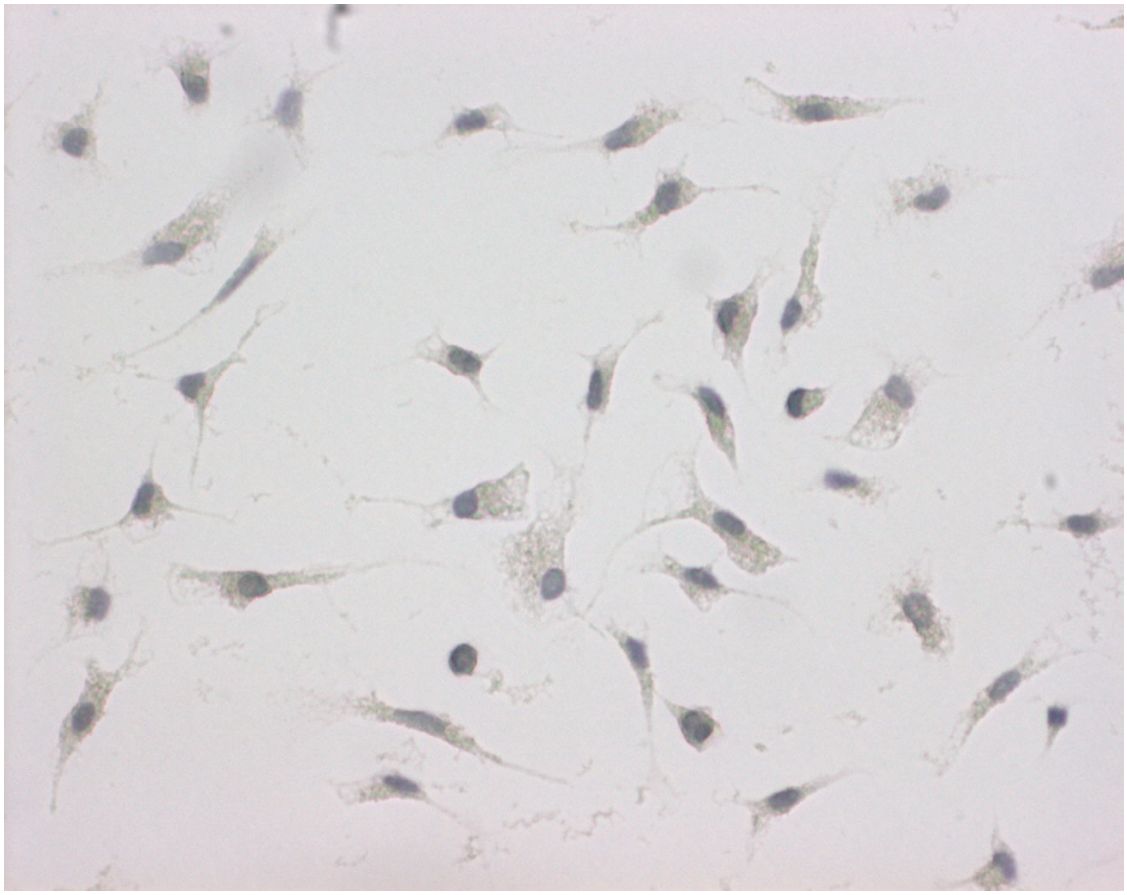


Figure 3-1. Cytochemical detection of non-specific esterase (NSE) in primary culture macrophages. After 24 hours in culture, more than 95% of the cells were positive for NSE staining. Magnification 400×

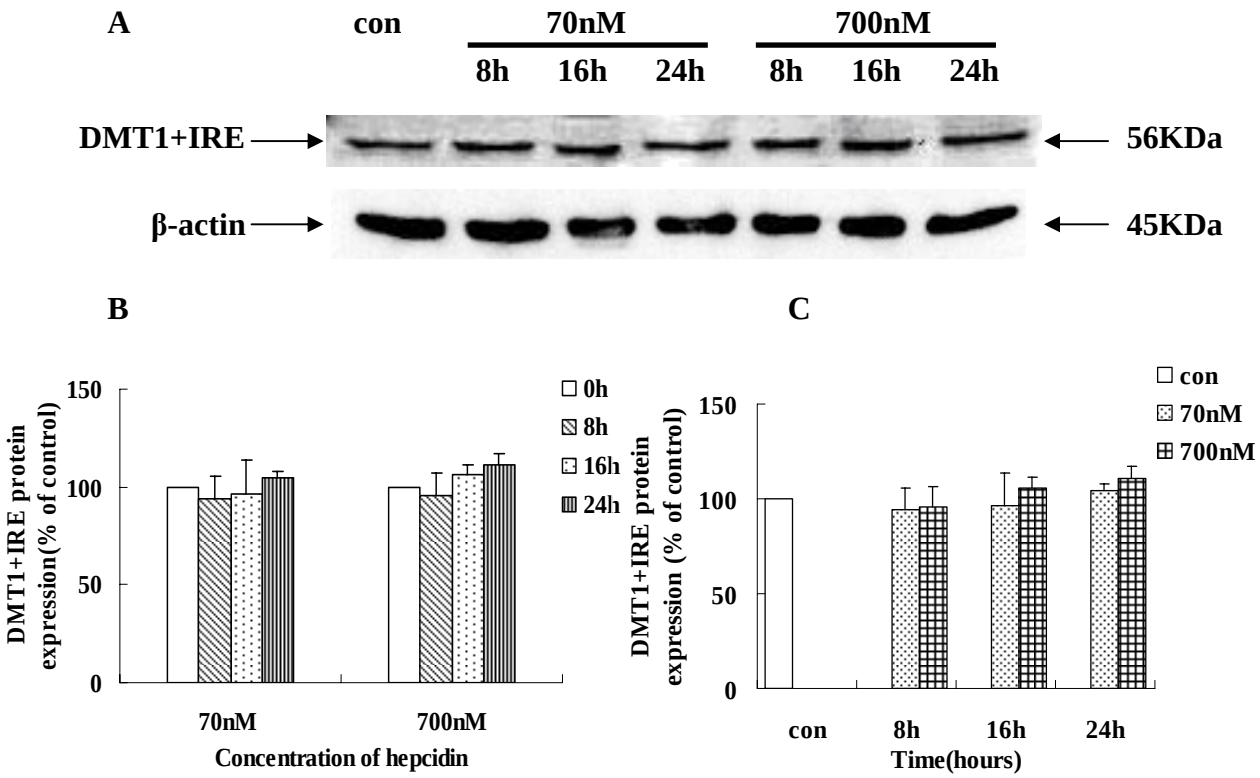


Figure 3-2. Effect of hepcidin on DMT1+IRE protein expression in macrophages. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~56kDa for DMT1+IRE and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the DMT1+IRE protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 3 independent experiments.

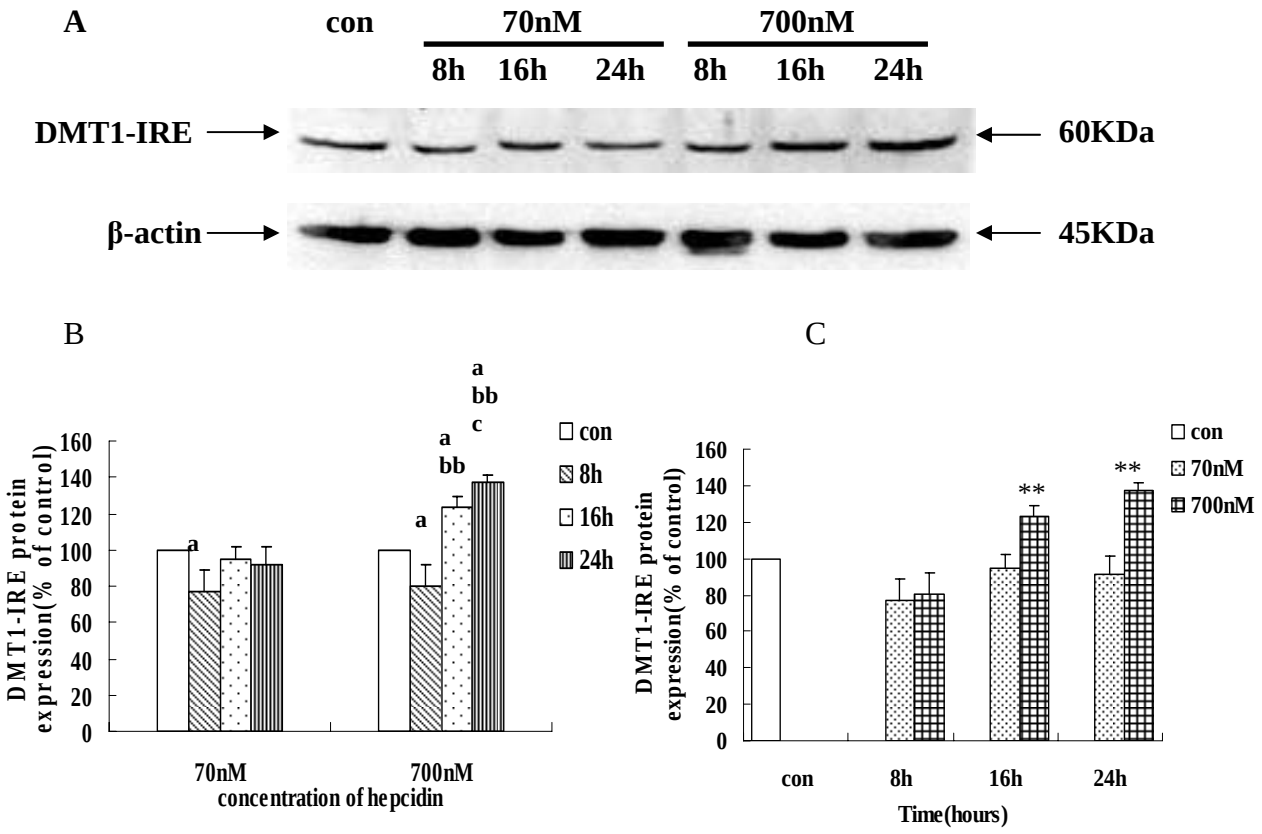


Figure 3-3. Effect of hepcidin on DMT1-IRE protein expression in macrophages. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~60kDa for DMT1-IRE and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the DMT1-IRE protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs controls; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other incubated with hepcidin for the same time (*P <0.05, **P <0.01).

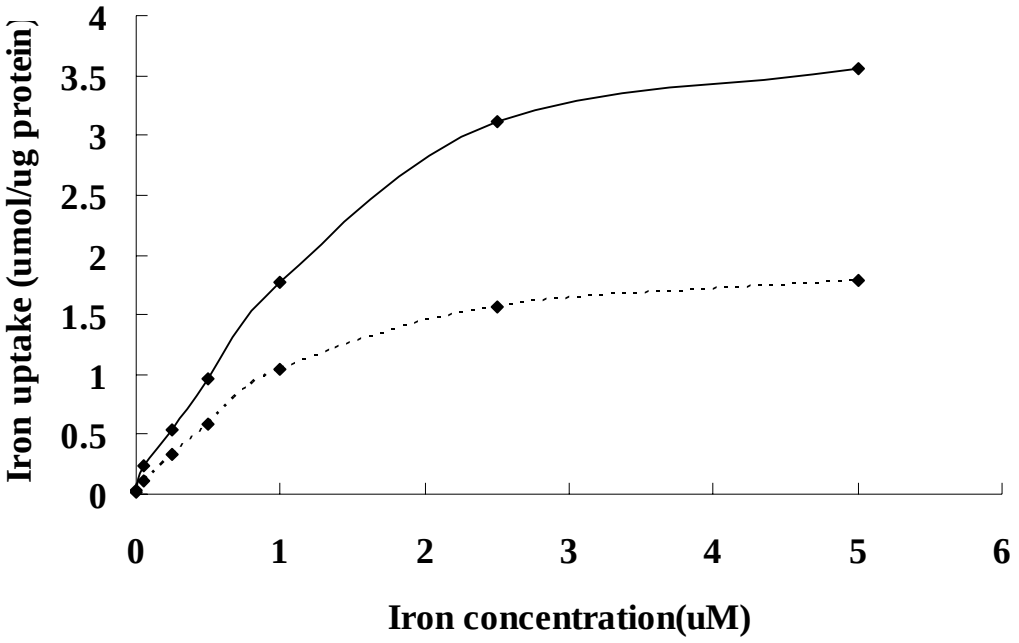


Figure 3-4. Effect of the various concentrations of the Fe(II) on the rate of iron uptake into cytosolic（◆ — ◆）and stromal （◆ - - ◆） fractions of macrophages. The cells were incubated with different concentrations of iron under the control conditions (0.27M sucrose, pH 6.5) for 30 minutes at 37^{o}C, and the measurement of iron uptake was as described in the Materials and Methods. Each point was the mean of three experiments.

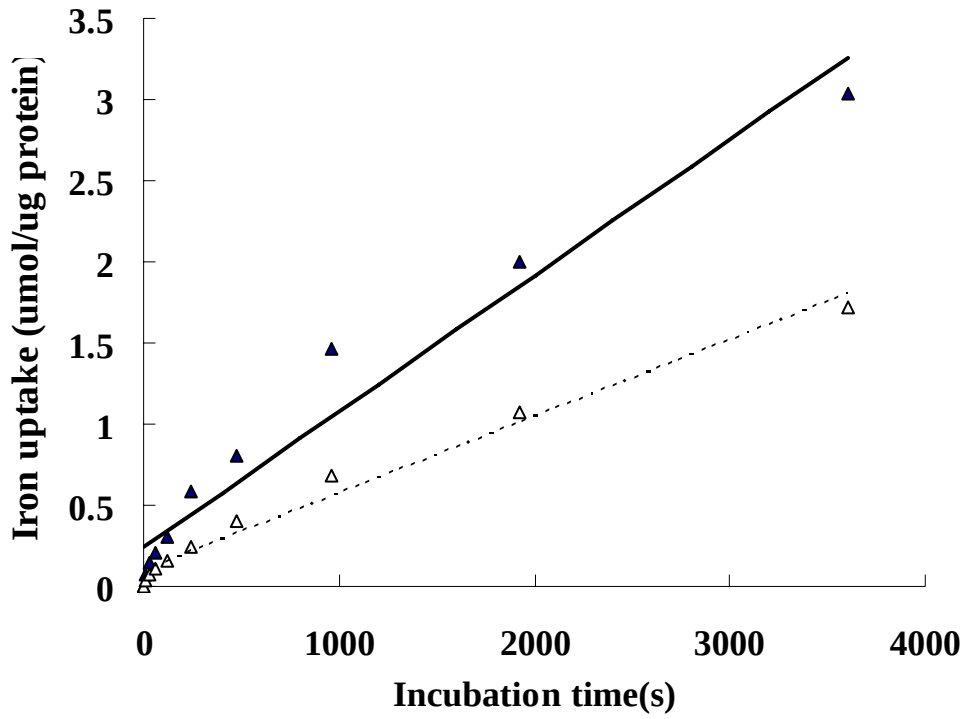
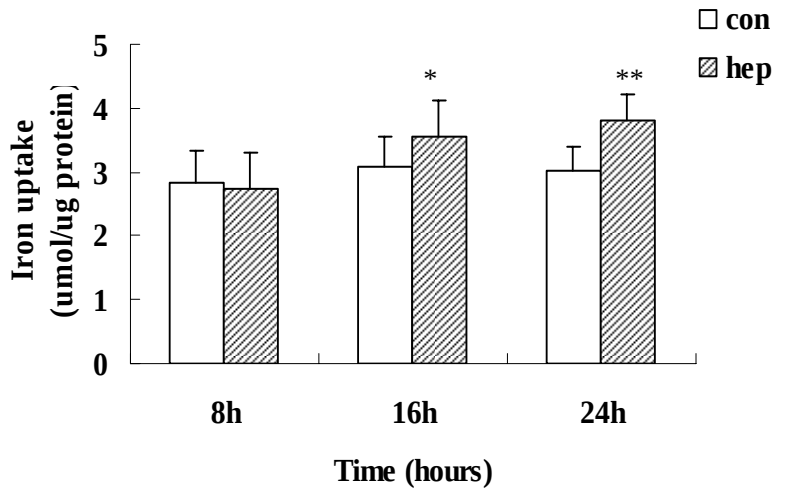


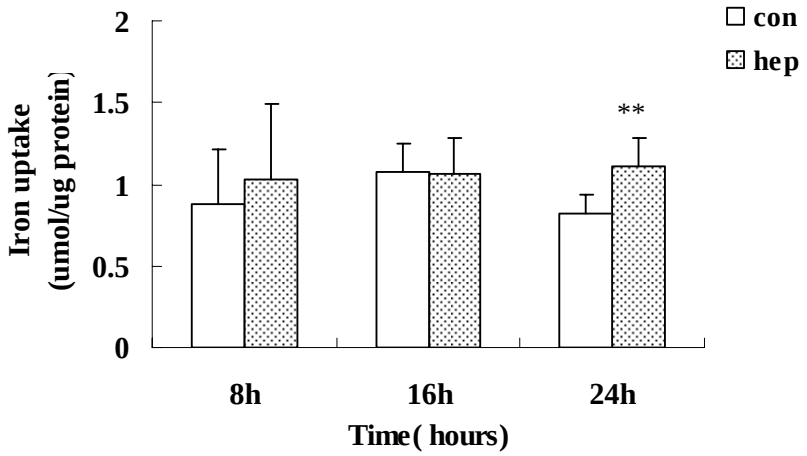
Figure 3-5. Time course of the Fe(II) uptake.

The macrophages were incubated with 2uM Fe(II) under the control conditions (0.27M sucrose, pH 6.5) at 37^{o}C. At the indicated time points, the cells were washed and the measurement of iron uptake into cytosolic（▲） and stromal（△）fractions of macrophages was as described in the Materials and Methods. Each point was the mean of three experiments.

A


con
hep
Iron uptake (umol/ug protein)
0
1
2
3
4
5
*
**
8h
16h
24h
Time (hours)

B


con
hep
Iron uptake (umol/ug protein)
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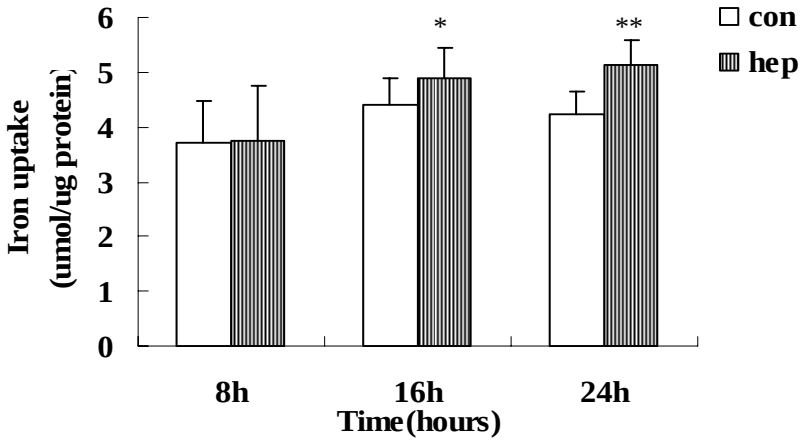
0.5
1
1.5
2
**
8h
16h
24h
Time(hours)

C


con
hep
Iron uptake (umol/ug protein)
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2
3
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5
6
*
**
8h
16h
24h
Time(hours)

Figure 3-6. Effect of hepcidin on the NTBI uptake in macrophages.

The macrophages were preincubated with or without 700nM hepcidin for 8 hour, 16 hours and 24 hours. At the indicated time points, the cells were washed and incubated with 2uM Fe(II) under the control conditions (0.27M sucrose, pH 6.5) at 37^{o}C for 30 minutes. The iron taken into cytosolic (A) and stromal (B) fractions was measured as described in the Materials and Methods. The total of the iron uptake (C) was the sum of the two parts. All these data were presented as mean$\pm$SD of 6 independent experiments performed in triplicate. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

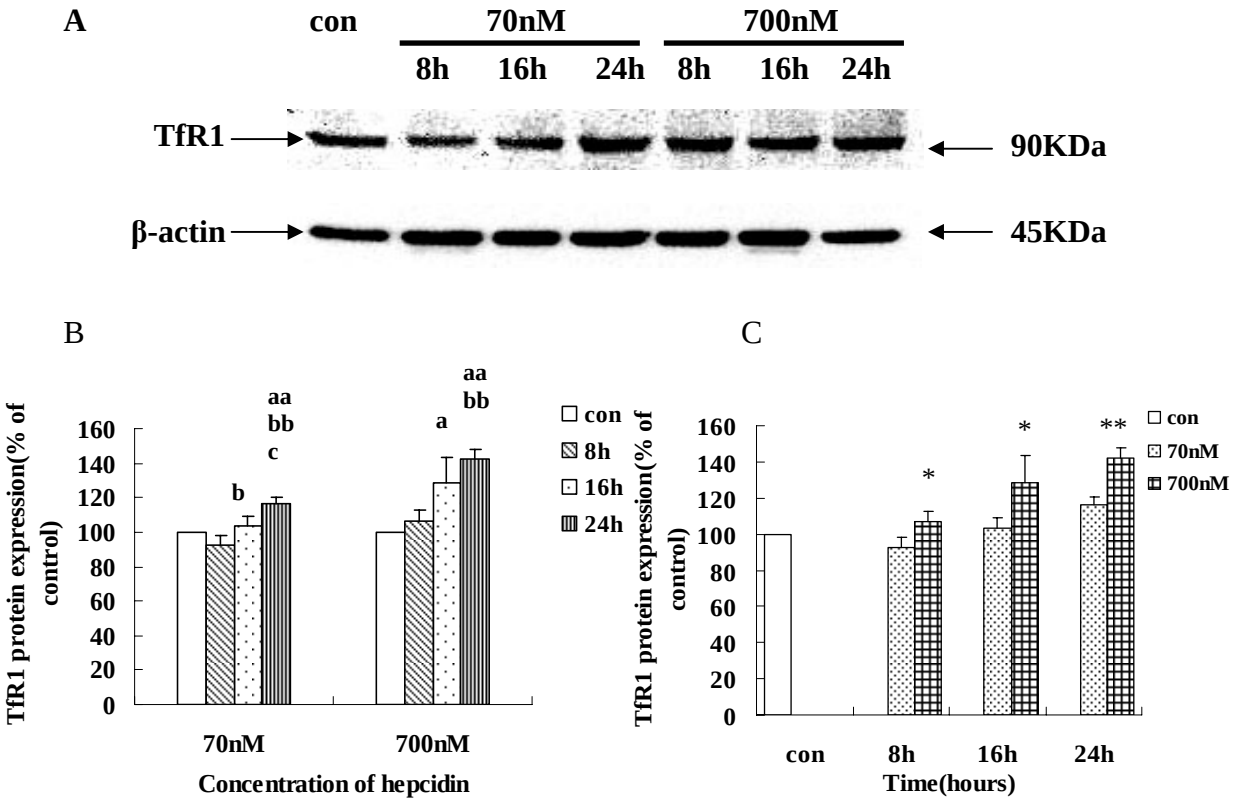
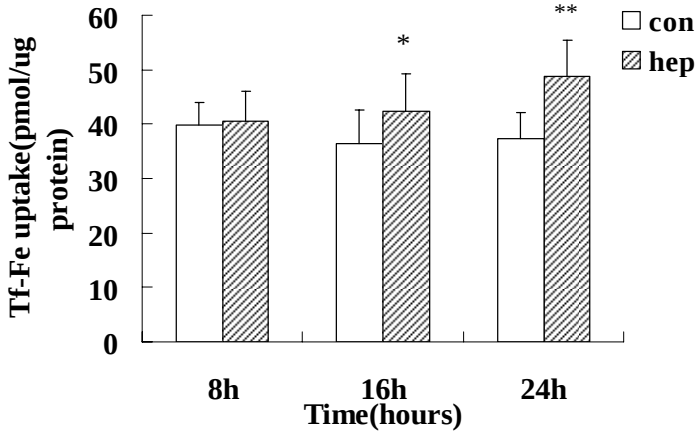


Figure 3-7. Effect of hepcidin on TfR1 protein expression in macrophages.

Western blot analysis was performed as described in Materials and Methods. The *M*r of ~90kDa for TfR1 and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the TfR1 protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs controls; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other incubated with hepcidin for the same time (*P <0.05, **P <0.01).

A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Tf-Fe uptake(pmol/ug protein)
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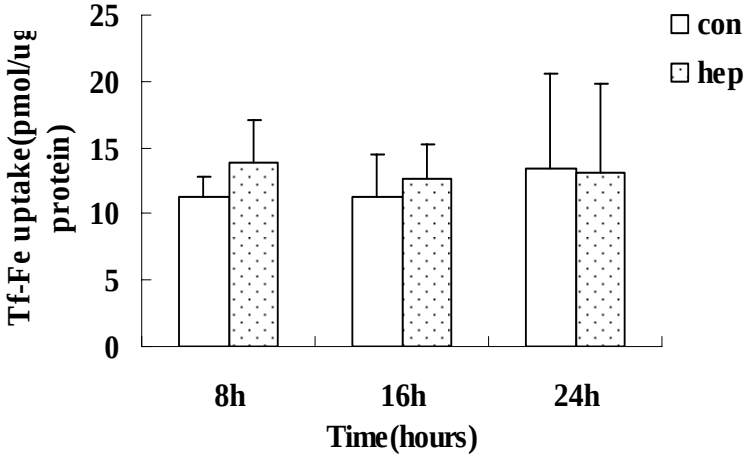
con
hep
8h
16h
24h
Time(hours)

B


25
20
15
10
5
0
Tf-Fe uptake(pmol/ug protein)
con
hep
8h
16h
24h
Time(hours)

C

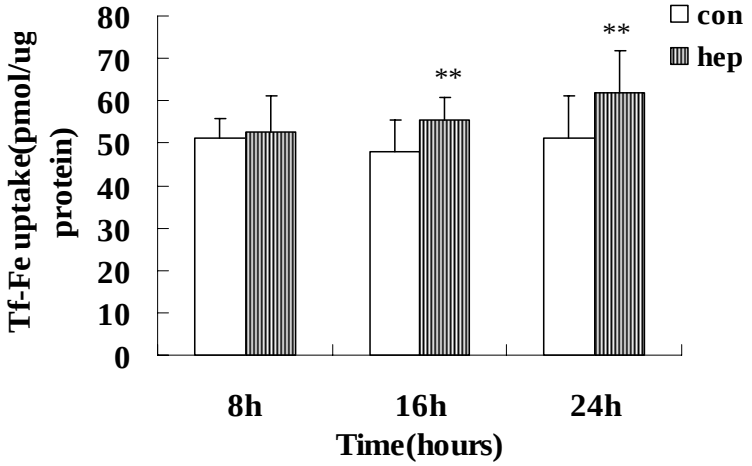

80
70
60
50
40
30
20
10
0
Tf-Fe uptake(pmol/ug protein)
**
**
con
hep
8h
16h
24h
Time(hours)

Figure 3-8. Effect of hepcidin on the Tf-Fe uptake in macrophages.

The macrophages were preincubated with or without 700nM hepcidin for 8 hours, 16 hours and 24 hours. At the indicated time points, the cells were removed the endogenous transferrin and incubated with serum-free culture medium with 10ug/ml Tf-Fe at 37^{o}C for 60 minutes. The iron taken into cytosolic (A) and stromal (B) fractions was measured as described in the Materials and Methods. The total of iron uptake (C) was the sum of the two parts. All these data were presented as mean$\pm$SD of 6 independent experiments performed in triplicate. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

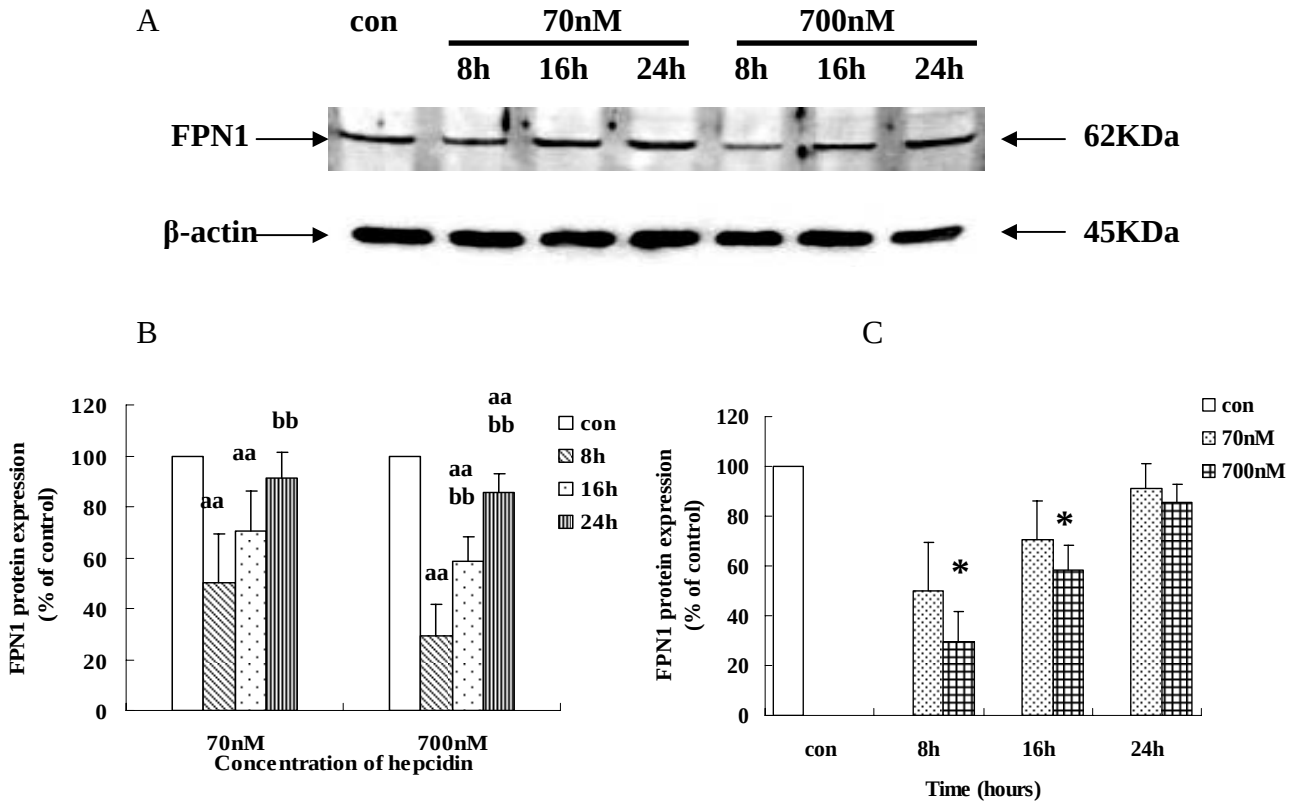


Figure 3-9. Effect of hepcidin on FPN1 protein expression in macrophages.

Western blot analysis was performed as described in Materials and Methods. The *M*r of ~62kDa for FPN1 and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the FPN1 protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs controls; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other incubated with hepcidin for the same time (*P <0.05, **P <0.01).

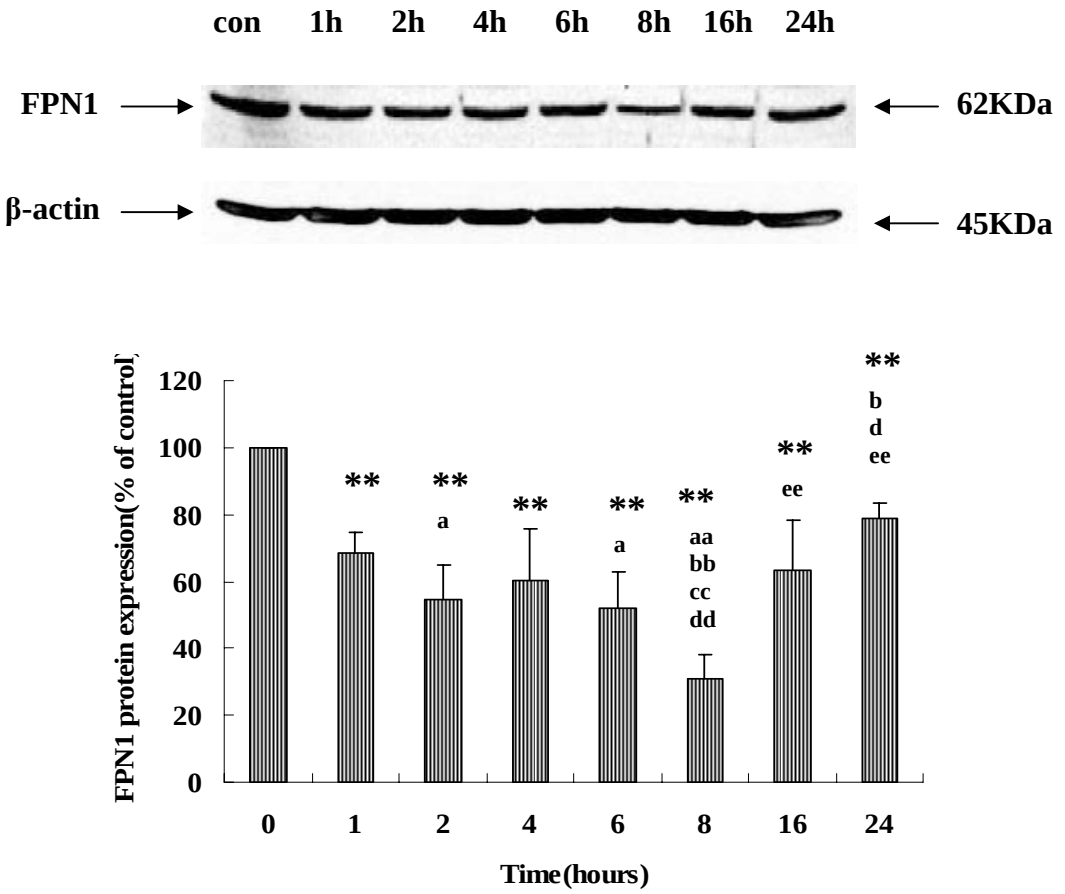


Figure 3-10. Effect of hepcidin on FPN1 protein expression in macrophages. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~62kDa for FPN1 and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the FPN1 protein expression after 700nM hepcidin treatment for different time (B) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other which incubated with hepcidin. Asterisks above data bars indicated that these groups were statistically different from the control group (**P* <0.05, ***P* <0.01).

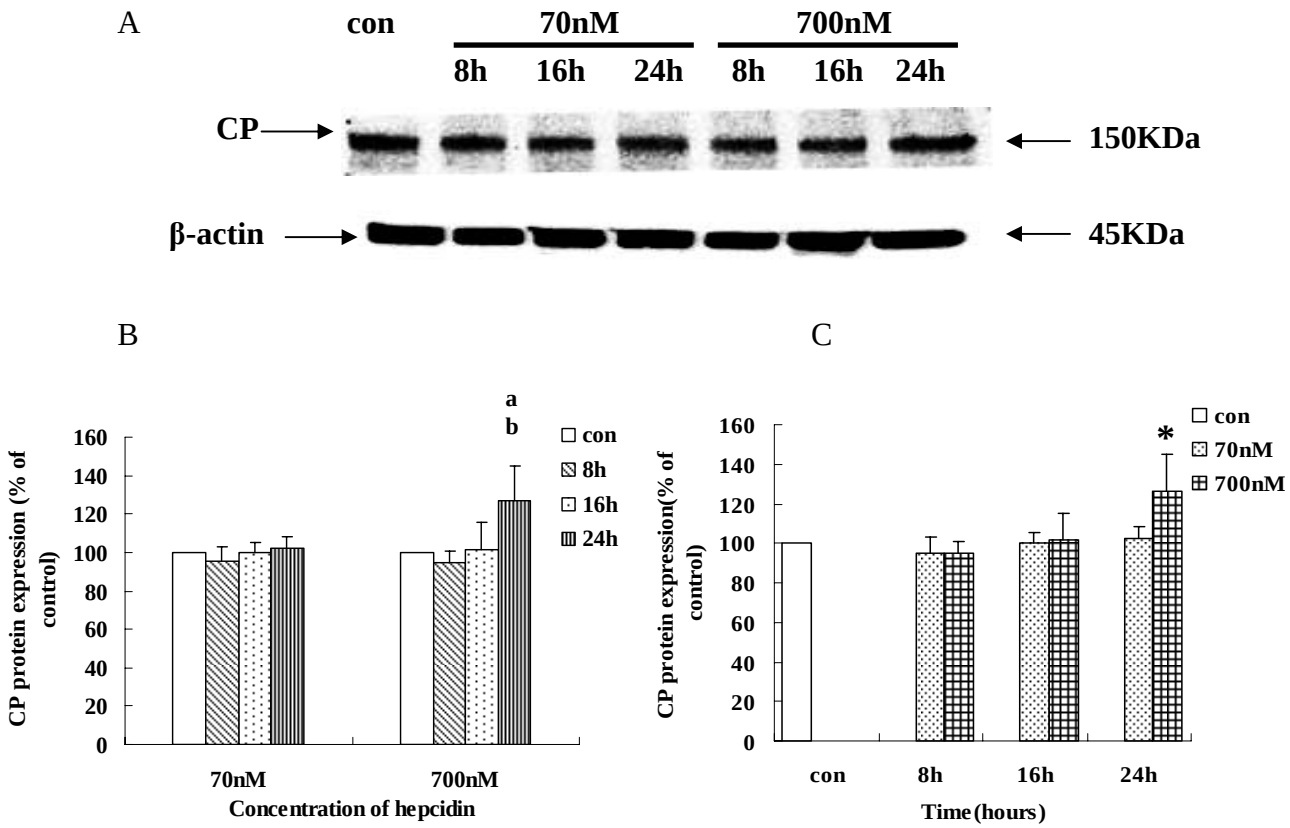
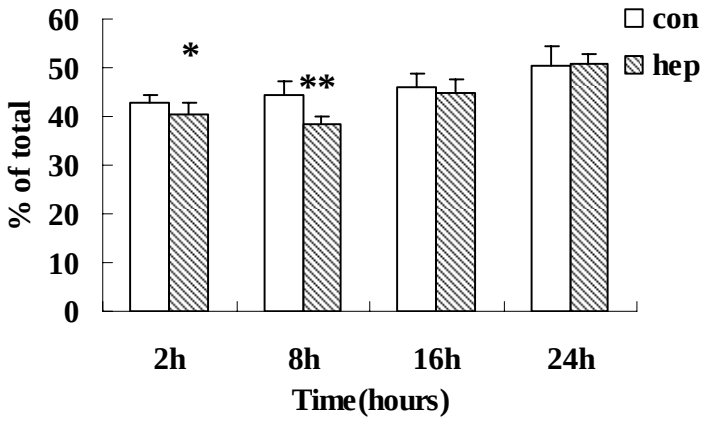


Figure 3-11. Effect of hepcidin on CP protein expression in macrophages.

Western blot analysis was performed as described in Materials and Methods. The *M*r of ~150kDa for CP and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the CP protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: P<0.05, aa: P<0.01 vs controls; b: P<0.05, bb: P<0.01 vs 8 hours group; c: P<0.05, cc: P<0.01 vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other incubated with hepcidin for the same time (**P* <0.05, ***P* <0.01).

A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% of total
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con
hep
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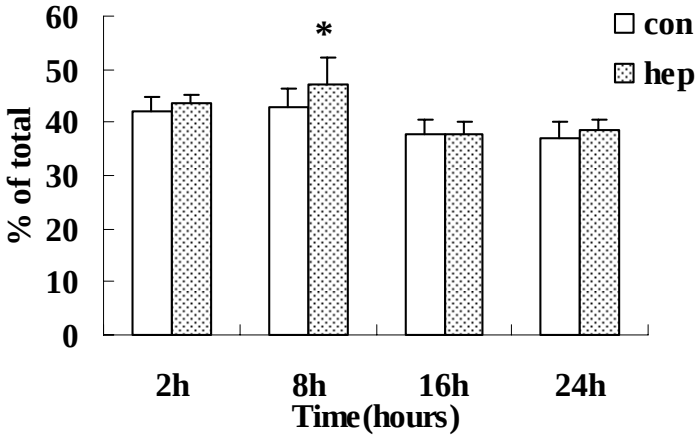
8h
16h
24h
Time(hours)

B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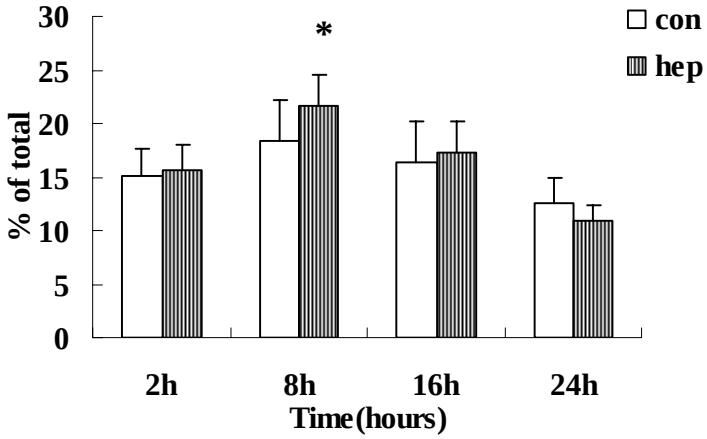
Time(hours)

C


30
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16h
24h
Time(hours)

D

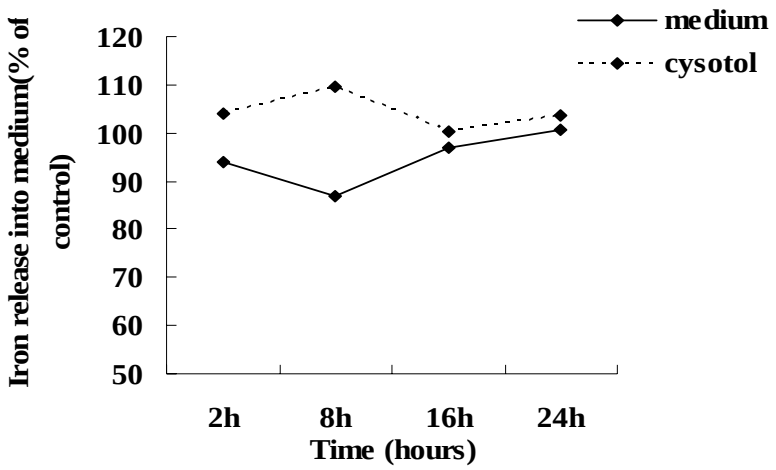


Figure 3-12. Effect of hepcidin on the iron release by the macrophages.

The cells were preloaded with iron by incubation with 2uM ^{55}Fe(II) in sucrose (pH 6.5) for 30 minutes at 37^{o}C and washed with ice-cold PBS. They were then incubated with or without 700nM hepcidin at 37^{o}C. At different time points during iron release, the medium was collected and the cells were lysed. Radio-labelled iron release into the media (A) and in the cytosolic (B) and stromal (C) fractions was measured as described in the Materials and Methods. Results were expressed as a percentage of total iron present in the system (=media+cytosol+stromal). The iron distributions (D) between the medium（◆ — ◆） and the cytosol（◆ - - ◆） were expressed as a percentage of the control. All these data were presented as mean±SD of 6 independent experiments performed in triplicate. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

CHAPTER 4

EFFECT OF LIPOPOLYSACCHARIDE ON THE IRON TRANSPORT PROTEINS EXPRESSION AND IRON UPTAKE AND RELEASE IN THE PERITONEAL MACROPHAGES

4.1 ABSTRACT

Hepcidin is a β-defensin like peptide and a principle regulator of systemic iron homeostasis. In concordance with its dual functions, its expression is modulated by systemic iron requirements and is in response to infectious and inflammatory stimuli. Here we used an *in vivo* rat endotoxemia model to analyze the relationship between lipopolysaccharide (LPS), hepcidin and iron metabolism in peritoneal macrophages. The temporal associations between liver hepcidin mRNA, iron transporters protein level in macrophages and serum iron parameters were studied in rats after LPS injection. Hepatic hepcidin mRNA was dramatically induced and peaked within 4 h after LPS injection. Two hours later, the FPN1 protein expression and iron release decreased, the TfR1 protein expression and Tf-Fe uptake increased in the

macrophages. Concurrently the serum iron decreased to the minimal level. Taken together, the time course suggested a possible causal relationship between hepcidin, iron transporters expression and serum iron concentration.

KEY WORDS

Hepcidin, Lipopolysaccaride, Macrophages, DMT1, Ferroportin1, Transferrin receptor, Ceruloplasmin, Iron uptake, Iron release

4.2 INTRODUCTION

Chronic and acute inflammation are well-characterized conditions in which iron metabolism is altered. These changes in iron metabolism are characterized by a drop in serum iron, an increase in the rate of plasma iron disappearance, a decline in the rate of plasma iron turnover, RES cell iron sequestration and hyperferritinemia (Jurado, 1997; Patruta and Horl, 1999; Weiss, 1999). On the other hand, patients with iron overload have been reported to be more sensitive to bacterial infections, thus suggesting a relationship between iron and infection (Boelaert, 1996; Brock, 1987).

At the opposite end of the iron disorder spectrum are conditions in which iron absorption and distribution from the iron stores are not sufficient to meet the needs of hemoglobin synthesis in the bone marrow, despite adequate dietary iron. These 'iron refractory' anemias include most prominently anemia of inflammation (AI, also called anemia of chronic disease). AI is a normocytic and normochromic anemia (Jurado, 1997; Nicolle, 1984) that develops in the setting of acute or chronic infections or inflammatory disorders. Its defining diagnostic feature is low serum iron (hypoferremia) in the presence of a considerable amount of iron in the bone marrow macrophages. When chronic, AI can become hypochromic and microcytic, resembling iron deficiency anemia. It has been suspected for some time that AI may be a side-effect of the host defense response to infection (Jurado, 1997).

For a long time, inflammation has been implicated in causing these confusing findings, until the recent discovery of a new peptide, hepcidin. It acts as an iron-regulatory hormone, and constitutes an important link between host defense, inflammation and iron metabolism.

As an antimicrobial peptide, hepcidin is active against Gram-positive (e.g. Bacillus subtilis) and Gram-negative (e.g. Neisseria cinerea) bacteria as well as yeasts (e.g.

Saccharomyces cerevisiae) in vitro (Krause et al., 2000). It contains basic amino acids that confer a positive total charge and a tendency to assume amphipathic secondary structures. This property enables the antimicrobial peptides to permeate the membranes of invading microorganisms. Hepcidin does not show any sequence similarity to any of the known antimicrobial-peptides, but it resembles structurally the defensin family because of the four disulfide bridges in its tertiary structure. So hepcidin is part of the innate immune system and thus constitutes the first line of defense against infections.

In agreement with the potential role for hepcidin in the host defense, hepcidin mRNA was increased in the livers of the LPS-treated mice and in the LPS-treated hepatocytes (Pigeon et al., 2001). In vitro stimulation of fresh human hepatocytes with a panel of cytokines showed a strong induction of hepcidin mRNA by IL-6, but not IL-1α or TNF-α (Nemeth et al., 2003), indicating that IL-6 might be the mediator of hepcidin induction by inflammation.

In humans, increased urinary hepcidin levels were detected in patients with chronic infections or severe inflammatory diseases (Nemeth et al., 2003). Furthermore, IL-6 infusion in human volunteers rapidly induced hepcidin and hypoferremia (Nemeth et

al., 2004a), whereas IL-6 knockout mice injected with turpentine failed to increase hepcidin and developed hypoferremia. Additionally, in a mouse model of inflammation, injections of turpentine increased hepcidin mRNA and decreased serum iron (Nicolas et al., 2002b). Importantly, the turpentine-induced hypoferremia was not observed in the hepcidin-deficient mice (Nicolas et al., 2002b), demonstrating that hepcidin is required for iron sequestration during inflammatory conditions.

Like some other antimicrobial peptides, hepcidin has dual function. In addition to its antimicrobial activity, it acts as an iron regulatory hormone which negatively regulates intestinal iron absorption and macrophage iron release. High levels of hepcidin may result in low serum transferrin saturation and high ferritin levels as presented in ACD. So hepcidin may be considered a principal iron regulatory hormone, a key mediator of AI, and a bridge between innate immunity and iron metabolism (Verga Falzacappa et al., 2006).

4.3 MATERIALS AND METHODS

4.3.1 Materials

Please refer to chapters 2 and 3.

4.3.2 Animals

Please refer to chapter 2.

4.3.3 Methods

4.3.3.1 Sampling of Blood and Tissues

Animals i.p with LPS at dose of 1ug/g body weight were anesthetized with diethyl ether at the indicated time. Blood samples were then collected into syringes from the heart, and aliquots were taken immediately for hemoglobin (Hb) concentration and hematocrit (Hct) determination. The remaining blood was cooled to 4^{o}C and centrifuged at 3,000 g for 10 min at 4°C. The serum was analyzed for serum iron, total iron-binding capacity (TIBC) and Tf saturation. After taken the peritoneal macrophages, the rat were perfused with ice-cold phosphate-buffered saline (Milli-Q

water prepared and DEPC treated, pH 7.4) through the left ventricle. The liver was also rapidly removed, aliquots were rinsed with cold saline to remove blood, and used immediately for total RNA extraction. The remaining tissues were wrapped with aluminum and immediately frozen below –70°C for storage after treatment with fluid nitrogen.

4.3.3.2 Measurement of Hemoglobin (Hb) Concentration

The hemoglobin concentration was determined by the cyanmethaemoglobin method. 10ml standard human Hb or samples mixed with 2.5 ml of Drabkin's solution were left to stand at room temperature for at least 10 min. The absorbance of the test sample and the human Hb standard at the wavelength of 540 nm was recorded against the Drabkin's solution. The standard curve was plotted and the concentration of each unknown was determined according to the following equation. Hb conc. (g/100ml) = (A540 sample/A540 standard)×conc. Standard (g/100ml)

4.3.3.3 Measurement of Hematocrit (Hct)

A microhematocrit capillary tube was filled with blood to 75%~90% of the tube

length. Then the tube was placed in a Hawksley microhematocrit and centrifuged at 12000rpm for 5min. After centrifugation, the Hct was read using the Microhematocrit Reader. The result was expressed as a percentage.

4.3.3.4 Serum Iron, UIBC, TIBC Measurement

Serum iron and total iron binding capacity were determined using a commercial kit (Stanbio laboratory Co., Boerne, USA). 1 ml iron HA buffer reagent (or Tris Buffer) and 0.1 ml iron-free water, iron standard and serum were respectively added to cuvettes and mixed. They were incubated for 1 minute at room temperature. The absorbance at 560 nm of the test sample and standard vs blank were recorded as the INITIAL A or INITIAL B. 20 µL iron color reagent was added into each cuvette, mixed thoroughly and placed in a water bath at 37°C for 5 minutes. Then the absorbance at 560 nm of the test sample and standard vs blank was recorded as the FINAL A or FINAL B.

The serum iron concentration of each sample was determined according to the following equations:

A (B) test = FINAL A (B) test - INITIAL A (B) test

A (B) standard = FINAL A (B) standard – INITIAL (B) Standard

Serum Total iron (µg/dl) = Atest/Astandard × 500.

Serum UIBC (µg/dl) = 500 – (Btest/Bstandard × 500)

Serum TIBC (µg/dl) = Serum Total iron + Serum UIBC

4.3.3.5 RNA Isolation

Total RNA was isolated from liver using Trizol® Reagent for real-time RT-PCR according to the manufacturer's instructions. Any genomic DNA potentially present in the RNA processing was removed by incubating the RNA with RNase free DNase I. The relative purity of the isolated RNA was assessed spectrophotometrically and the ratio of A260 nm to A280 nm exceeded 1.8 for all preparations or 28S RNA bands = twice the amounts of the 18S RNA.

4.3.3.6 Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction (Real-time PCR)

Total RNA (1ug) was reversely transcribed in a 20ul reaction using RT-for-PCR Kit (Promega Corporation, Madison, WI, USA) with oligo dT primers to the manufacturer's instructions. The primers for real-time PCR of hepcidin were based on the cDNA

sequence reported by Christelle Pigeon (Pigeon et al., 2001). The forward primer sequence is 5'- acagaaggcaagatggcact -3' (13-32 nt); the reverse primer sequence is 5'- gaagttggtgtctcgcttcc -3' (194-213 nt). Amplification was performed (using the Smart cycler, Cepheid Corporate, CA, USA) with initial denaturation at 95^{o}C for 5min, followed by 40 cycles at 95 ^{o}C (15s), 59^{o}C (15s) and 72 ^{o}C (30s). The β-actin cDNA (5'-primer, 5'- gtcgtaccactggcattgtg -3'; 3'-primer, 5'- ctctcagctgtggtggtgaa -3') was amplified simultaneously as the internal control. The PCR products were analyzed on a 1.5% agarose gel using LumiAnalyst Image (Roche, Mannheim, Germany). The expected size of hepcidin and β-actin was 201 bp and 181 bp, respectively.

The gene relative expression levels were calculated using method arithmetic formulas. The control (non-treated) sample was used as a calibrator. The amount of target, normalized to an endogenous housekeeping gene (β-actin) and relative to the calibrator, is then given by $\triangle\triangle C_T$, where $\triangle\triangle C_T = \triangle C_T$ (sample) — $\triangle C_T$ (calibrator), and $\triangle C_T$ is the C_T of the target gene subtracted from the C_T of the housekeeping gene. For the untreated control sample, $\triangle\triangle C_T$ equals zero and 2^0 equals one, so that the fold change in gene expression relative to the untreated control equals one, by definition. For the treated samples, evaluation of $\triangle\triangle C_T$ indicates the fold change in gene expression relative to the untreated control.

4.3.3.7 Western Blot Analysis

Please refer to chapters 2 and 3.

4.3.3.8 Non-transferrin Bound Iron (NTBI) Uptake Assay

Please refer to chapters 2 and 3.

4.3.3.9 Transferrin Binding Iron Uptake Assay

Please refer to chapters 2 and 3.

4.3.3.10 Iron Release Assay

Please refer to chapters 2 and 3.

4.3.4 Statistical Analysis

Statistical analyses were performed using SPSS software for Windows (version 10.0).

Data were presented as mean ± SD. The difference between the means was determined by One-Way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. Differences with $P<0.05$ were considered significant.

4.4 RESULTS

4.4.1 Changes in the Hepatic Hepcidin After LPS Administration

LPS, at the dose of 1 ug/g body weight, induced a rapid increase in hepatic hepcidin mRNA expression. Before LPS, there was a low basal level of hepcidin expression in the liver. By 2 h after LPS injection, hepcidin expression increased about 2 folds. The increase continued with a peak at 4 h (hepcidin mRNA changed 9.54±0.54 folds, mean±SD, n=4 independent experiments) and then the expression subsided (Fig4-1). From 12h to 24h, the hepcidin mRNA even decreased to a level lower than the control. An expansion of the study to 36 h showed that there was a second increase of hepcidin mRNA expression but which was lower than that at 4 h (Fig 4-1).

4.4.2 Effect of LPS on Biochemical Parameters

Because iron disorders and anemia are always accompanied by inflammation, we next examined iron distribution, Hb and HCT after LPS treatment. After LPS administration, the serum iron concentration decreased to 70% and 50% of the control level at 2h and 4 h, respectively (Fig 4-2 and Table 4-1). With LPS treatment for 6h, two hours after the maximal level of liver hepcidin excretion was reached, serum iron fell to its nadir value. The average of the pretreatment serum iron concentration was 192.80±8.78 ug/dl, but 6 hours after LPS injection, the serum iron concentration fell to 16.94±14.20 ug/dl ($P<0.01$). At the same time, the transferrin saturation decreased to 3.3%. After 6h, both the serum iron and the transferrin saturation began to increase, but they were still lower than the control after LPS injection for 36h. To determine whether the hypoferremic effect would result in anemia, we tested the blood parameters after LPS treatment for different durations. As shown in Table4-2, there were no significant differences between the control (i.p with saline) and the LPS-treated groups (i.p with LPS).

4.4.3 Effect of LPS on DMT1 Protein Expression and NTBI Uptake in Macrophages

Since treatment of cultured macrophages with hepcidin resulted in changes of iron

metabolism as shown in our previous studies, we next examined whether LPS regulated the iron transporters expression and iron uptake or release of peritoneal macrophages in vivo, and investigated the relationship between these changes and the hepcidin level.

When investigating DMT1-IRE protein level by means of western blot, which is shown in Fig 4-3, a protein band with a molecular mass of ~60KDa reacted strongly with the anti-DMT1-IRE antibody. After being normalized to β-actin, we found that its expression was decreased to 70% of the control ($P<0.05$) after LPS administration for 2 hours. Then the protein level began to increase in the following hours. After LPS treatment for 24 hours and 36 hours, the DMT1-IRE protein expression recovered to the pre-LPS levels.

We then detected the changes of the NTBI uptake in macrophages after LPS treatment for 2 hours. At the desired time point, the cells were subjected to $^{55}Fe(II)$ and incubated at $37^{o}C$ for 30 minutes. As shown in Fig4-4, there were no significant differences between the untreated and the LPS-treated groups in both cytosolic and stromal fractions ($P>0.05$).

4.4.4 Effect of LPS on TfR1 Protein Expression and Tf-Fe Uptake in Macrophages

When studying the effect of cytokines on the TfR expression, Ludwiczek (Ludwiczek et al., 2003) found that LPS could up-regulate the mRNA of TfR1 in U937 cells. This finding was consistent with our results. In our experiments, we examined the TfR1 protein expression after LPS treatment in macrophages.

Contrasted to the DMT1-IRE, the TfR1 protein expression in the macrophages showed a different pattern after LPS administration. LPS induced a significant increase of the TfR1 protein expression at 6h. Then the protein decreased after LPS administration for 12 hours, and fell to the normal level at 24h. To see if these regulations of TfR1 expression by LPS might affect the TfR-mediated iron acquisition, we examined the uptake of ^{55}Fe-transferrin of macrophages. After LPS treatment for 6 hours, the peritoneal macrophages were collected and subjected to Tf-Fe uptake assay. The Fig4-6 showed that LPS significantly increased the uptake of ^{55}Fe-transferrin when compared to the controls. After separating the cytosolic and stromal fractions, we found that the increase in the former was significant ($P<0.05$).

4.4.5 Effect of LPS on FPN1, CP Protein Expression and Iron Release in Macrophages

Yeh et al (Yeh et al., 2004) reported that LPS could decrease the mRNA of FPN1 in rat liver and intestine. Similarly, in a mouse acute inflammation mode induced by lipopolysaccaride, Yang et al found that the FPN1 expression in RES cells of the spleen, liver and bone marrow was down-regulated by inflammation (Yang et al., 2002). The FPN1 expressed on the cultured splenic macrophages also induced to a decrease by the LPS. We had previously demonstrated that hepcidin could decrease the expression of FPN1 in peritoneal macrophages in vitro. In order to better define the effect of hepcidin, we used LPS to induce the production of hepcidin and then examined the FPN1 expression of macrophages in vivo. Similar to that observed in vitro, treatment with LPS resulted in a down-regulation of FPN1 expression. Different from the result in vitro, LPS induced biphasic changes in FPN1 expression. The FPN1 protein was significantly decreased to about 40% after the LPS administration for 2h and followed by an increase to 70% at 4h. Then at 6h this protein decreased again to a level similar to that at 2h. An expansion of the study to 36h showed that the FPN1 protein increased at 12h and recovered to the control level at 24h and 36h.

In parallel, we also studied the effect of LPS on the expression of CP. Both interleukin-1α and LPS could increase the hepatic ceruloplasmin mRNA content and ceruloplasmin biosynthesis in normal animals (Gitlin et al., 1992). But in our studies there were no significant changes in the expression of CP, until the LPS treatment for 12h, then the protein level of CP decreased to 68% of the control. After the LPS administration for 24h, the CP protein level fell to its lowest level and recovered at 36h. The changes during 12h and 24h were similar to the changes of hepcidin mRNA. It is not clear why CP in peritoneal macrophages behaves differently from that in other type cells. Courselaud et al found that CCAAT/enhancer-binding protein alpha (C/EBPα) and beta (C/EBPβ) mediated the CP transcription induced by IL-6. On the other hand, C/EBPα is likely to be a key regulator of the hepcidin gene transcription (Courselaud et al., 2002). These findings proposed a possible link between hepcidin and CP.

Accordingly, the FPN1 expression was well-detected in the untreated control samples and significantly down-regulated by LPS treatment. We then studied whether the effects of LPS on FPN1 expression may have implications on the release of ^{55}Fe(II). The peritoneal macrophages were harvested from rats treated with LPS for 2 hours and 6 hours. As was shown in Fig 4-9, LPS had no effect on the iron

release after 2h treatment; while with LPS treatment for 6 hours, there was a diminished release of the metal from these cells into medium ($P<0.05$).

4.5 DISCUSSION

Infection and inflammation could markedly induce hepcidin synthesis in mice, fish and humans. Our data also demonstrated that LPS induced a biphasic change of hepcidin expression in rat liver, which is similar to Yeh's results (Yeh et al., 2004). Recent findings showed that cytokine IL-6 is apparently the key inducer of hepcidin synthesis during inflammation (Nemeth et al., 2004a). And STAT-3 seemed to be involved in the activation of hepcidin promoter to inflammation stimuli (Wrighting and Andrews, 2006).

During inflammation induced by subcutaneous injections of turpentine, normal mice show a marked decrease in serum iron (hypoferremia) (Nemeth et al., 2004a; Nicolas et al., 2002b). This response is completely ablated in the hepcidin-deficient mice and in the IL-6-deficient mice. In humans, the hepcidin increase elicited by IL-6 infusion is accompanied by a decrease in serum iron and transferrin saturation of more than 30% (Nemeth et al., 2004a). It therefore appears that the IL-6-hepcidin axis is

critically important for this response, and hepcidin is the main mediator of hypoferremia of inflammation.

In our studies, the maximal hepcidin excretion was detected at 4 hours after LPS injection. On the other hand, the serum iron concentration fell to the lowest level at 6 hours, two hours later than the peak of hepcidin expression. Kemna et al (Kemna et al., 2005) showed that the acute phase response was boosted by a dramatic increase in IL-6, which peaked at 3-4 hours after LPS injection. The time-course of IL-6 increase in relation to hepcidin induction and serum iron decrease coincided with that observed in subjects with direct injection of IL-6 (Nemeth et al., 2004a).

In vivo studies of animal models of acute inflammation secondary to LPS or turpentine administration have demonstrated several mechanisms for the decline in serum iron. LPS and turpentine induce an accelerated clearance of iron from blood, which is thought to be due to an increase in the transferrin-dependent uptake of iron by hepatocytes and other cells (Alvarez-Hernandez et al., 1989; Beguin et al., 1989; Feldman et al., 1981a; Feldman et al., 1981c; Hershko et al., 1974; Moldawer et al., 1989). Release of lactoferrin by inflammatory cells may also be a contributing factor to this acute decline (Gutteberg et al., 1989), but this hypothesis has been challenged

recently (Gordeuk et al., 1988; Uchida et al., 1991). Other changes in iron metabolism because of inflammatory stimuli include a decline in RES and hepatocyte cell iron turnover, and a decrease in RES cell iron turnover which in turn results in an accumulation of iron in the RES compartment (Beguin et al., 1989; Feldman et al., 1981b; Feldman et al., 1981c; Fillet et al., 1989; Hershko et al., 1974). The mechanism for the RES iron sequestration is not known. Until recently, several labs reported that LPS could suppress FPN1 synthesis and block iron efflux from macrophages during inflammation.

The data presented here showed that acute LPS administration to rats resulted in a down-regulation of FPN1 expression in the peritoneal macrophages. There were two decreases of FPN1 protein in our studies, with the first one happened at the 2 hours after LPS administration. It was possible that the first reduction of FPN1 expression might result not from the action of hepcidin but from the direct stimulation by LPS through the Toll-like receptor 4 (TLR4)-dependent signaling processes (Yang et al., 2002). The proposal of this possibility was based on the observation that hepcidin induced by LPS began to increase at the same time point. However, these results could not rule out hepcidin as the responsible agent. Liu et al (Liu et al., 2005) found that hepcidin could also express on the mouse peritoneal macrophages and splenic

macrophages after LPS treatment, whereas during inflammation, locally produced hepcidin might contribute to the regulatory pool of hepcidin by greatly increasing the local hepcidin concentration around liver Kupffer, spleen macrophages and other mononuclear-phagocyte system (MPS) cells. MPS-produced hepcidin may act on cells extracellularly at the plasma membrane where concentration would be high, or result in sorting of FPN1 directly from the secretory pathway to lysosomes. There are other reasons that locally produced hepcidin may be important in the regulation of FPN1.

The second decrease of FPN1 protein was perhaps regulated by LPS through hepcidin. Concurrent with the first rise in hepcidin, FPN1 protein on the macrophages decreased after LPS treatment for 6 hours. These inverse changes also occurred in rats treated with smaller doses of LPS and in rats at different postnatal ages (Yeh et al., 2004), which were consistent with the causal relationship between hepcidin and FPN1. Our previous work in vitro also revealed that treatment with hepcidin rapidly induced a dramatic reduction in the FPN1 protein level. The reduction of FPN1 expression is consistent with the hypothesis that FPN1 is the regulatory target of hepcidin.

Hepcidin was recently shown to regulate cellular iron efflux *in vitro* by binding to the iron efflux channel ferroportin1 and inducing its internalization and degradation (Nemeth et al., 2004b). This mechanism could explain the rapid development of hypoferremia observed in the LPS-injected subjects. Other reports have also pointed to a fast onset of hypoferraemia with LPS (Bertini et al., 1989; Gutteberg et al., 1989). These observations make it unlikely that the LPS-mediated FPN1 down-regulation in the RES is responsible for the initial hypoferraemia observed with LPS. The initial hypoferraemia probably results from an increased rate of transferrin-mediated uptake of blood iron. It is more likely that the FPN1-mediated RES cell iron exit block may serve to maintain the hypoferraemia instead of initiating it. Daily, macrophages export around 20 mg of iron through ferroportin1, and the iron is taken up largely by the developing erythrocytes in the bone marrow. However, the plasma transferrin compartment contains only 2–4 mg of iron which must therefore turn over every few hours. Accordingly, it is expected that blocking macrophage iron efflux would decrease the plasma iron concentration within hours (Kemna et al., 2005). Interestingly, there was a decease of hepcidin mRNA during 12h-24h after LPS injection. This may be a feedback inhibition from the high hepcidin concentration in blood.

DMT1 is another potential target for hepcidin. Although the DMT1 mRNA levels are unchanged in the USF2 knockout mice (Nicolas et al., 2001), the changes in DMT1, FPN1 and the ferric reductase Dcytb expression have been noted to occur with changes in hepcidin activity (Anderson et al., 2002b; Frazer et al., 2002), suggesting that these proteins which are critical to intestinal iron absorption may be regulated by hepcidin. In our studies, the change of DMT1-IRE seemed to have little relation with hepcidin, because the increase of hepcidin induced by LPS began at 2 hours. This decrease may be due to the direct effect of LPS. Wang et al (Wang et al., 2005b) found that LPS could increase expression of DMT1 in bronchial epithelial cells. Our observations appeared to be different from what was found in other type cells. First, increase in DMT1 level after LPS treatment was detected in other type cells but decrease in peritoneal macrophages. Second, down-regulation of the surface DMT1 in peritoneal macrophages did not appear to be effect as reported in other systems. Possible explanations may include differences in subcellular localization and/or post translational modification such as glycosylation, which could affect the stability of DMT1.

Macrophages are able to take up iron via the TfR-mediated pathway. The expression of TfR mRNA is slightly stimulated by pro-inflammatory stimuli such as IFN-γ, LPS

and TNF-α when applied alone, which most likely involves a transcriptional mechanism since the IRE-binding activity of IRPs is not significantly changed by these cytokines as compared to the controls (Ludwiczek et al., 2003). Consistent with our in vitro data, we found that LPS could increase the uptake of Tf-Fe into cells, which may be due to the up-regulation of TfR1 protein expression. Moreover, concurrent with the first rise in hepcidin, the TfR1 expression increased after four hours. It was possible that this increase might result from the action of hepcidin. The increased rate of transferrin-mediated uptake of blood iron maybe partly responsed to the hypoferremia induced by inflammation.

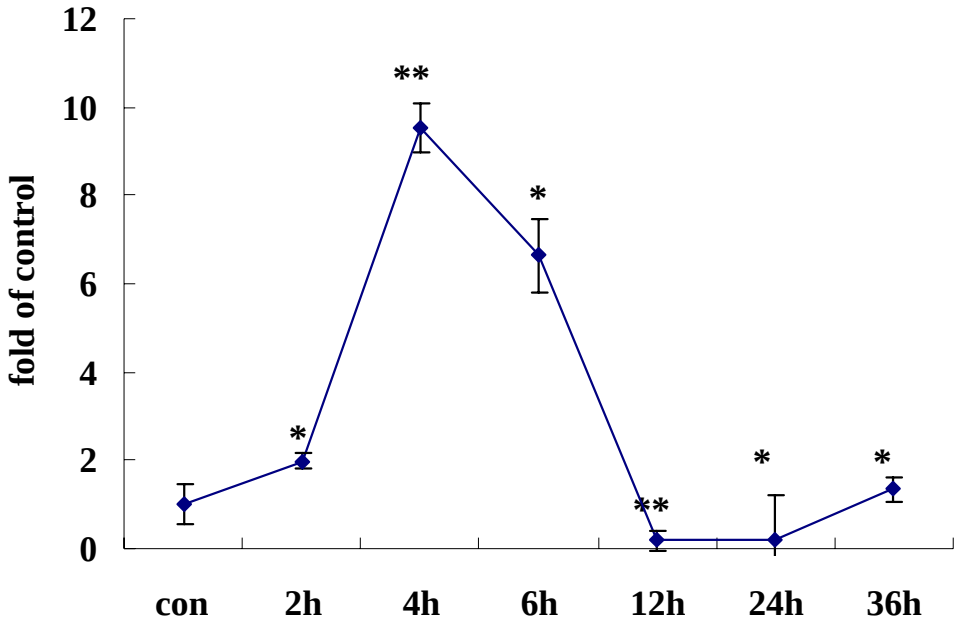
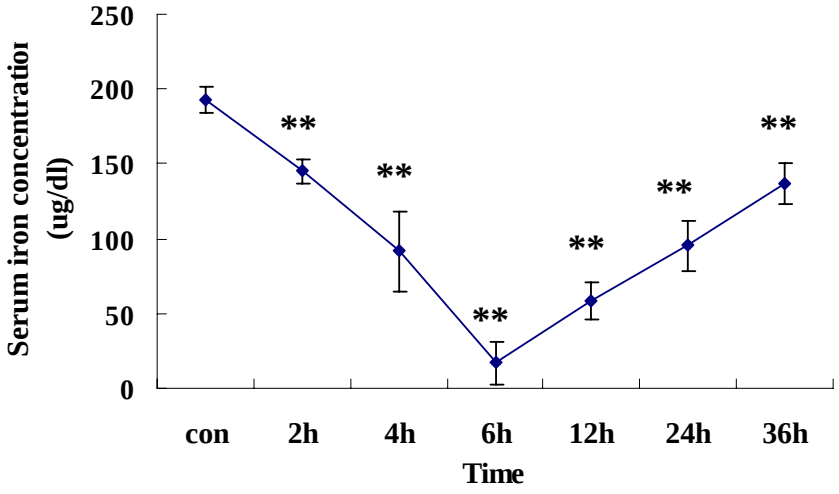


Figure 4-1. Regulation of hepcidin mRNA expression in liver by LPS.

The rats were administrated LPS (1ug/g body weight i.p) or steriled saline (control), then killed at the indicated time points. Total RNA were isolated from the liver. Hepcidin mRNA expression was analysed by quantitive real-time PCR (described in Materials and Methods) and data were normalised to mRNA expression of a house-keeping gene, β-actin. Data were presented as fold change ± SD (n=4 samples / time point), whereby the hepcidin mRNA expression in control was set to 1. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

A


Serum iron concentration (ug/dl)
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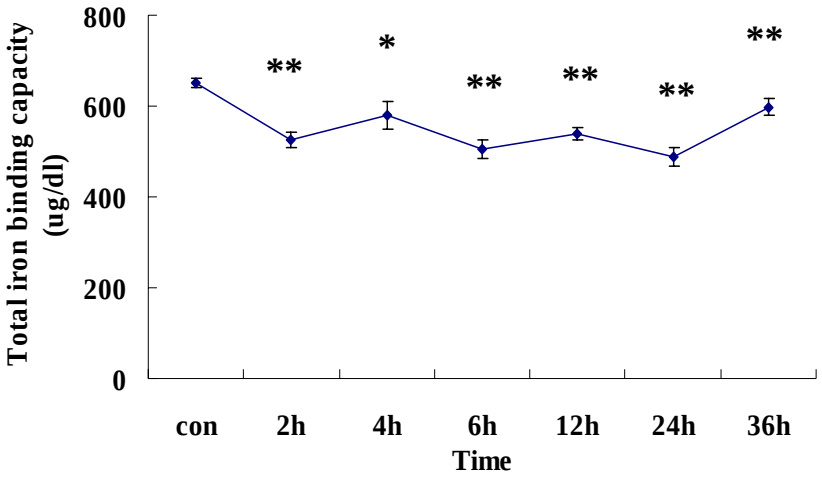
B


Total iron binding capacity (ug/dl)
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Time

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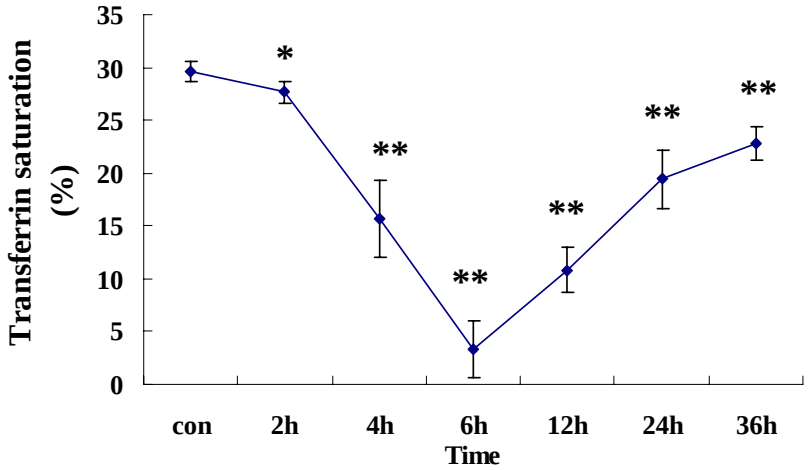
Transferrin saturation (%)
35
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25
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con
2h
4h
6h
12h
24h
36h
Time

Figure 4-2. Change of serum iron parameters after LPS administration.

The rats were administrated with LPS (1ug/g body weight i.p) or steriled saline (control), blood was collected at the indicated time points. Serum iron (A) and total iron binding capacity (B) were determined as described in Materials and Methods. The transferrin saturation (C) was calculated from the above data. The data were presented as mean $\pm$ SD of 4 independent experiments. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

Table 4-1. Serum Iron, TIBC and transferrin saturation parameters in the control and LPS treated rats.

n=6	Serum iron (ug/dl)	TIBC(ug/dl)	Transferrin saturation(%)
con	192.83±8.78	650.93±10.68	29.61±0.97
2h	145.18±7.85**	525.07±8.28**	27.64±1.10*
4h	91.71±26.53**	580.44±29.81*	15.66±3.66**
6h	16.94±14.20**	505.67±19.56**	3.30±2.72**
12h	58.23±12.13**	538.17±11.59**	10.80±2.13**
24h	95.29±17.03**	489.15±20.03**	19.41±2.75**
36h	136.74±13.87**	598.28±19.63**	22.82±1.64**

Table 4-2. Hematological variables in the control and LPS-treated rats.

n=6	Hb (g/dl)	Hct (%)
con	15.96±0.93	45.60±1.02
2h	14.97±0.49	42.90±0.22
4h	15.89±1.76	44.80±0.45
6h	16.17±0.22	46.90±0.42
12h	16.59±1.04	46.33±0.29
24h	16.96±0.40	48.00±1.09
36h	15.41±1.15	46.38±1.89

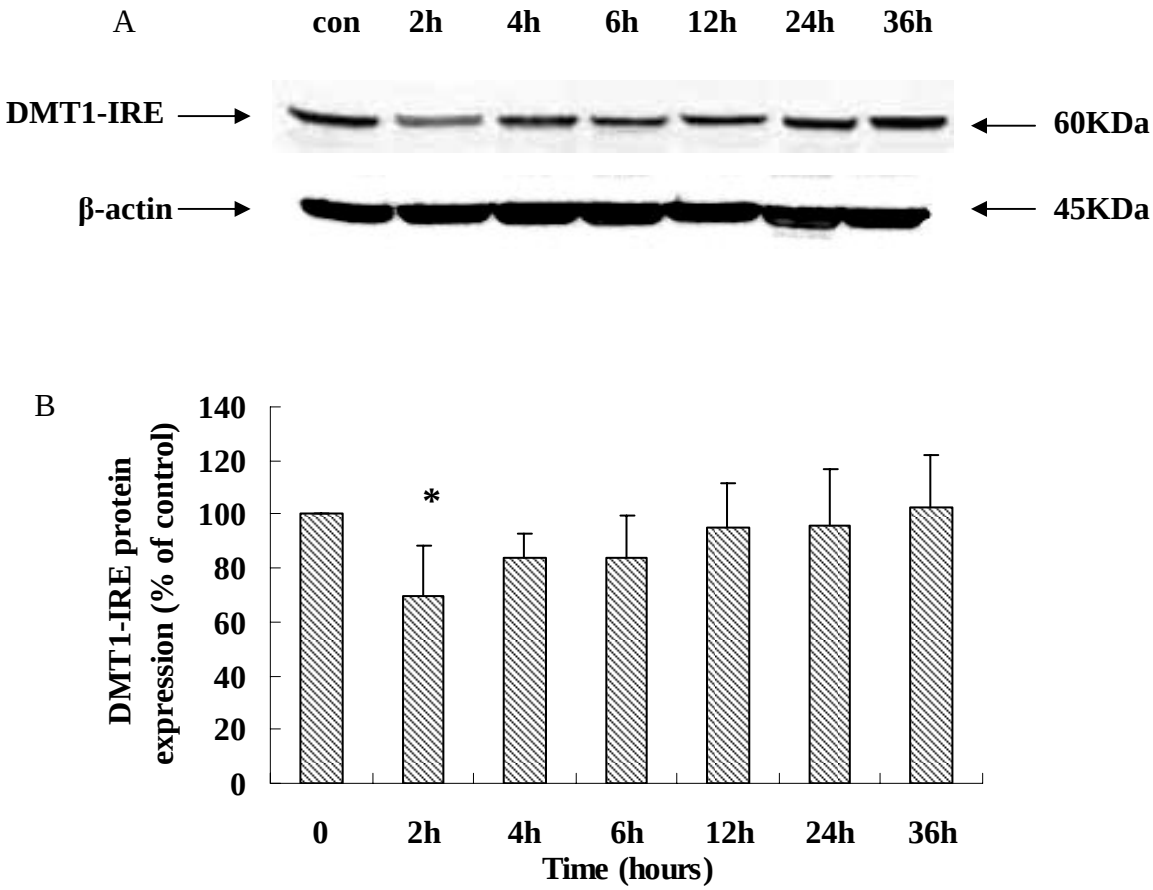


Figure 4-3. Effect of LPS on DMT1-IRE protein expression in macrophages.

The rats were administrated with LPS (1ug/g body weight i.p) or steriled saline (control), the peritoneal macrophages were then collected at the indicated time points and the total protein was extracted. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~60kDa for DMT1-IRE and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the DMT1-IRE protein expression after LPS treatment for different time (B) was normalized for β-actin and expressed as a percentage of the control level. The data were presented as mean $\pm$ SD of 4 independent experiments. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05).

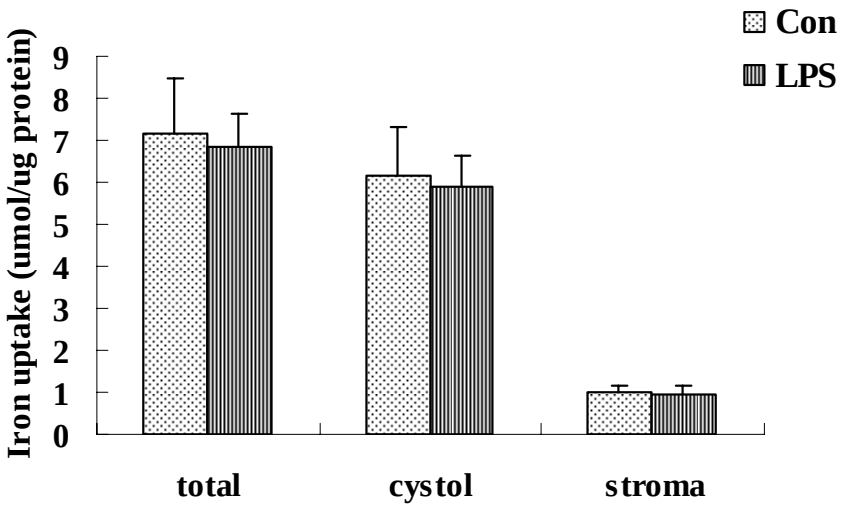


Figure 4-4. Effect of LPS on NTBI uptake in macrophages.

The rats were administrated with LPS (1ug/g body weight i.p) or steriled saline (control), the peritoneal macrophages were then collected after 2 hours. The cells were washed and incubated with 2uM Fe(II) under the control conditions (0.27M sucrose, pH 6.5) at 37^{o}C for 30 minutes. The iron taken into cytosolic and stromal fractions were measured as described in the Materials and Methods. The total iron uptake was the sum of the two parts. All these data were presented as mean$\pm$ SD of 4 independent experiments performed in sextuple.

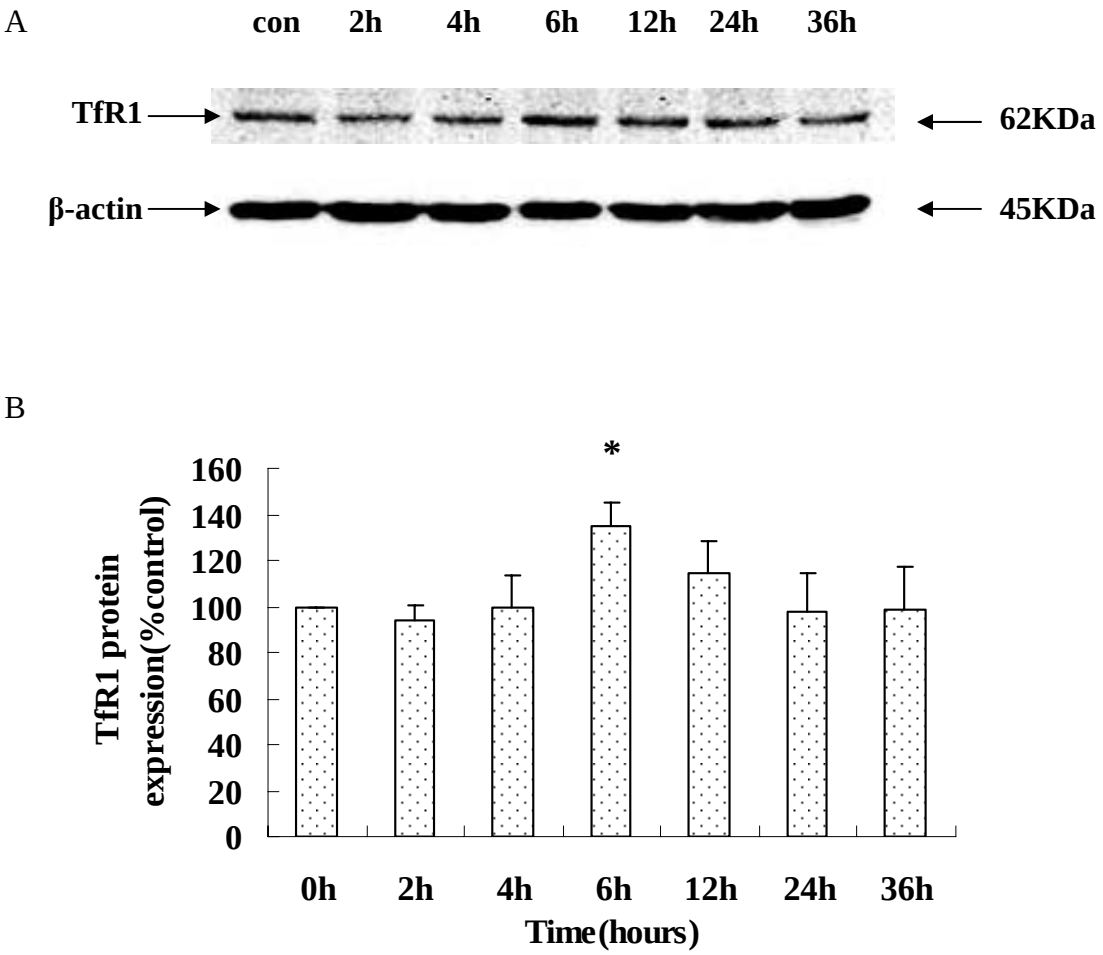


Figure 4-5. Effect of LPS on TfR1 protein expression in macrophages.

The rats were administrated with LPS (1ug/g body weight i.p) or steriled saline (control), the peritoneal macrophages were then collected at the indicated time points and the total protein was extracted. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~90kDa for TfR1 and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the TfR1 protein expression after LPS treatment for different time (B) was normalized for β-actin and expressed as a percentage of the control level. The data were presented as mean $\pm$ SD of 4 independent experiments. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05).

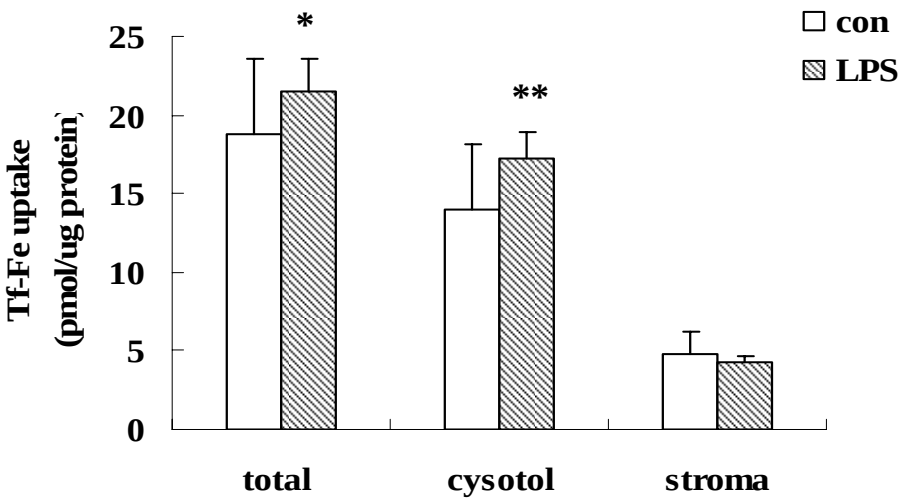


Figure 4-6. Effect of LPS on Tf-Fe uptake in macrophages.

The rats were administrated with LPS (1ug/g body weight i.p) or steriled saline (control), the peritoneal macrophages were then collected after 6 hours. Then the cells were removed the endogenous transferrin and incubated with serum-free culture medium with 10ug/ml Tf-Fe at 37^{o}C for 60 minutes. The iron taken into cytosolic and stromal fractions were measured as described in the Materials and Methods. The total iron uptake was the sum of the two parts. All these data were presented as mean$\pm$SD of 4 independent experiments performed in sextuple. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

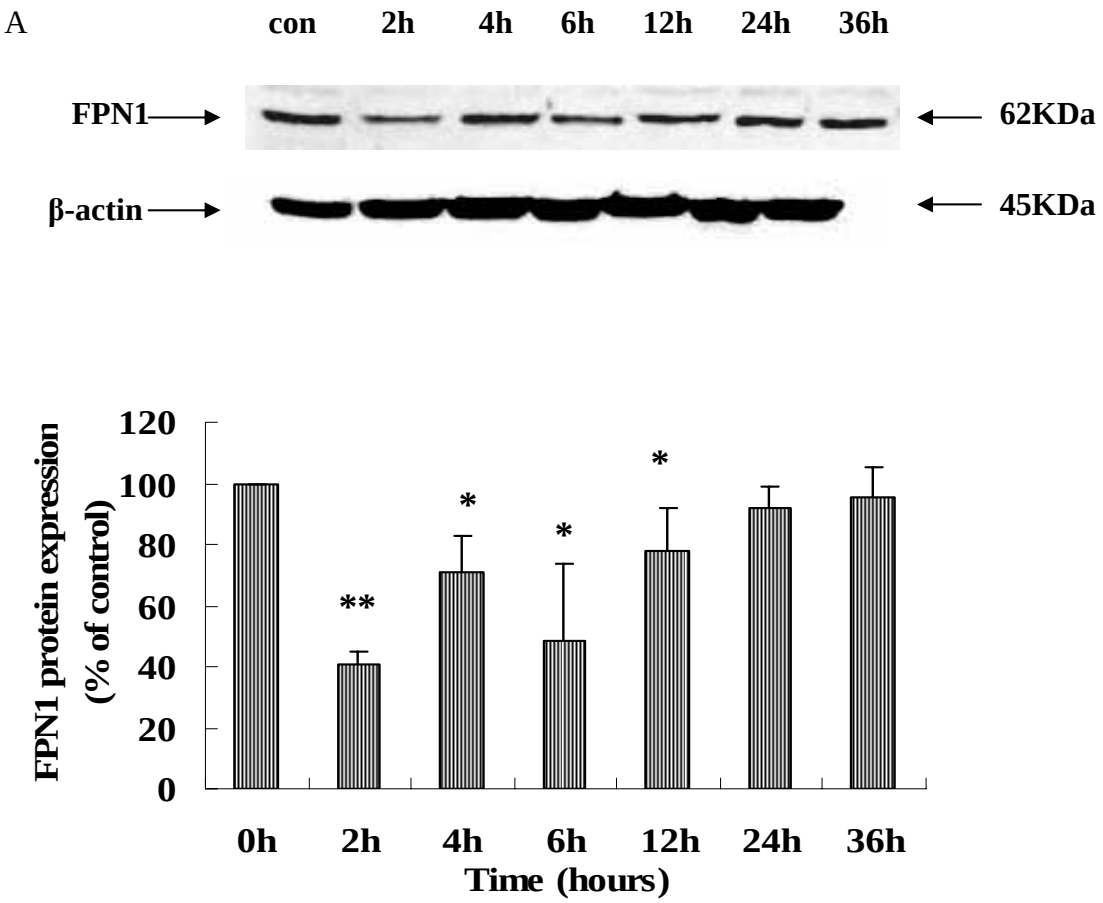


Figure 4-7. Effect of LPS on FPN1 protein expression in macrophages.

The rats were administrated with LPS (1ug/g body weight i.p) or steriled saline (control), the peritoneal macrophages were then collected at the indicated time points and the total protein was extracted. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~62kDa for FPN1 and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the FPN1 protein expression after LPS treatment for different time (B) was normalized for β-actin and expressed as a percentage of the control level. The data were presented as mean±SD of 4 independent experiments. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

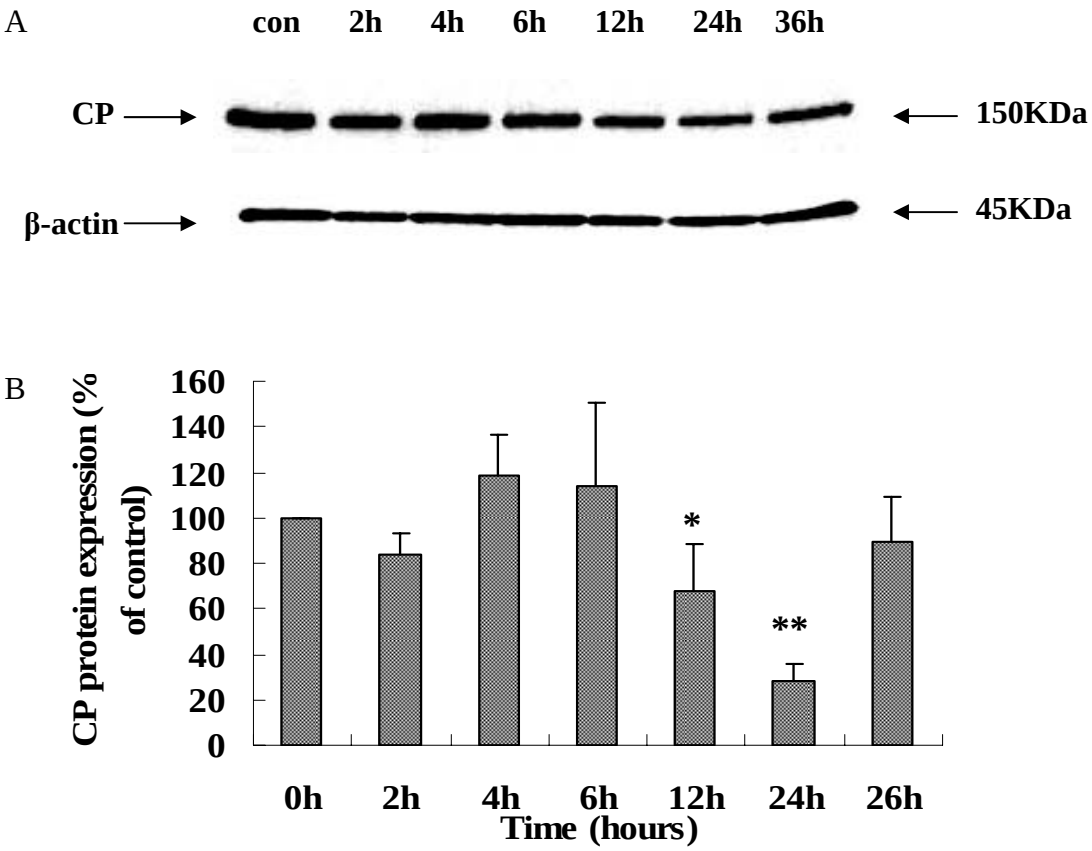


Figure 4-8. Effect of LPS on CP protein expression in macrophages.

The rats were administrated with LPS (1ug/g body weight i.p) or steriled saline (control), the peritoneal macrophages were then collected at the indicated times points and the total protein was extracted. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~150kDa for CP and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the CP protein expression after LPS treated for different time (B) was normalized for β-actin and expressed as a percentage of the control level. The data were presented as mean±SD of 4 independent experiments. Asterisks above data bars indicated that these groups were statistically different from the control (**P* <0.05, ***P* <0.01).

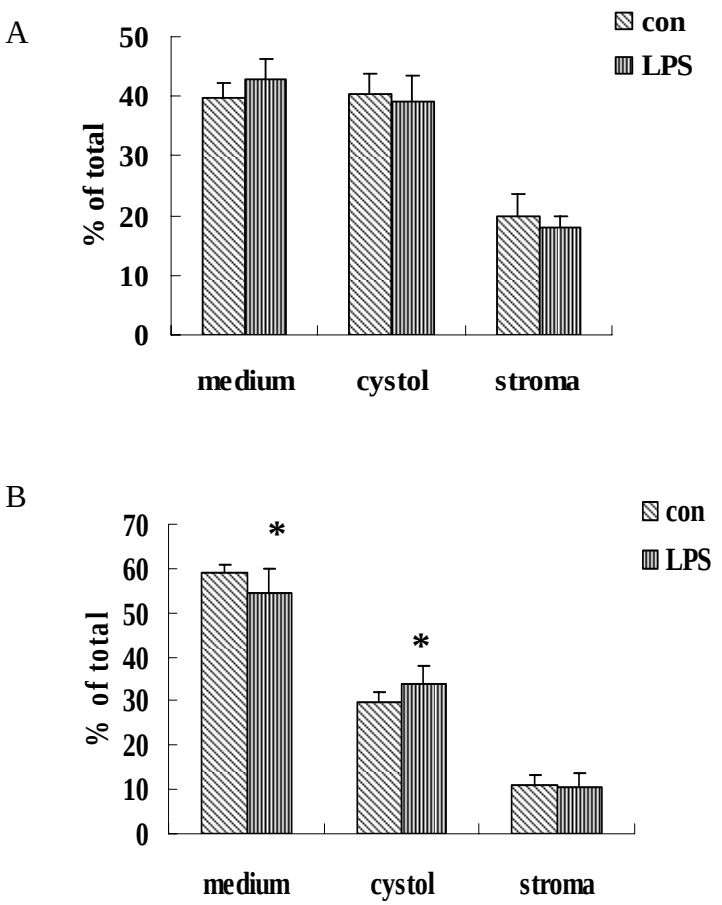


Figure 4-9. Effect of LPS on the iron release in the macrophages.

The rats were administrated with LPS (1ug/g body weight i.p) or steriled saline (control), the peritoneal macrophages were then collected after 2 h (A) and 6h (B). The cells were preloaded with iron by incubation with 2uM ^{55}Fe(II) in sucrose (pH 6.5) for 30 minutes at 37^{o}C, and then washed with ice-cold PBS. They were then incubated in the serum-free medium at 37^{o}C for 60 minutes. At the end of release, the medium was collected, and the cells were lysed. Radio-labelled iron released into the media and in the cytosolic and stromal fractions was measured as described in the Materials and Methods. Results were expressed as percentage of total iron present in the system (=media+cytosol+stromal). All these data were presented as mean$\pm$SD of 4 independent experiments performed in sextuple. Asterisks above data bars indicated that these groups were statistically different from control (*P <0.05).

CHAPTER 5

EFFECT OF HEPCIDIN ON THE IRON TRANSPORT PROTEINS EXPRESSION AND IRON UPTAKE AND RELEASE IN THE H9C2 CELLS

5.1 ABSTRACT

The study described in Chapter 3 demonstrated that hepcidin could regulate iron metabolism in the peritoneal macrophages. However, it is unknown whether organ- or tissue-specific mechanism is involved in this effect of hepcidin. To understand this issue, experiments were carried out to evaluate the effect of hepcidin on the protein expression of DMT1, TfR1, FPN1, CP, Heph and iron uptake and release in rat H9C2 cardiomyocytes. By Western blot, the relative amount of DMT1-IRE in H9C2 cells was decreased after 700nM hepcidin treatment for 8 hours, accompanied by the reduction of NTBI uptake by the H9C2 cells. The level of TfR1 was also reduced by 700nM hepcidin in a time-dependent manner as well as the 70nM hepcidin treatment for 24 hours. The change of Tf-Fe uptake in H9C2 cells was parallel with the TfR1

protein level. Hepcidin at a high concentration (700nM) significantly decreased the Tf-Fe uptake into the cells. Hepcidin at high concentration also induced a rapid decrease in the FPN1 protein expression after it treatment for 8 hours. After 16 and 24 hours, the FPN1 protein level recovered and returned to the pre-treatment level. On the other hand, hepcidin at both concentrations had no effect on the expression of Heph protein, and just increased the CP protein level with 700nM hepcidin treatment for 24 hours. The change of iron release from H9C2 cells was consistent with the level of FPN1, which significantly decreased after 700nM hepcidin treatment for 8 and 16 hours. Combining these data with the result from the peritoneal macrophages, we suggested that the effect of hepcidin on iron metabolism was concentration-dependent and time-dependent, and organ- or tissue-specific.

KEY WORDS

Hepcidin, H9C2, DMT1, Ferroportin1, Transferrin receptor, Ceruloplasmin, Iron uptake, Iron release

5.2 INTRODUCTION

Iron is an essential trace element in humans. Like other cells, the heart cells require iron for many aspects of their physiology. However, excess iron can catalyze to generate the reactive oxygen species, which are harmful to heart. Studies show that a number of cardiovascular diseases is associated with iron-mediated injury. These include heart ischemia-reperfusion injury (Berenshtein et al., 1997; Chen et al., 2002), hemochromatosis (Pereira et al., 2001; Sullivan and Zacharski, 2001), bata-thalassemia (Aessopos et al., 2001) and so on. While iron chelation therapy can significantly alleviate heart reperfusion injury, the prognosis of thalassemia patients, and endothelial function of patients with coronary artery diseases (Duffy et al., 2001; Horwitz and Rosenthal, 1999; Horwitz et al., 1998; Taher et al., 2001).

At present, it is unknown how iron increases to a pathological level in the heart under abnormal circumstances. Recently, studies of the hepcidin level in the hereditary haemochromatosis (HH) and beta-thalassemia give some clues to investigate the mechanism of iron homeostasis of heart.

Hepcidin is expressed predominantly in the liver, but is also detectable in heart (Pigeon et al., 2001). Mutations in the hepcidin gene have recently been shown to result in juvenile haemochromatosis (JH), which is a more severe form of iron

loading disorder than HH. In JH, symptomatic organ involvement occurs as early as the second decade of life. Although liver involvement is a constant feature in genetic hemochromatosis, diabetes, hypogonadotropic hypogonadism, cardiomyopathy, arrhythmias and heart failure are far more frequent in JH than in the adult-onset form. Heart failure and/or arrhythmias are the most frequent causes of death (De Gobbi et al., 2002; Vas et al., 2005). Another gene mutation, HJV, also results in JH. HJV and its mRNA have a broad spectrum of expression, including skeletal muscle, liver and heart (Papanikolaou et al., 2004). The expression of HJV pattern suggests that its role in regulating iron allocation could be extended to other tissues beyond the liver. Babitt et al. (Babitt et al., 2006) demonstrated that HJV's ability to function as a BMP co-receptor could be fundamental for its role in regulating the hepcidin expression and subsequent systemic iron homeostasis.

Based on the expression and the role of hepcidin, HJV and the severe heart failure in JH, we hypothesis that the heart is perhaps another target of hepcidin. Our earlier studies provided direct evidence that hepcidin could regulate the iron metabolism of peritoneal macrophages through changing the related iron transporters level. At present, there is no data about the effect of hepcidin on heart iron hemeostasis. Therefore, in the current study, we chose the H9C2 cell, a kind of cardiomyocytes

cell line, as the cell model to investigate the effect of hepcidin on iron uptake and release and on the expression of the DMT1, TfR1, FPN1, CP and Heph protein. The understanding of these aspects is fundamental for elucidating the mechanism of iron balance in the heart. Most importantly, data on the expression and regulation of DMT1, TfR1, FPN1, CP and Heph in the heart would help explain the heart iron metabolism and the etiology of the iron-related cardiovascular diseases.

5.3 MATERIALS AND METHODS

5.3.1 Materials

Please refer to chapters 2 and 3.

5.3.2 Methods

5.3.2.1 Cryopreservation of Cells

The cultured cells were detached from the substrate using dissociation agents. Then the detached cells were placed in complete growth medium and centrifuged at ~200

×g for 5 min. After withdrawing the supernatant down to the smallest volume without disturbing the cells, the cell pellet was resuspended in freezing cold medium to concentration of 5×10^6 to 1×10^7 cells/ml. Aliquot of the cells solution were placed into cryogenic storage vials. The vials were put on ice or in a 4^oC refrigerator and freezing began within 5 min. The cells were slowly frozen at rate of $1\ ^oC$ /min. This could be done by programmable coolers or by placing the vials inside an insulated box placed in a $-70\ ^oC$ to $-90\ ^oC$ freezer, then transferred the vials for storage in liquid nitrogen.

5.3.2.2 Thawing of Cryopreserved Cells

Removed the cryopreserved cells from storage and thawed quickly in a $37\ ^oC$ water bath. Placed 1 or 2 ml of frozen cells into 25 ml of complete growth medium. Mixed them very gently. Then centrifuged these cells at 80 g for 2 to 3 minutes. After discarding the supernatant, gently resuspend the cells in the complete growth medium and performed viable cell count. The cells were cultured for 12 to 24 hours, and the medium is replaced with fresh complete growth medium to remove cryopreservatives.

5.3.2.3 H9C2 Cardiomyocytes Culture

The H9C2 cardiomyocytes were obtained from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's modified Eagle's medium (DMEM/Glutamax; Life Technologies), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100U/ml of sodium penicillin G and 100 µg/ml of streptomycin sulfate. The medium was changed every 3 days. The subculture was prepared by removing the medium, adding 1-3 ml of fresh 0.25% trypsin or 2mM EDTA solution (for protein extraction) for several minutes. The culture was allowed to stand at room temperature for 10 to 15 minutes. Fresh medium was added, aspirated and dispensed until the cells were detached. Then the cells were transferred to a 15 ml centrifuge tube containing 3-5 ml fresh medium and centrifuged at 1000 rpm for 5 minutes at 4^{o}C. The supernatant was discarded and the pellet triturated in 2 ml fresh medium. The cell number was determined by trypan blue exclusion under the microscope, and the required number of cells was placed into the flasks (for maintenance), or in 6-well plates and 35mm culture dishes. All the apparatus and mediums used for cell culture were sterilized before use.

5.3.2.4 Western Blot Analysis

Please refer to chapters 2 and 3.

5.3.2.5 Non-transferrin Bound Iron (NTBI) Uptake Assay

Please refer to chapters 2 and 3.

5.3.2.6 Transferrin Binding Iron Uptake Assay

Please refer to chapters 2 and 3.

5.3.2.7 Iron Release Assay

Please refer to chapters 2 and 3.

5.3.3 Statistical Analysis

Statistical analyses were performed using SPSS software for Windows (version 10.0).

Data were presented as mean±SD. The difference between the means was determined by One-Way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. Differences with $P<0.05$ were considered significant.

5.4 RESULT

5.4.1 Effect of Hepcidin on DMT1–IRE Protein Expression in H9C2 Cells

To test the effect of hepcidin on iron metabolism in H9C2 cells, we first analyzed the DMT1-IRE protein synthesis. The H9C2 cardiomyocytes grown in the 6-well culture plates were pre-incubated with different amount of hepcidin (70nM and 700nM) for 8, 16 and 24 hours. At the desired time points, the cell proteins were extracted for Western blot analysis. As shown in Fig5-1A, there were major bands with a molecular weight (*M*r) of～60 kDa for DMT1-IRE and ～45 kDa for β-actin, which were in good agreement with the molecular weights observed in the peritoneal macrophages. After quantification by densitometry and correction of total protein loading by using β-actin expression, there was no significant difference observed between the control groups and the groups treated with 70nM hepcidin for 8, 16 and 24 hours (P>0.05). However, when the cells were incubated with 700nM hepcidin for

8 hours, the DMT1-IRE protein level decreased to 75% compared with the control ($P<0.05$). Following 16 and 24 hours, this protein expression recovered and returned to the control level ($P>0.05$). These findings implied that a low concentration of hepcidin was not able to affect the DMT1-IRE protein expression in H9C2 cells.

5.4.2 Effect of Hepcidin on the Non-transferrin Bound Iron (NTBI) Uptake in H9C2 Cells

At present, it is unknown how iron increases to pathological level in the heart under abnormal circumstances. However, the overtaking of NTBI mediated by DMT1 in the heart cells might be a key cause in heart injury, because the toxicity of NTBI is much higher than that of Tf-Fe as judged by its ability to promote hydroxyl radical formation. Therefore, in this study, we further investigated the NTBI uptake by the H9C2 heart cells with or without pre-incubation of 700nM hepcidin for 8, 16 and 24 hours. After incubating with 2uM $^{55}Fe(II)$ at $37^{o}C$ for 30 minutes, the cells were separated into cytosolic and stromal fractions and subjected to detection of the CPM value of each part. Compared to the control of each time point, the NTBI uptake into cytosolic fraction was significantly decreased in the 8 hours group (2.65±0.28 umol/ug protein, control; 2.25±0.25 umol/ug protein, hepcidin treatment, $P<0.05$),

whereas there were no significant changes in the 16 and 24 hours groups.

5.4.3 Effect of Hepcidin on TfR1 Protein Expression in H9C2 Cells

Tf-TfR is the main iron uptake route in most kinds of cells. Changes in the TfR protein induced by hepcidin with concentrations ranging from 70nM to 700nM for different time periods were detected by Western blot in H9C2 cardiomyocytes. A single band was observed with its antibody, with a molecular weight (*M*r) of ～90 kDa. As illustrated in Fig5-3, there was a time-dependent decrease of TfR1 protein after high concentration of hepcidin (700nM) treatment. There were no significant changes in the TfR1 protein expression with 70nM hepcidin incubation for 8 and 16 hours. However, when hepcidin was incubated for 24 hours, the TfR1 protein level decreased to 65% of the control. On the other hand, the TfR1 expression was gradually decreased after 700nM hepcidin treatment for 8 hours (90.26±3.86% of the control), 16 hours (78.33±12.45% of the control) and reached the minimal level at 24 hours (36.88±4.35% of the control).

5.4.4 Effect of Hepcidin on the Tf-Fe Uptake in H9C2 Cells

Since the TfR1 protein expression was affected by hepcidin, we further investigated the Tf-Fe uptake in the H9C2 cells after pre-treatment with 700nM hepcidin for different time. As described in 'Methodology', the pre-treated cells were subjected to incubate with iron-saturated Tf (10ug/ml) at each time point for 60 minutes at 37^{o}C. The intracellular radioactivity (cytosol) and the membrane-pellet (stoma) were separated and measured respectively. The results were summarized and presented in Fig5-4. It could be seen that the intracellular radioactivity declined gradually and finally reached a lower level after hepcidin pre-incubation. There was a slight but not significant decrease in the radioactivity in cytosolic fraction after 8 hours pre-incubation with hepcidin. In the groups with hepcidin pre-treatment for 16 and 24 hours, the radioactivity in cytosolic fraction reduced from 12.19±0.87 pmol/ug protein to 10.63±1.38 pmol/ug protein and from 13.96±3.55 pmol/ug protein to 10.41±0.52 pmol/ug protein, respectively ($P<0.05$). The total radioactivity of the H9C2 cells was the sum of cytosol and stroma fractions and its declining tendency was same as cytosol. It was clearly demonstrated that hepcidin could regulate both the TfR1 protein expression and Tf-Fe uptake in a dose- and time-dependent manner in H9C2 cells.

5.4.5 Effect of Hepcidin on FPN1, CP and Heph Protein Expression in H9C2 Cells

To determine the effect of hepcidin on the synthesis of FPN1, Heph and CP proteins, the total protein in cell extracts derived from cultured H9C2 cells treated with hepcidin (70nM and 700nM) for 8, 16 and 24 hours were analyzed using the Western blot. A single major band was observed with these antibodies, with M_r of ～62 kDa for FPN1 (Fig5-5 A), ～150 kDa for CP (Fig5-6 A), ～135 kDa for Heph (Fig5-7 A) and ～45 kDa for β-actin, which were in good agreement with the expected molecular weights based on published papers. As illustrated in Fig5-5, hepcidin could significantly reduce the expression of FPN1 at 700nM. The amount of FPN1 protein decreased to 45% of the control after 700nM hepcidin incubation for 8 hours. Following the 16 hours incubation, the FPN1 protein level recovered to 68% and returned to 88% of the control ($P<0.05$). However, hepcidin had no effect on the expression of FPN1 at the concentration of 70nM.

The protein expression of Heph was not significantly affected by hepcidin at either high or low concentrations. As shown in Fig5-6, the change of CP protein synthesis was not parallel to the FPN1 protein. After 700nM hepcidin treatment for 24 hours,

the CP protein increased to 131% of the control ($P<0.05$), whereas no significant change of CP protein were seen in the other treatment by hepcidin in the H9C2 cells.

5.4.6 Effect of Hepcidin on the Iron Release in H9C2 Cells

Our previous study showed that hepcidin could significantly change iron release from peritoneal macrophages *in vivo* and *in vitro*. In the present study, similar results were observed in the H9C2 cells. Before experiments, the cells were pre-loaded with 2uM Fe(II) for 30 minutes. After cooling the cells on ice to stop the uptake and washing them 3 times with cold PBS solution, hepcidin was added to the final concentration of 700nM and incubated for 8, 16 and 24 hours. Exposure of iron loading H9C2 cells to hepcidin for 8 hours induced a significant decrease of the radioactivity released into the medium compared to the control (21.63±2.49% of total, control; 17.87±0.99% of total, hepcidin treatment for 8 hours). In the groups treated with or without hepcidin for 16 hours, the iron released into medium decreased to 87% of the control. As to the 24 hours incubation with hepcidin, the percentage release into the medium was still lower than the control, but it was not significant ($P>0.05$). Corresponding to the change of radioactivity in the medium, the iron retention in the cells was increased following the hepcidin treatment.

5.5 DISCUSSION

To our knowledge, this is the first report about the effect of hepcidin on iron metabolism in the cardiac myocytes. The study described in Chapter 3 demonstrated that hepcidin could regulate the iron uptake and release in the peritoneal macrophages through regulating the related iron transporters on the macrophages. However, it is unknown whether any organ- or tissue-specific mechanism was involved in the effect of hepcidin. To understand this issue, we investigated the change of these iron transport proteins expression and the iron uptake and release in cardiac myocytes following hepcidin treatment.

Iron deposit in the heart is cell type-specific, primarily confined to the myocyte (Fitchett et al., 1980), and iron shows a greater tendency to deposit in cardiac than in smooth or skeletal muscle (Buja and Roberts, 1971). This appears to be recapitulated in culture. The basis of this selectivity is unknown. Our earlier studies provided direct evidence for the existence of DMT1, FPN1, Heph, CP and TfR mRNAs and proteins in the rat heart. Subcellular distribution showed that they were mainly located in the myocytes. In addition, iron status was found to have significant effect on the expression of DMT1, TfR1, FPN1, Heph and CP mRNAs or proteins in the

heart.

The presence of transmembrane iron importer DMT1 in cardiac myocytes provides important insights into the understanding of the cause of excessive accumulation of iron and the pathogenesis of iron-induced diseases in the heart. In the uptake of NTBI of heart cells, which might be a key cause of heart injury, DMT1 might play a key role (Tsushima et al 1999).

In this study, we first detected the DMT1-IRE protein level and NTBI uptake after hepcidin treatment. Our data presented here showed that hepcidin had slight effect on the expression of DMT1-IRE. The DMT1-IRE protein level decreased to about 75% of the control after 700nM hepcidin incubation for 8 hours. Under the same condition, the NTBI uptake by H9C2 cells also decreased. Similar studies were observed in the $CaCO_2$ epithelial cells. There was a reduction in both iron transport and DMT1 protein expression after hepcidin treatment (Laftah et al., 2004). In good agreement with our results, there were strong inverse correlation data between hepcidin expression and the duodenal level of DMT1 and Dcytb proteins in various pathophysiological situations (Frazer et al., 2004). However, the mechanism involved in the regulation of the DMT1 protein expression is not yet understood.

Different from NTBI, the membrane TfR mediated endocytosis or the internalization of the complex of Tf bound iron is the major route of cellular iron uptake (Li et al., 2003; Li and Qian, 2002). The TfR1 expression is higher in primary cultured rat cardiac myocytes than in fibroblast (Liu et al., 2003). Our earlier immunohistochemistry result showed that there was prominent TfR1 staining in the cardiac myocytes of the myocardium.

Unlike DMT1-IRE, we found that hepcidin had significant effect on the expression of TfR1 in H9C2 cells. When cells were treated with hepcidin (70nM or 700nM) for 8 and 16 hours, there were no or little changes in the TfR protein expression. But when the incubation time was prolonged to 24 hours, the expression of TfR decreased to 65% (70nM) and 36% (700nM) of control respectively. To determine whether this change affected the iron uptake of H9C2 cells, we detected the Tf-Fe uptake after hepcidin incubation. The data indicated that hepcidin could significantly decrease the transferrin bound iron uptake at 24 hours, which was consistent with the change of TfR1 protein.

At present, there is no evidence to show that hepcidin could direct interact with TfR1. A possible explanation for the change of TfR1 protein after hepcidin treatment might

be that of a secondary response to the intracellular iron level. Like DMT1+IRE and many other mRNAs encoding proteins which are involved in iron uptake and storage, the TfR1 mRNA contains IRE within its 3' UTR, which is a binding site for the iron-regulatory protein (IRP). IRE mediates the changes of the level of these proteins in response to iron availability. Under low iron status, IRP would bind to and stabilize the IRE of TfR mRNA, leading to an increase in the TfR1 mRNA expression and protein synthesis. In addition, high iron status would result in a decrease in the IRE/IRP binding and hence the destabilization of the TfR mRNA, leading to a suppression of the TfR1 mRNA expression and protein synthesis. The IRE/IRP mechanism is in principle protective by decreasing further transferrin-dependent iron acquisition while enhancing storage in ferritin (Parkes et al., 2000).

The export of iron from different cell types involves ferroportin1, the sole known exporter of iron in vertebrates. It also requires ferroxidase (hephaestin and ceruloplasmin) to deliver ferric iron to the transferrin. Our study showed that hepcidin could induce a rapid decease of the FPN1 protein level in H9C2 cells when 700nM hepcidin was incubated for 8 hours. Moreover, the iron release of H9C2 cells decreased after 700nM hepcidin treatment for 8 hours. The response of FPN1 to

hepcidin presented a similar change between the heart cells and the peritoneal macrophages, suggesting that there might be a similar mechanism of the regulation of FPN1 by hepcidin. It was as follows: (a) hepcidin directly binds to ferroportin1; (b) the binding of hepcidin causes FPN1 to be internalized and degraded; and (c) the loss of ferroportin1 from the cell membrane ablates the cellular iron export.

Now very little is known about the mechanisms involved in regulation of the hephaestin (Heph) protein expression. Previous studies showed that variations in iron status had a small but not significant effect on Heph expression in the duodenum (Frazer et al., 2001; Frazer et al., 2003; Rolfs et al., 2002; Yamamoto et al., 2002), whereas Sakakibara and Aoyama's studies showed that dietary iron-deficiency increased Heph mRNA expression in the small intestine of rats (Sakakibara and Aoyama, 2002). In a notable study on *sla* mice, the cellular iron levels were increased in the intestinal enterocytes yet the systemic iron levels were low. The pattern of the increased Heph mRNA expression was similar to that of the iron-deficient mice, and the Heph protein was also up-regulated. Thus, Heph mRNA and protein levels correlated with the systemic instead of the local iron status (Chen et al., 2003). Our results showed that hepcidin had no effect on the Heph protein level in both concentrations. A possible explanation was that the change of the

cellular local iron status had little influence on the Heph protein synthesis.

Ceruloplasmin, a soluble ferroxidase produced by the liver, plays a key role in iron homeostasis by favoring cellular iron release and iron incorporation into transferrin. Surprisingly, the change of ceruloplasmin was not consistent with FPN1 in our study. While the ferroportin1 exporter was down-regulated in the H9C2 after hepcidin treatment for 8 hours, there were no significant changes in ceruloplasmin. However, when 700nM hepcidin was incubated for 24 hours, the FPN1 protein recovered to the normal level, whereas the ceruloplasmin protein expression increased to 130% of the control. The inverse correlation between FPN1 and CP was also seen in the *Usf2-/-* mice. Viatte et al (Viatte et al., 2005) pointed out that the increased ferroportin1 and decreased ceruloplasmin levels were expected to be the consequence of hepcidin deficiency instead of liver iron overload, since in the experimentally iron-loaded mice, the level of hepatic ferroportin1 was unaltered while the ceruloplasmin level was increased. The mechanism involved in the regulation of the CP protein expression by hepcidin needs further investigation.

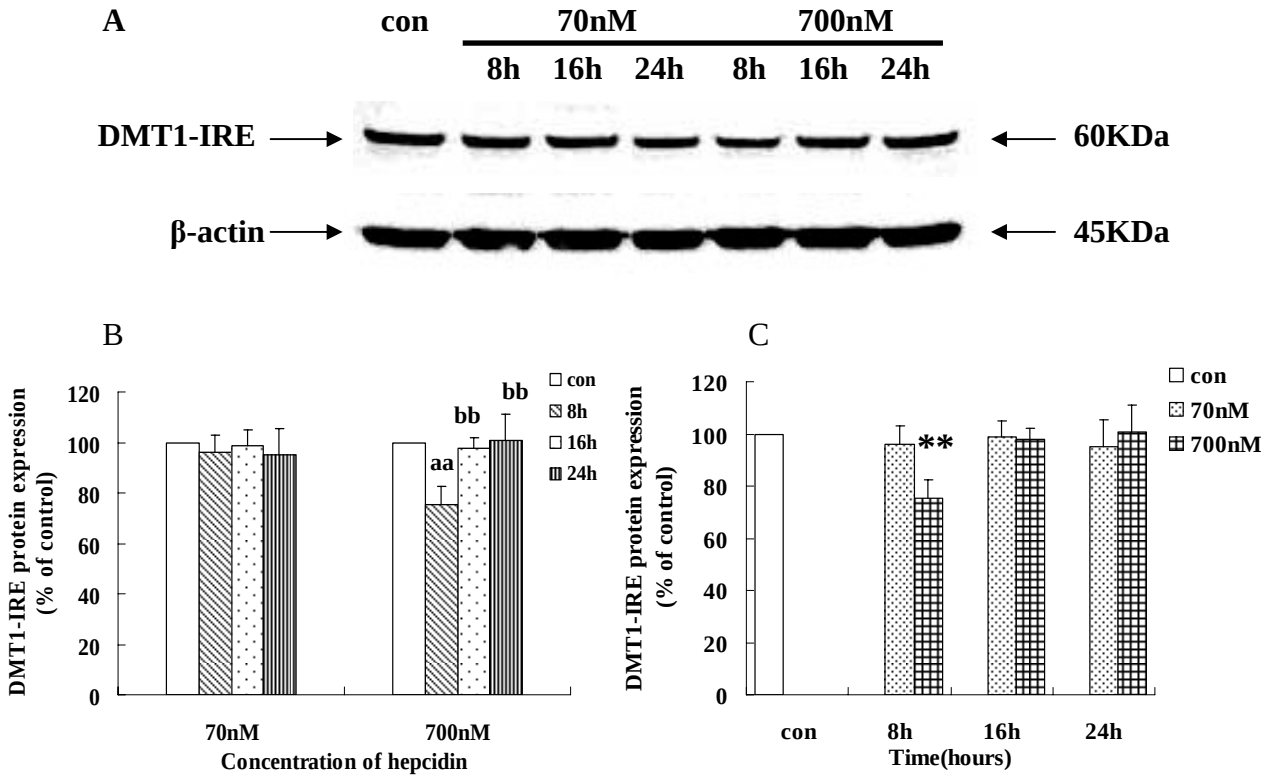
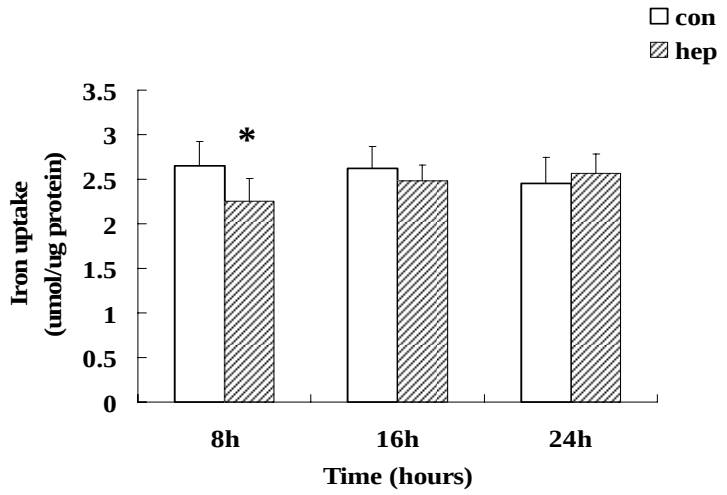


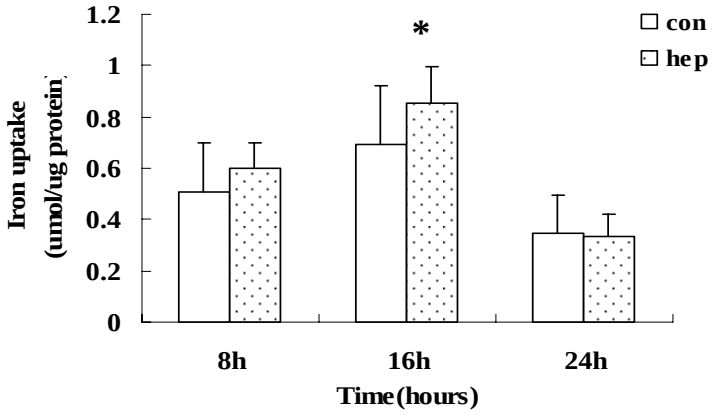
Figure5-1. Effect of hepcidin on DMT1-IRE protein expression in H9C2 cardiomyocytes.

Western blot analysis was performed as described in Materials and Methods. The *M*r of ~60kDa for DMT1-IRE and β-actin of ~45kDa (A) appeared as a single band on each gel. The result of the DMT1-IRE protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs controls; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other while incubated with hepcidin for the same time (**P* <0.05, ***P* <0.01).

A


con
hep
Iron uptake
(umol/ug protein)
3.5
3
2.5
2
1.5
1
0.5
0
*
8h
16h
24h
Time (hours)

B


con
hep
Iron uptake
(umol/ug protein)
1.2
1
0.8
0.6
0.4
0.2
0
*
8h
16h
24h
Time(hours)

C

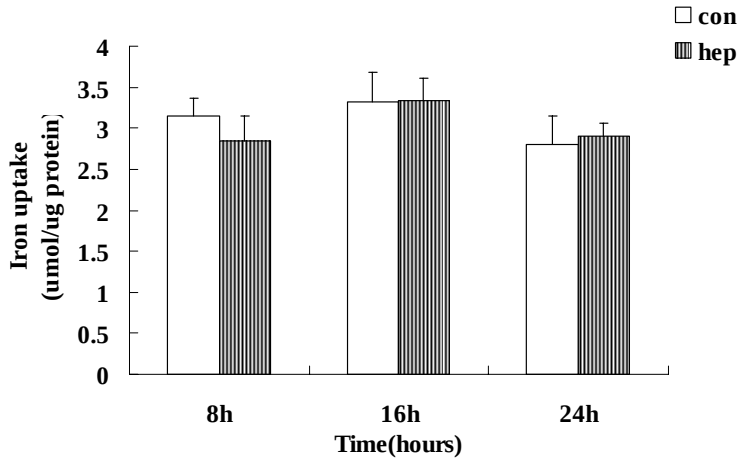

con
hep
Iron uptake
(umol/ug protein)
4
3.5
3
2.5
2
1.5
1
0.5
0
8h
16h
24h
Time(hours)

Figure 5-2. Effect of hepcidin on the NTBI uptake in H9C2 cardiomyocytes.

The H9C2 cardiomyocytes were preincubated with or without 700nM hepcidin for 8, 16 and 24 hours. At the indicated time points, the cells were washed and incubated with 2uM Fe(II) under the control conditions (0.27M sucrose, pH 6.5) at 37^{o}C for 30 minutes. The iron taken into cytosolic (A) and stromal (B) fractions were measured as described in Materials and Methods. The total iron uptake (C) was the sum of the two parts. All these data were presented as mean$\pm$SD of 6 independent experiments performed in triplicate. Asterisks above the data bars indicated that these groups were statistically different from control (*P <0.05).

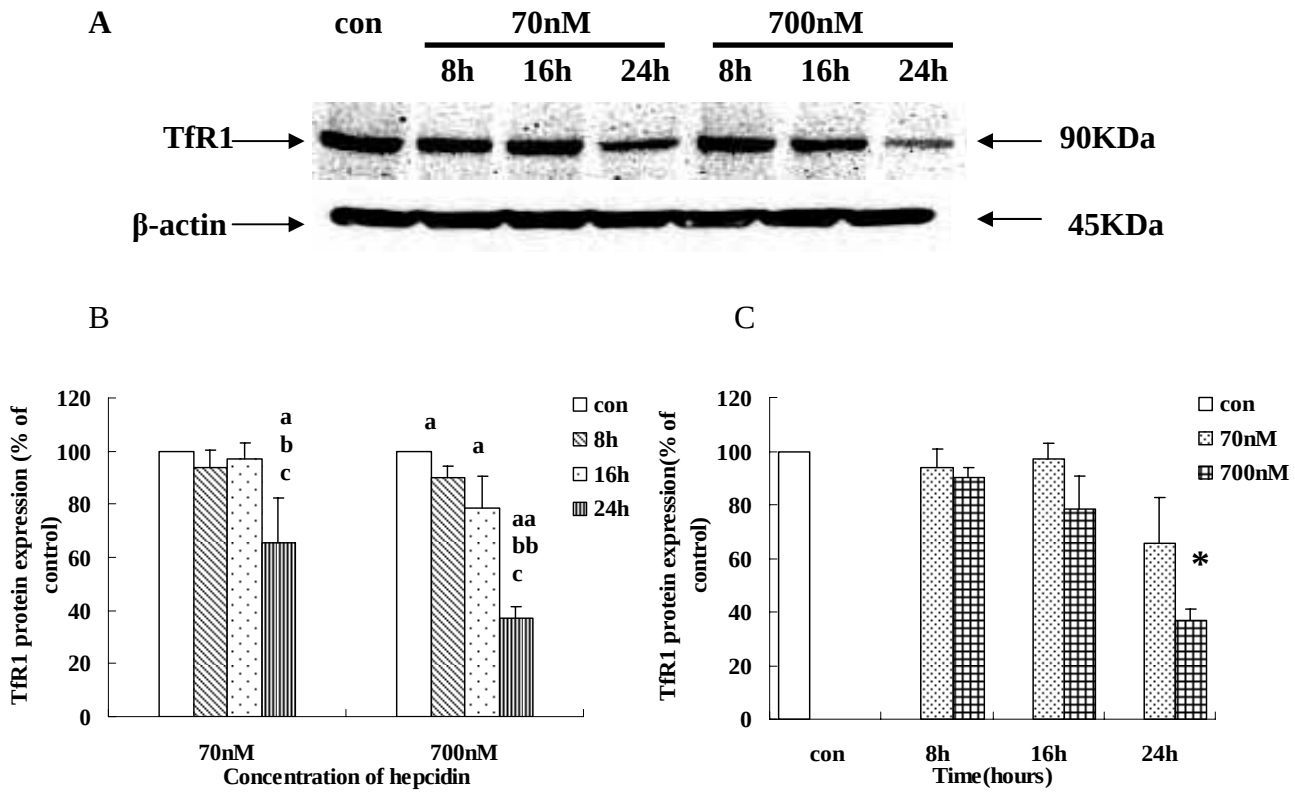
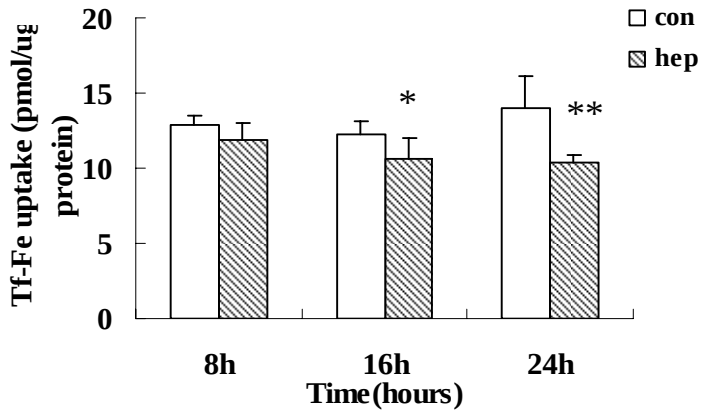
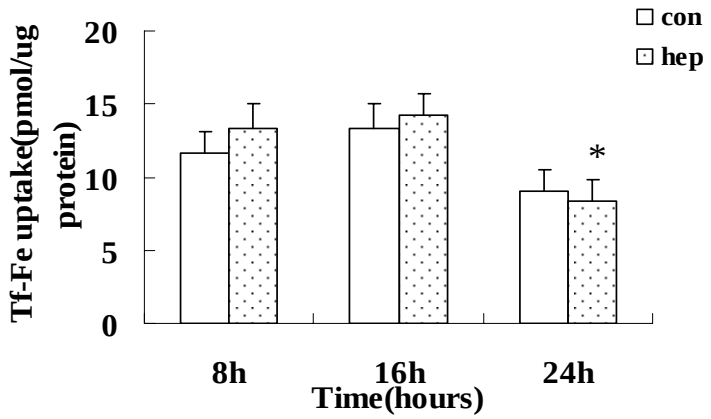


Figure 5-3. Effect of hepcidin on TfR1 protein expression in H9C2 cardiomyocytes. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~90kDa for TfR1 and β-actin of ~45kDa (A) appeared as a single band on each gel. The result of the TfR1 protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs control; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicate that these groups were statistically different from each other while incubated with hepcidin for the same time (**P* <0.05, ***P* <0.01).

A


20
15
10
5
0
Tf-Fe uptake (pmol/ug protein)
con
hep
*
**
8h
16h
24h
Time(hours)

B


20
15
10
5
0
Tf-Fe uptake(pmol/ug protein)
con
hep
*
8h
16h
24h
Time(hours)

C

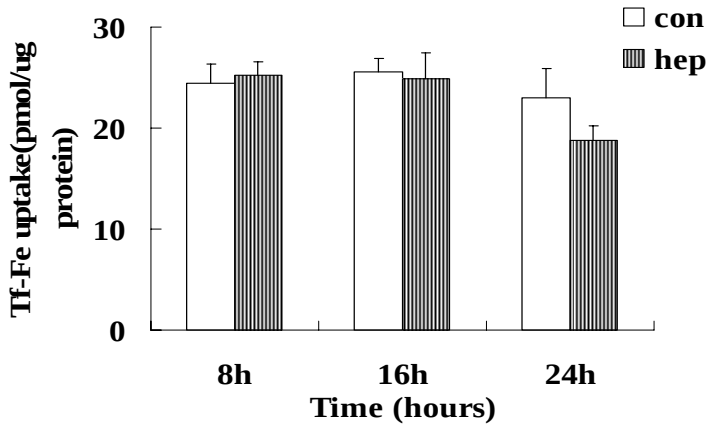

30
20
10
0
Tf-Fe uptake(pmol/ug protein)
con
hep
8h
16h
24h
Time (hours)

Figure 5-4. Effect of hepcidin on the Tf-Fe uptake in H9C2 cardiomyocytes.

The H9C2 cardiomyocytes were preincubated with or without 700nM hepcidin for 8 hours, 16 hours and 24 hours. At the indicated time points, the endogenous transferrin was removed from the cells and incubated with serum-free culture medium with 10ug/ml Tf-Fe at 37^{o}C for 60 minutes. The iron taken into cytosolic (A) and stromal (B) fractions was measured as described in Materials and Methods. The total iron uptake (C) was the sum of the two parts. All these data were presented as mean$\pm$SD of 6 independent experiments performed in triplicate. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

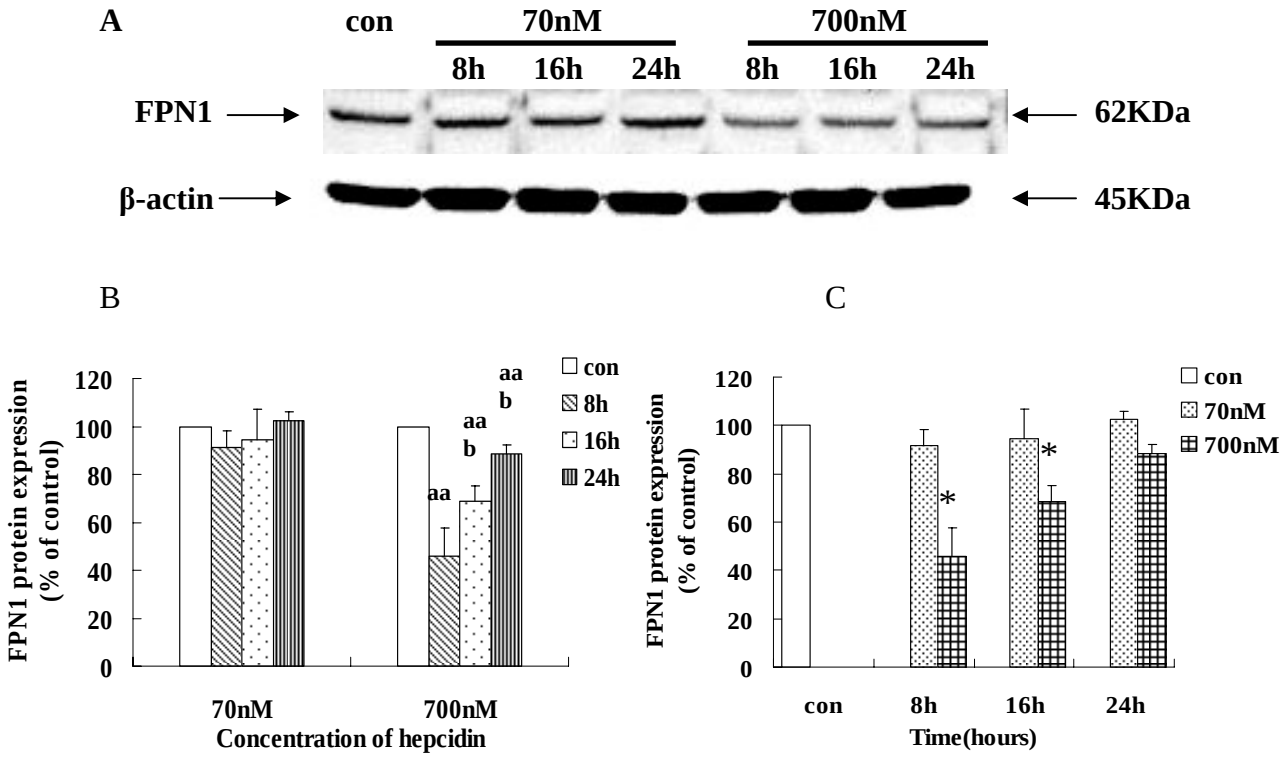


Figure 5-5. Effect of hepcidin on FPN1 protein expression in H9C2 cardiomyocytes. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~62kDa for FPN1 and β-actin of ~45kDa (A) appeared as a single band on each gel. The result of the FPN1 protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs control; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other while incubated with hepcidin for the same time (**P* <0.05, ***P* <0.01).

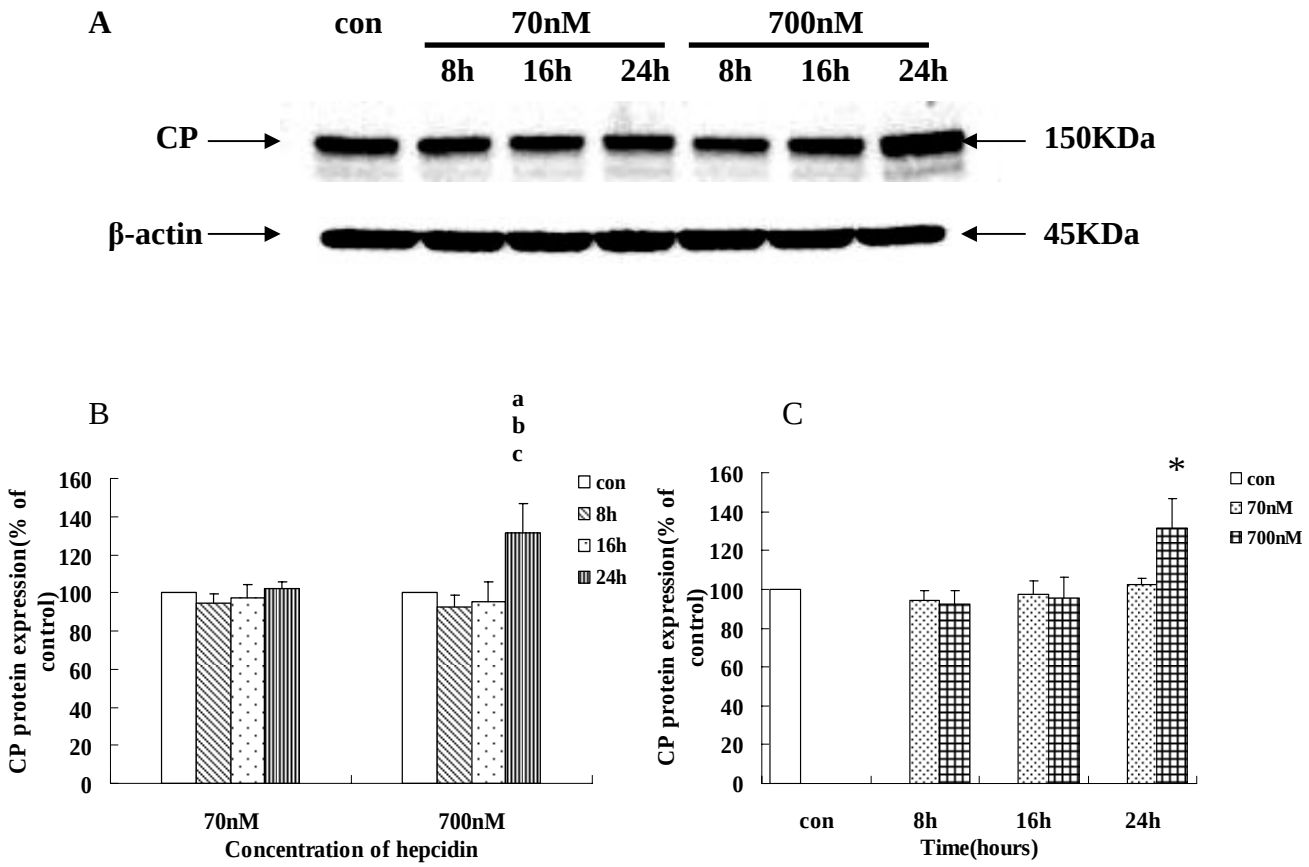


Figure 5-6. Effect of hepcidin on CP protein expression in H9C2 cardiomyocytes. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~150kDa for CP and β-actin of ~45kDa (A) appeared as a single band on each gel. The result of the CP protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs control; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other while incubated with hepcidin for the same time (**P* <0.05, ***P* <0.01).

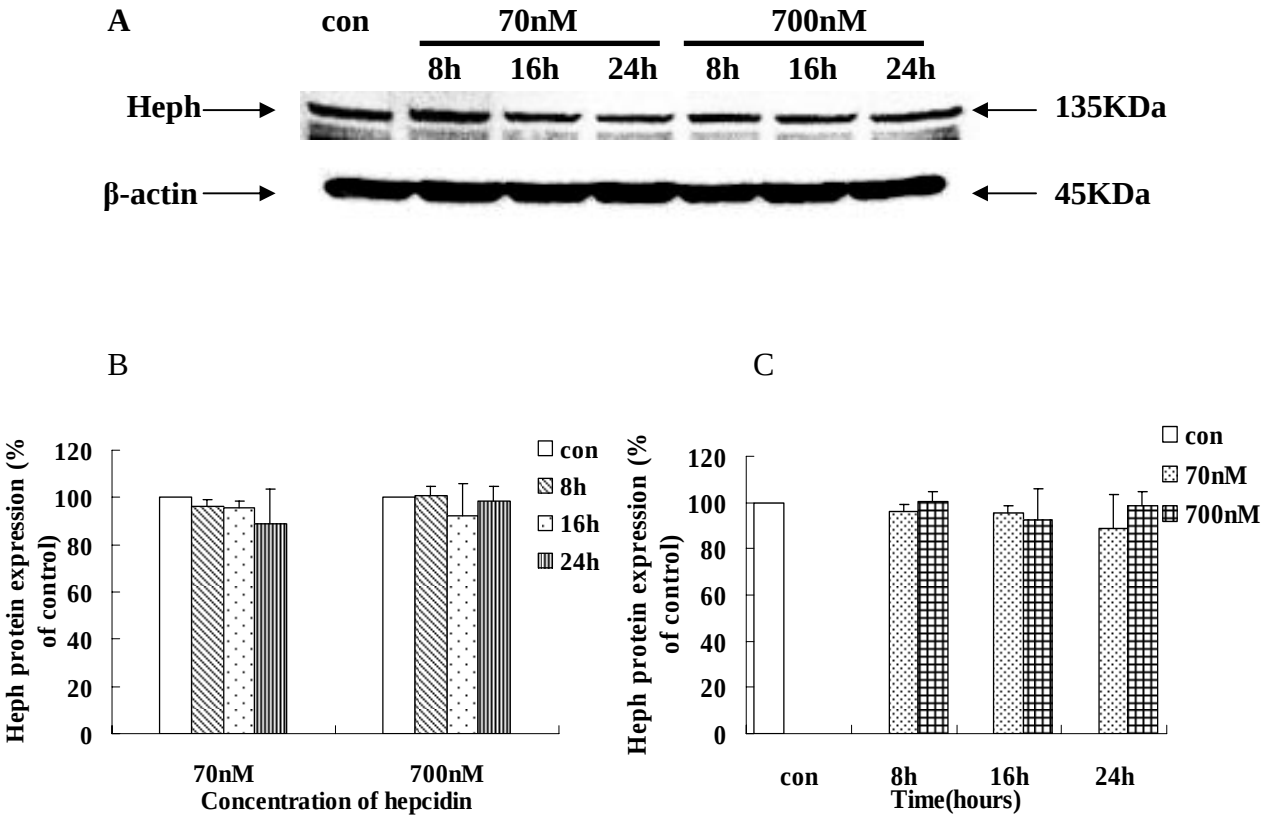
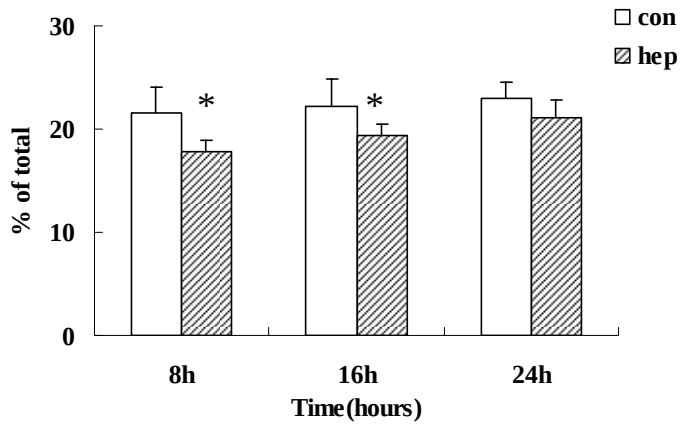
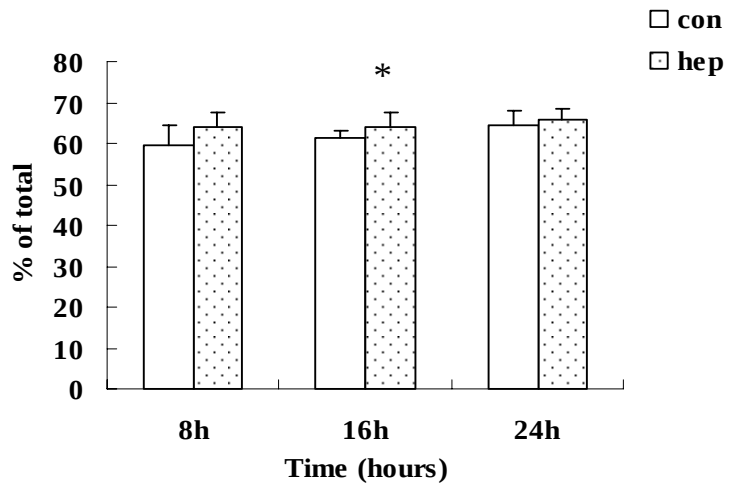


Figure 5-7. Effect of hepcidin on Heph protein expression in H9C2 cardiomyocytes. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~135 kDa for Heph and β-actin of ~45kDa (A) appeared as a single band on each gel. The result of the Heph protein expression after various concentrations (B) of hepcidin treatment for different time (C) was normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Asterisks above the data bars indicated that these groups were statistically different from each other (*P <0.05).

A


con
hep
30
20
10
0
% of total
*
*
8h
16h
24h
Time(hours)

B


con
hep
80
70
60
50
40
30
20
10
0
% of total
*
8h
16h
24h
Time (hours)

C

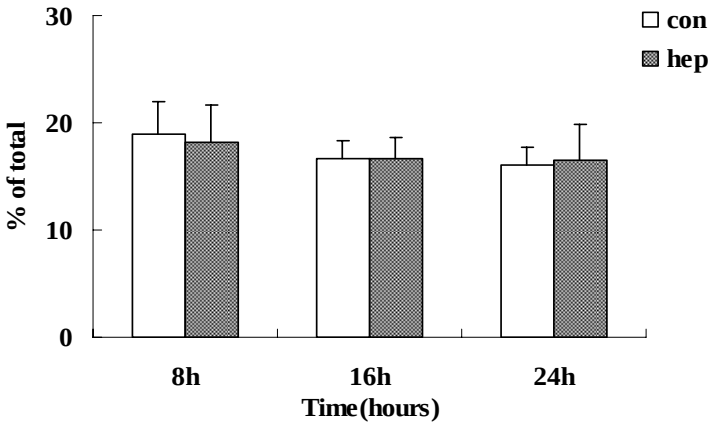

con
hep
30
20
10
0
% of total
8h
16h
24h
Time(hours)

Figure 5-8. Effect of hepcidin on the iron release by the H9C2 cardiomyocytes.

The cells were preloaded with iron by incubation with 2uM ^{55}Fe(II) in sucrose (pH 6.5) for 30 minutes at 37^{o}C, and washed with ice-cold PBS. They were then incubated with or without 700nM hepcidin at 37^{o}C. At different time points during the iron release, the medium was collected, and the cells were lysed. Radio-labelled iron release into the medium (A) and in the cytosolic (B) and stromal (C) fractions were measured as described in Materials and Methods. Results were expressed as the percentage of total iron present in the system (=media+cytosol+stromal). All these data were presented as mean$\pm$SD of 6 independent experiments performed in triplicate. Asterisks above the data bars indicated that these groups were statistically different from the control (*P <0.05).

CHAPTER 6

REGULATION OF HEPCIDIN GENE EXPRESSION BY LPS AND THE EFFECT OF HEPCIDIN ON THE IRON METABOLISM IN THE C6 GLIOMA CELLS

6.1 ABSTRACT

It has been discovered recently that hepcidin plays an essential role in iron metabolism outside the CNS. The study described in Chapter 3 and 5 also demonstrated that hepcidin could regulate the iron transporters expression and iron uptake and release in peritoneal macrophages and H9C2 cardiomyocytes directly. However, it is unknown whether hepcidin has the same role on the CNS iron homeostasis. To understand this issue, experiments were conducted to evaluate the regulation of hepcidin expression in the CNS and the effect of hepcidin on the iron metabolism of the C6 brain glioma cells. By real-time RT-PCR, the hepcidin mRNA was expressed in different brain regions, including the cortex, hippocampus, striatum and substantia nigra. In addition, the hepcidin mRNA level in the cortex and

substantia nigra were significantly regulated by LPS. The wide distribution of hepcidin in CNS indicated that hepcidin might be involved in the iron homeostasis there, putatively together with other iron transporting proteins. In our study, the DMT1-IRE and TfR1 protein expression were down-regulated by hepcidin in the C6 glioma cells. In addition, after hepcidin treatment, both the NTBI and Tf-Fe uptake by C6 cells were decreased at the corresponding time points. Hepcidin had no effect on the expression of Heph protein, but it could induce a rapid decrease of the FPN1 protein expression and slightly increased the CP protein. Further analysis of the iron release from C6 glioma cells by the isotope trace method found that hepcidin reduced the iron release from the cell into the medium paralleled with the FPN1 protein change. Based on these results, we suggested that LPS could regulate the hepcidin gene expression with a region-specific pattern and hepcidin could regulate iron metabolism in the brain cells.

KEY WORDS

Hepcidin, Lipopolysaccaride, C6 glioma cells, DMT1, Ferroportin1, Transferrin receptor, Ceruloplasmin, Iron uptake, Iron release

6.2 INTRODUCTION

Iron is essential for the growth and survival of all body tissues. Numerous enzymes are dependent on iron as a vital component for oxidative respiration. The brain has the highest rate of oxidative metabolism compared to other organs, therefore its iron requirement is high. Served as a cofactor for enzymes, iron is involved in neurotransmitter synthesis, energy metabolism and myelination of axons (Connor and Menzies, 1996). On the other hand, iron is an effective catalyst in free radical reaction and excess iron in the brain can be potentially harmful via the generation of reactive oxygen species. An abnormally high level of iron in the brain has recently been demonstrated in a number of NDs, such as AD, PD and HSS, which are the most prevalent and devastating illnesses in the present population. It is generally accepted that iron accumulates in specific brain areas as a function of age (Zecca et al., 2004). Iron accumulation has indeed been reported to occur in cases of Parkinson's disease (PD) and Alzheimer's disease (AD) (Qian and Wang, 1998). For example, iron was found to accumulate within the neuromelanin granules of neurons in the substantia nigra of patients with PD (Good et al., 1992). In PD, iron has been suggested to contribute to oxidative stress (Bush, 2000) and has been considered as an important factor in the pathogenesis of PD.

The hepcidin gene has been identified and cloned in human, pig, rat, mouse, flounder, and long-jaw mudsucker (Park et al., 2001). Hepcidin is expressed predominantly in the liver, but it is also detectable in the brain and spinal cord (Pigeon et al., 2001). Hepcidin mRNA is increased in iron-overloaded mice and the amount of its mRNA decreases if mice are fed on low iron diet (Pigeon et al., 2001). Moreover, hepcidin mRNA is increased in the liver of LPS-treated mice and in LPS-treated hepatocytes (Pigeon et al., 2001). In vitro stimulation of fresh human hepatocytes with a panel of cytokines showed a strong induction of hepcidin mRNA by IL-6, indicating that IL-6 may be the mediator of hepcidin induction by inflammation. Down-regulation of hepcidin has been reported as a result of acute hemolysis, bleeding and hypoxic conditions (Nicolas et al., 2002b).

In human, mutations of hepcidin cause severe hereditary juvenile hemochromatosis (Roetto et al., 2003). In addition, hepcidin expression decreased in patients suffering from primary idiopathic hemochromatosis, suggesting that hepcidin is a key regulator of body iron metabolism (Bridle et al., 2003). It is important to note that hemochromatosis may represent a risk factor for AD (Moalem et al., 2000), indicating that imbalances in iron homeostasis of the brain contribute to the neurodegenerative processes. Iron homeostasis is important for the central nervous

system (CNS) because iron deficiency (Lozoff et al., 1991) as well as iron overload (Qian and Wang, 1998) can cause severe brain damage.

Our earlier studies showed that hepcidin could regulate the iron metabolism of several peripheral cells, such as peritoneal macrophages and cardiomyocytes. At present, the widespread distribution of hepcidin within the CNS indicate that it maybe play an important role in the brain iron homeostasis. Even so, the molecular mechanisms of the hepcidin activity, the mechanism of its regulation, and the linkage between hepcidin and other iron transport proteins in the CNS are still unknown. Therefore, in this study, experiments were designed to investigate the expression of hepcidin mRNA in different brain regions after LPS treatment. In addition, we used the C6 glioma cells as brain cell model to investigate the effect of hepcidin on the iron uptake and release and on the protein expression of the DMT1-IRE ,TfR1, FPN1, CP and Heph.

6.3 MATERIALS AND METHODS

6.3.1 Materials

Please refer to chapters 2 and 3.

6.3.2 Animals

Please refer to chapter 2.

6.3.3 Methods

6.3.3.1 Sampling of Tissues

Animals i.p with LPS at the dose of 1ug/g body weight were anesthetized with diethyl ether at the indicated time. After perfusion with ice-cold phosphate-buffered saline (Milli-Q water prepared and DEPC treated, pH 7.4) through the left ventricle, the brain was rapidly removed and immediately dissected into four brain regions: cortex, hippocampus (Hippo), striatum and substantia nigra (SNigra) according to modified methods published by Focht et al. (Focht et al., 1997), and used immediately for total RNA extraction. The remaining tissues were wrapped with aluminum, and immediately frozen below –70°C for storage after treatment with fluid nitrogen.

6.3.3.2 RNA Isolation

Please refer to chapters 2 and 4.

6.3.3.3 Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction (Real-time PCR)

Please refer to chapters 2 and 4.

6.3.3.4 Cryopreservation of Cells

Please refer to chapters 2 and 5.

6.3.3.5 Thawing of Cryopreserved Cells

Please refer to chapters 2 and 5.

6.3.3.6 C6 Glioma Cell Culture

The C6 glioma cells were obtained from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's modified Eagle's medium (DMEM/Glutamax; Life Technologies), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and 100U/ml of sodium penicillin G and 100 µg/ml of streptomycin sulfate. The medium was changed every 3 days. The subculture was prepared by removing the medium, and add 1-3 ml of fresh 0.25% trypsin or 2mM EDTA solution (for protein extraction) for several minutes. Fresh medium was added, aspirated and dispensed until the cells were detached. Then the cells were transferred to a 15 ml centrifuge tube containing 3-5 ml of fresh medium and centrifuged at 1000 rpm for 5 minutes at 4^{o}C. The supernatant was discarded and the pellet triturated in 2 ml of fresh medium. The cell number was determined by trypan blue exclusion under the microscope, and the required number of cells was placed into the flasks (for maintenance), 6-well plates or 35mm culture dishes. All the apparatus and mediums used for cell culture were sterilized before use.

6.3.3.7 Western Blot Analysis

Please refer to chapters 2 and 3.

6.3.3.8 Non-transferrin Bound Iron (NTBI) Uptake Assay

Please refer to chapters 2 and 3.

6.3.3.9 Transferrin Binding Iron Uptake Assay

Please refer to chapters 2 and 3.

6.3.3.10 Iron Release Assay

Please refer to chapters 2 and 3.

6.3.4 Statistical Analysis

Statistical analyses were performed using SPSS software for Windows (version 10.0). Data were presented as mean±SD. The difference between the means was determined by One-Way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. Differences with $P<0.05$ were considered significant.

6.4 RESULTS

6.4.1 Effect of LPS on the Hepcidin mRNA Expression in the Cortex, Hippocampus, Striatum and Substantia Nigra of Rat Brain

To investigate whether hepcidin is expressed in the CNS and whether it corresponds to LPS like peripheral organs, we carried out a cDNA synthesis from mRNA isolated from different rat brain regions. The rats were i.p with LPS at the dose of 1 ug/g body weight. At the indicated time points, the brain cortex, hippocampus, striatum and substantia nigra were excised for real-time RT-PCR analysis. As shown in Fig6-1 A, the hepcidin mRNA in the cortex began to increase at 4 hours after LPS treatment, and reached the maximal level at 12 hours, which was about 11.4 folds of the control group ($P<0.01$). Then the expression of hepcidin mRNA subsided. By 24 hours after LPS, the hepcidin mRNA fell to lower than the control (0.59 folds, $P>0.05$). However, an expansion of the study to 36 hours showed that there was a second increase of hepcidin mRNA expression at 36 h after LPS administration, which was lower than that at 12 hours.

Different from the cortex, there was some but no significant difference of hepcidin

mRNA expression in the hippocampus and striatum after LPS administration (Fig 6-1 B and C). We further analyzed the hepcidin mRNA in the substantia nigra after LPS treatment. LPS induced a rapid increase of hepcidin mRNA expression in the substantia nigra. Before LPS, there was a low basal level of hepcidin expression in the substantia nigra. By 2 hours after LPS, hepcidin expression increased to about 4.64 folds of the control ($P<0.01$). The increase continued with a peak expression at 6 hours (9.51 folds of the control), then the expression decreased and fell to the minimal level (0.61 fold of control) at LPS treatment for 12 hours. There was a second increase of the hepcidin mRNA from 24 hours to 36 hours after LPS treatment, but the level was lower than that at 6h (Fig6-1 D). These data implied that hepcidin widely expressed in the CNS and peripheral administration of LPS could regulate the hepcidin mRNA expression in some brain regions.

6.4.2 Effect of Hepcidin on the DMT1-IRE Protein Expression in the C6 Glioma Cells

Our earlier studies showed that hepcidin could regulate the DMT1 protein expression on the peripheral cells such as the peritoneal macrophages and cardiomyocytes. To determine whether hepcidin had the same effect in the CNS, we investigated the

expression of the DMT1-IRE on the C6 glioma cells after hepcidin incubation. The concentrations of hepcidin were 70nM and 700nM, and the incubated time was for 8, 16 and 24 hours respectively. As illustrated in Fig6-2 A , a single major band was observed with the specific antibody with *Mr* of ～60 kDa DMT1-IRE and ～45 kDa for β-actin. It was found that the amount of DMT1 protein decreased to 87.08±5.85 % (70nM) and 73.21±9.24% (700nM) of the control respectively, while the C6 cells were incubated with hepcidin for 8 hours. After the C6 cells were treated with 70nM hepcidin for 16 and 24 hours, the DMT1-IRE protein level increased and had no significant difference from the control (P>0.05). On the other hand, with 700nM hepcidin treatment for 16 and 24 hours, the DMT1-TIRE protein increased to 87.13 ±4.83% (P<0.05) and 95.23±9.72% (P>0.05) of the controls respectively (Fig6-2 B and C).

6.4.3 Effect of Hepcidin on the NTBI Uptake by the C6 Glioma Cells

Analysis of animals with mutations in DMT1 has shown that DMT1 is a membrane transporter that functions in controlling iron entry across the apical surface of absorptive cells in the duodenum and in the export of iron out of the transferrin cycle endosomal vesicles in reticulocytes. From these findings, it is suggested that DMT1

is important in both TfR and non-TfR mediated iron uptake and transport processes. In this study, we further investigated the NTBI uptake of C6 glioma cells after hepcidin pre-treatment. The cells were pre-incubated with or without 700nM hepcidin for 8, 16 and 24 hours, then incubated with 2uM ^{55}Fe(II) at 37^{o}C for 30 minutes. After separating the cytosolic and stromal fractions, both of them were subject to detection of radioactivity. As shown in Fig6-3 A, a significant decrease of the radioactivity in the cytosol was observed between the untreated group (3.58$\pm$ 0.26 umol/ug protein) and the hepcidin treated for 8 hours group (3.09$\pm$0.39 umol/ug protein)($P<0.05$). With hepcidin treatment for 16 and 24 hours, there was no significant decrease in the control and the hepcidin treament groups in both cytosolic and stromal fractions.

6.4.4 Effect of Hepcidin on the TfR1 Protein Expression in the C6 Glioma Cells

To determine whether hepcidin modulated the TfR1 protein expression, C6 glioma cells grown in 35mm culture dishes were treated with 70nM and 700nM hepcidin for 8, 16 and 24 hours. Western blot analysis was quantified by densitometry and total protein loading was corrected using β-actin expression. Representative Western blot

showing one band with an expected molecular weight ～90 kDa and ～45 kDa of TfR1 and β-actin is presented in Fig6-4 A. The modulation of protein expression demonstrated that treatment with different dilutions of hepcidin for 24 hours decreased the TfR1 protein levels to 62.55±21.24% (70nM) and 39.80±4.32% (700nM) of the control. Levels in both of the hepcidin treatment for 8 hours groups at the two concentrations slightly decreased, but this was not significant to the control (Fig6-4 B and C). This result was similar to the change of TfR1 on the H9C2 cardiomyocytes.

6.4.5 Effect of Hepcidin on the Tf-Fe Uptake by the C6 Glioma Cells

Moreover, we studied whether the effects of hepcidin on TfR1 expression might have any implications on the Fe-Tf uptake of the C6 glioma cells. These cells were pre-incubated with or without (controls) 700nM hepcidin for 8, 16 and 24 hours before the addition of ^{55}Fe-Tf (10ug/ml) into the incubation medium and incubated for 60 minutes at 37^{o}C. The intracellular radioactivity (cytosol) and the membrane-pellet (stoma) were measured as described in Methodology. The result demonstrated that the pre-treatment of cells with hepcidin for both 8 hours and 24 hours resulted in a decrease of the cellular Tf-Fe uptake in C6 cells. As illustrated in

Fig6-5 C, the total radioactivity level fell from 20.66±5.33 pmol/ug protein (control) to 19.57±1.67 pmol/ug protein (treated for 8 hours)($P>0.05$), and from 19.09±3.21 pmol/ug protein (control) to 15.55±0.49 pmol/ug protein (treated for 24 hours) ($P<0.01$). Compared to the control, they decreased 5.28% and 18.54%, respectively. After separating the supernant (cytosol) and the membrane-pellet (stoma) to detect the radioactivity, we found that hepcidin pre-treatment for 8 hours and 24 hours could decrease the radioactivity in the cytosolic fraction to 96.88% and 73.76% of the control, while decreased the stromal radioactivity to 92.48% and 90.83% of the control. These data implied that the decrease of the total radioactivity in 8 hours was mainly due to the decrease the non-specific attachment on the cell membrane, whereas at 24 hours, hepcidin decreased the total radioactivity in cells through decreasing the Tf-Fe uptake into cells. On the other hand, the Tf-Fe uptake was increased after hepcidin pre-treatment for 16 hours, but it was mainly because of the increase of radioactivity in the stromal fraction.

6.4.6 Effect of Hepcidin on FPN1, CP, Heph Protein Expression in the C6 Glioma Cells

Our earlier studies indicated that hepcidin could regulate the FPN1 level and iron

release of the peripheral cells. To determine whether hepcidin had the same effect in the CNS, we chose the C6 cell, a rat glioma cell line, to investigate the synthesis of FPN1, CP, Heph protein and iron release in these cells. The western blot performed on the cell samples obtained from the hepcidin treatment for 8, 16 and 24 hours with concentrations of 70nM and 700nM. The results showed that hepcidin has significant effect on the expression of FPN1 protein in the C6 glioma cells. As shown in Fig6-6, 700nM hepcidin significantly decreased the FPN1 protein level after incubation for 8 hours. Then the FPN1 protein expression increased with hepcidin incubation to 16 hours, but it was still lower than the control, being about 74.10±11.4 % of the control. By 24 hours incubation, the FPN1 protein level recovered to the pre-treatment level ($P>0.05$). However, there were no significant changes after 70nM hepcidin treatment for different time. These results clearly revealed that hepcidin also could decrease the FPN1 protein in the CNS cells, and the optimum concentration was 700nM, which was same in the peripheral organs.

Analysis of the Western blot results showed that the expression of Heph and the control protein, β-actin were unchanged throughout these experiments. Moreover, the CP protein expression did not change after treatment with 70nM hepcidin for 8, 16 and 24 hours, or with 700nM hepcidin for 8 hours and 16 hours. Some increase in CP

expression was observed only after 24 hours incubation with 700nM hepcidin ($P<0.05$) (Fig6-7).

6.4.7 Effect of Hepcidin on Iron Release From the C6 Glioma Cells

Considering that hepcidin could regulate the FPN1 protein expression in the peripheral and CNS cells, we hypothesized that hepcidin might have the same effect on the iron release in the C6 glioma cells. To confirm this hypothesis, we loaded the C6 glioma cells with $^{55}Fe(II)$ first and then incubated them with or without 700nM hepcidin for 8, 16 and 24 hours at $37^{o}C$. The results showed that the radioactivity release into the medium was significantly decreased after hepcidin incubation for 8 hours (21.90±1.22%, control; 17.81±1.23%, hepcidin treatment) and 16 hours (22.50±1.38%, control; 20.54±1.10%, hepcidin treatment); while there were no significant changes after hepcidin incubation for 24 hours. This time-dependent change was consistent with the protein expression of FPN1.

6.5 DISCUSSION

To our knowledge, this is the first attempt to investigate the role of hepcidin in the

brain. Before this study, there were several reports on the neurologic dysfunction in hemochromatosis patients (e.g., cognitive decline, gait differences, cerebellar ataxia, and extrapyramidal dysfunction)(Costello et al., 2004). Moreover, Dekker et al (Dekker et al., 2003) showed an increased prevalence of mutations in the hemochromatosis gene in the Parkinson's disease patients. Recent magnetic resonance imaging (MRI) findings suggested there was an excess iron deposition in the basal ganglia of patients with hereditary hemochromatosis (Berg et al., 2000). It is important to note that hemochromatosis may represent a risk factor for AD (Moalem et al., 2000). Furthermore, associations of the hemochromatosis gene mutations with amyotrophic lateral sclerosis have been described (Goodall et al., 2005; Wang et al., 2004), indicating that imbalances in iron in the brain contribute to the neurodegenerative processes. Because both mice and humans with a mutation in the hemochromatosis gene (HFE-gene) express only low levels of hepcidin, this gene has been suggested to regulate the hepcidin levels (Bridle et al., 2003), although the underlying mechanism still needs to be elucidated.

Hepcidin is a protein that seems to act as an important iron-regulator protein in the peripheral organs (Nicolas et al., 2002b; Nicolas et al., 2002c). It is conceivable that it may operate as a regulator of iron metabolism in the CNS as well. In 2006 ,

Zechel et al (Zechel et al., 2006) first investigated the distribution of prohepcidin/hepcidin-1 in the CNS. Their results indicated that hepcidin is expressed at low levels, but nevertheless is widely distributed throughout the CNS. In our current study, we also detected the mRNA expression in several brain areas, including the cortex, hippocampus, striatum and substantia nigra. Moreover, we found that the level of hepcidin mRNA expression was changed after LPS administration but it varied in the brain regions. LPS could markedly induce the expression of hepcidin mRNA in the cortex and substantia nigra, but had no effect on the hepcidin mRNA expression in the hippocampus and striatum. This suggested that the regulation of hepcidin mRNA expression in the brain may be region-dependent.

There are several factors regulating the expression of hepcidin in liver and other peripheral organs, such as the iron level, hypoxia and anemia. Moreover, the synthesis and release of hepcidin are also rapidly mediated by bacterial lipopolysaccharide and cytokine release, especially interleukin-6 (Lee et al., 2004). However, the brain possesses a specialized barrier system that excludes plasma proteins, metals and polar substances from gaining free access to the brain interior. LPS, as a macromolecule, could not cross the blood-brain barrier (BBB) to directly regulate the hepcidin expression in the brain. Recently several studies found that

infection in the periphery stimulates the immune cells to produce inflammatory cytokines such as interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor α (TNFα). These cytokines activate the neural and humoral communication pathways that induce glial cells in discrete brain regions to produce the same inflammatory cytokines (Buttini and Boddeke, 1995; Maier et al., 1998; Quan et al., 1998; Rivest, 2003). Within the CNS, cytokines provide a sensory representation of the conditions in the periphery (Blalock, 1994) and induce neurochemical changes that elicit an adaptive behavioral response (Dantzer, 2001; Dantzer, 2004; Kelley et al., 2003; Pugh et al., 1998). It may explain why administration of LPS in the periphery could regulate the hepcidin mRNA expression in the CNS.

Our results indicated that hepcidin widely distributed in the CNS and was regulated by the LPS. Due to its widespread distribution within the CNS, hepcidin seems to play an important role there. Evidence for the role of hepcidin in the CNS could be found in a recent study. Wang et al. found that conditional Smad4 knockout mice displayed a dramatic reduction in the hepcidin levels as well as iron overload in the liver. Moreover, these mice also exhibited accumulation of iron in the pancreas, kidney, eye and brain (Wang et al., 2005a), which might indicate that the hepcidin levels in these areas were also reduced. However, the molecular mechanisms of the

hepcidin activity and the linkage between hepcidin and other iron regulator proteins in the CNS are still unknown.

Recently, important advances in the fields of physiology and pathophysiology of brain iron metabolism have been made with new findings on the role of DMT1, FPN1, Heph, CP and TfR1 in brain iron transport. At least in some NDs, brain iron misregulation is an initial cause of neuronal death and recent studies suggest that this misregulation might be led by either genetic or non-genetic factors (Qian and Shen, 2001). Iron accumulation in the brain occurs in a number of NDs. The membrane TfR mediated endocytosis or the internalization of Tf bound iron and the TfR is the major route of cellular iron uptake (Li et al., 2003; Li and Qian, 2002). DMT1 is a glycosylated protein composed of 12 transmembrane domains that has been shown to act as an iron transporter either by itself, or in conjunction with TfRs in the endomembrane compartment. Ferroportin1 is a newly discovered transmembrane iron export protein. It plays a key role in the Fe^{2+} transport across the basal membrane of enterocytes in the gut. It has been suggested that this protein may have the same role in Fe^{2+} transport across the abluminal membrane of the blood-brain barrier as in enterocytes. Our early immunocytochemistry studies of DMT1-IRE, TfR1 and FPN1, Heph, CP with special antibody confirmed that C6 glioma cells

were able to express these proteins. In this study, we used the C6 glioma cells, a rat glioma cell line as the cell model to investigate the effect of hepcidin on the iron metabolism in the central nervous cells.

Burdo et al, using antibodies that could distinguish the two isoforms, observed that DMT1-IRE protein was prevalent in the brain (Burdo et al., 2001). Therefore, they proposed that the predominant form of DMT1 in the brain might be the DMT1-IRE form. DMT1-IRE may be the major form to function as a brain iron transporter, whereas DMT1-IRE is iron-irrelative and only plays the role of other divalent metal transport in the brain. So in the present study, we investigated the regulation of the iron-uptake proteins DMT1-IRE, TfR1 and the iron uptake by hepcidin in C6 glioma cells. As demonstrated by Western blot analysis, DMT1-IRE protein expression was slightly suppressed by hepcidin at 700nM for 8 and 16 hours. In parallel, the NTBI uptake in C6 glioma cells was decreased after hepcidin treatment for 8 hours, whereas the TfR1 protein expression had no significant changes until hepcidin incubation for 24 hours with both concentrations. Also, the Tf-Fe uptake decrease was paralleled with the change of the protein expression. As described above, hepcidin treatment suppressed DMT1-IRE and TfR protein expression and decreased iron uptake in the C6 glioma cells. Therefore, the data suggested that hepcidin

treatment reduced the Fenton reactions which produced the more toxic hydroxyl radical (OH**.**), which in turn would inflict oxidative damage on neurons (Jenner and Brin, 1998). However the detailed mechanism requires further clarification.

The time-dependent change in the DMT1-IRE, TfR1 protein expression and iron uptake after hepcidin treatment was also observed in the H9C2 cardiomyocytes in Chapter 5. Although the detailed mechanism involved in the regulation by hepcidin is still unclear, these data at least suggested that the effect and regulation mechanism of hepcidin in heart and brain cells were same.

The existence of FPN1, CP and Heph protein in the rat brain implies that these proteins might play roles in brain iron metabolism. Functional studies demonstrated that FPN1 plays a key role in the Fe^{2+} transport across the basolateral membrane of enterocytes in the gut by a mechanism that requires an auxiliary ferroxidase activity of Heph or CP (Donovan et al., 2000; Griffiths and Cox, 2000; Kaplan and Kushner, 2000; McKie et al., 2000; Vulpe et al., 1999). In a recent paper, it has been proposed that FPN1 (or FPN1/Heph and FPN1/CP pathways) might have the same role in the Fe^{2+} transport across the abluminal (or basolateral) membrane of the BBB as it works in enterocytes (Qian and Shen, 2001). Also, this protein might be required for the

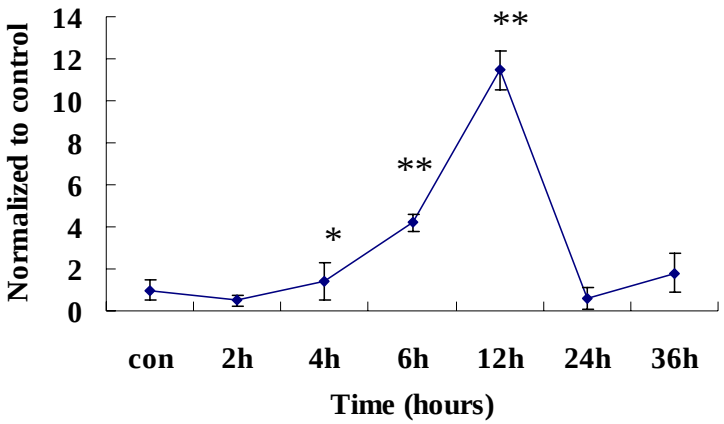
intracellular iron balance in some other brain cells, functioning as an iron exporter.

It is worth noting that the immunoreactivity of prohepcidin/hepcidin-1 in the CNS can be found in areas which are known to be immunoreactive for FPN1 (Jiang et al., 2002; Wu et al., 2004). Within the brain, the GFAP-positive cells express FPN1 protein, nevertheless, FPN1 is expressed mainly by neurons (Wu et al., 2004). For example, in the cerebellum, FPN1 protein can be detected in small amounts in the granular layer, whereas the cell bodies of the Purkinje cells displayed a strong immunoreactivity for FPN1 (Wu et al., 2004). The overlap of the distribution of hepcidin and FPN1 in the brain areas gave us a clue to investigate their relationship in brain. In our study, we found that hepcidin induced a rapid and marked decrease of FPN1 protein expression, but had no effect on the Heph protein level. Only when we extended the incubation time to 24 hour with 700nM hepcidin did CP protein slightly increased. Although the mechanism involved in the regulation of the expression of CP was still unclear, there was some evidence to explain the effect of hepcidin on the FPN1 protein. Nemeth et al. (Nemeth et al., 2004b) showed that a direct binding and internalization of ferroportin1 by hepcidin in hepatocytes cause trapping of iron inside these cells. It might be speculated that within the brain, hepcidin also binds to the iron exporter ferroportin1, induces its internalization and degradation, and

thereby blocks cellular iron efflux. The iron release experiment in our study supported the proposal that hepcidin could decrease the FPN1 expression and further regulate its function in the iron release from the C6 glioma cells.

Iron is an essential cofactor for many proteins that are involved in the normal function of neuronal tissues. However, iron accumulation in the brain can contribute to disorders of the CNS (Zecca et al., 2004). The understanding of these basic issues on hepcidin in brain iron metabolism is critical not only for elucidating the physiology of brain iron homeostasis but also probably for understanding the pathophysiological mechanisms that lead to iron overload in the brain.

A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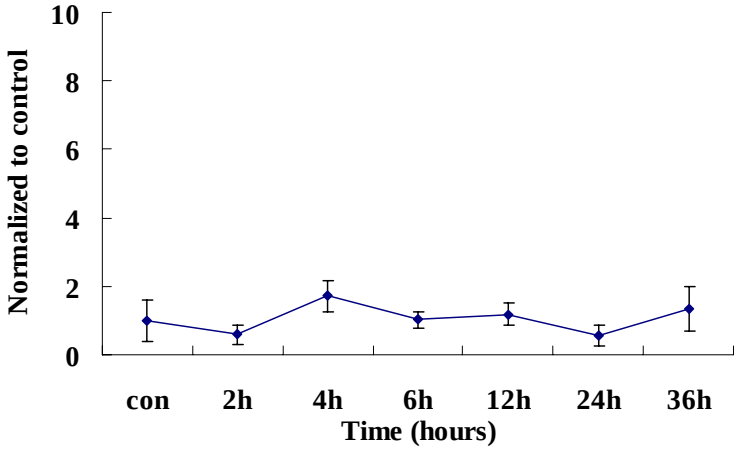
Time (hours)

B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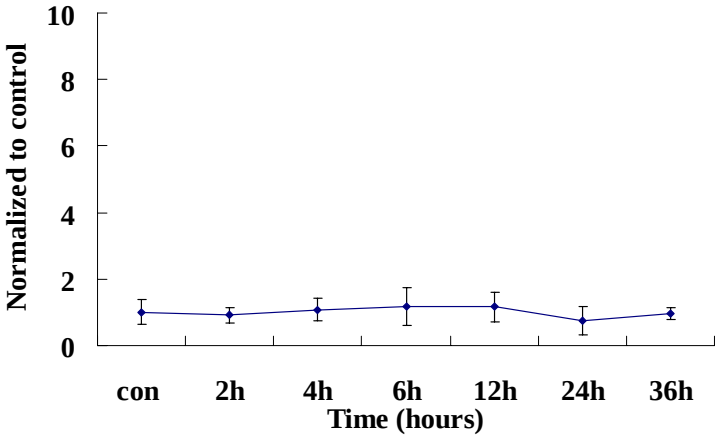
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Time (hours)

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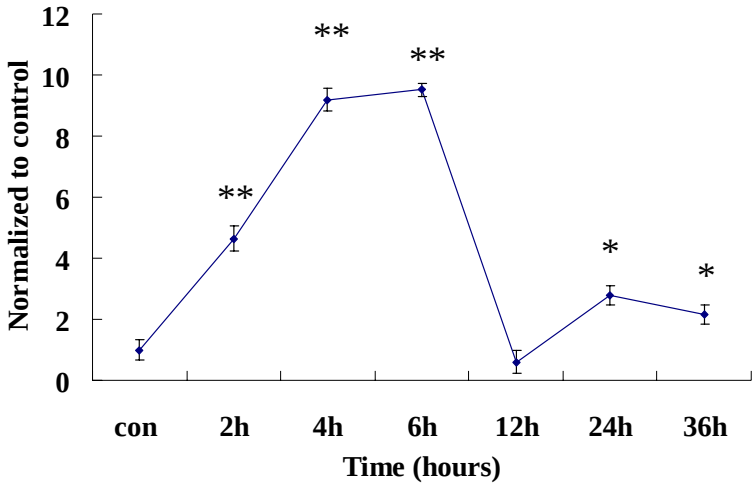

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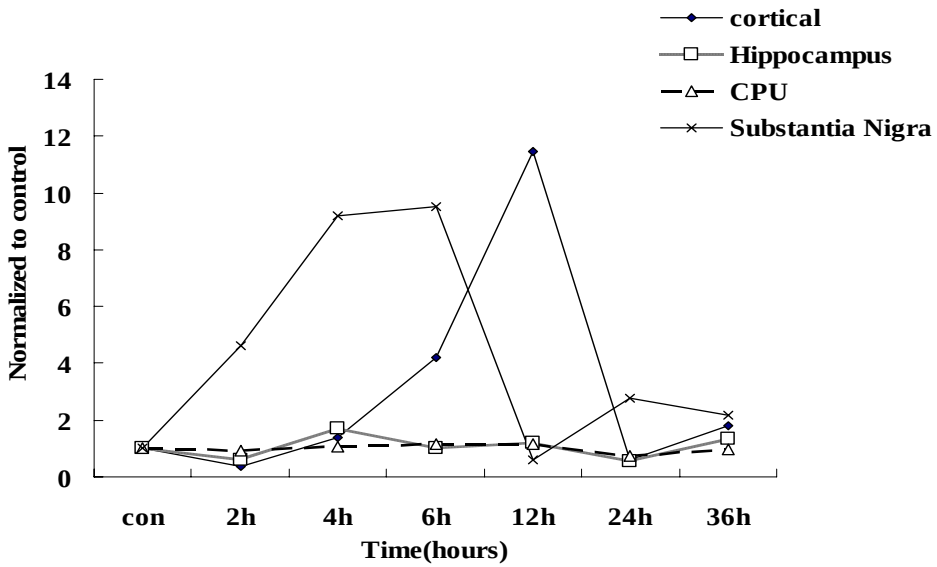


Figure 6-1. Effect of LPS on the hepcidin mRNA expression in the Cortex, Hippocampus, Striatum, and Substantia Nigra of Rat brains.

Real-time RT-PCR analysis was performed as described in Materials and Methods to detect the expression of hepcidin mRNA in the cortex (A), hippocampus (B), striatum (C) and substantia nigra (D) of rat brains after LPS administration for different time. Expression of hepcidin mRNA was quantified by normalizing to β-actin mRNA and expressed as folds relative to the untreated control. Data were presented as the mean ± SD of the values from 3 independent experiments. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

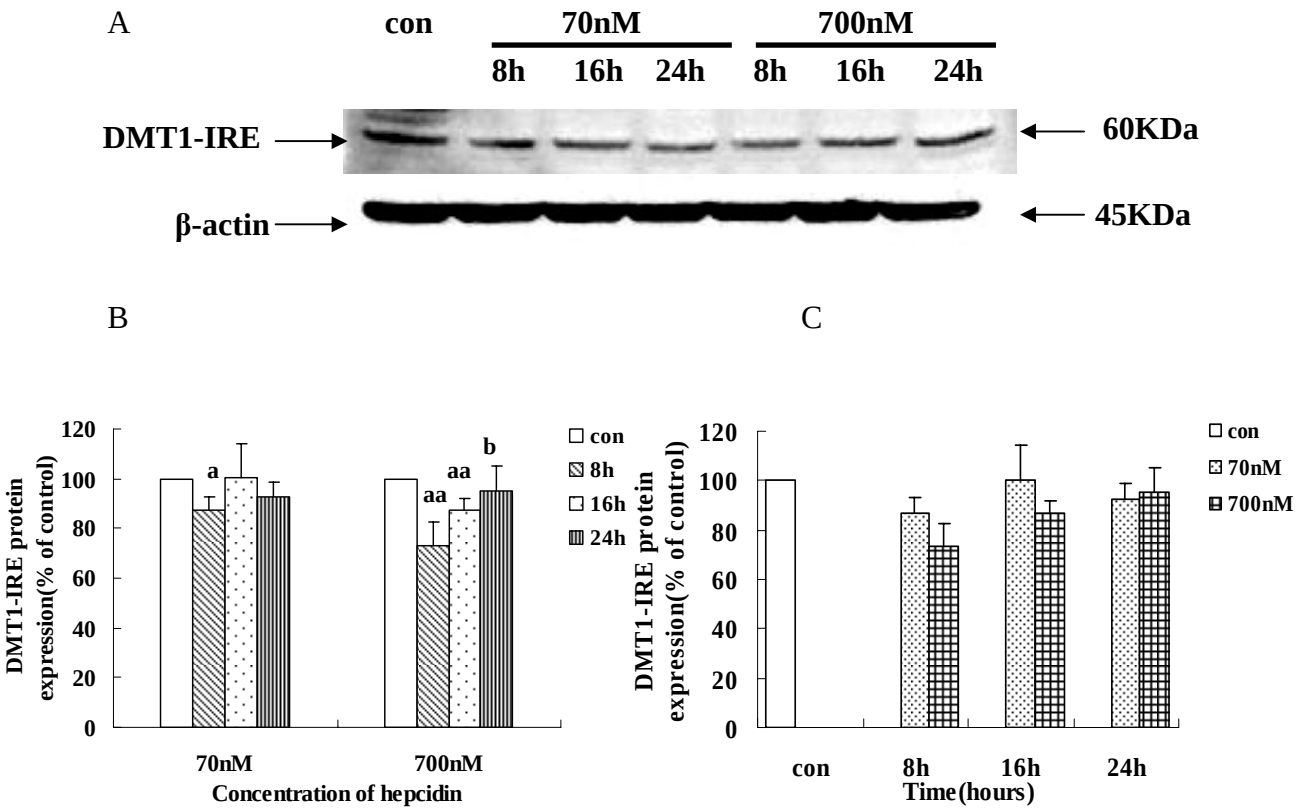
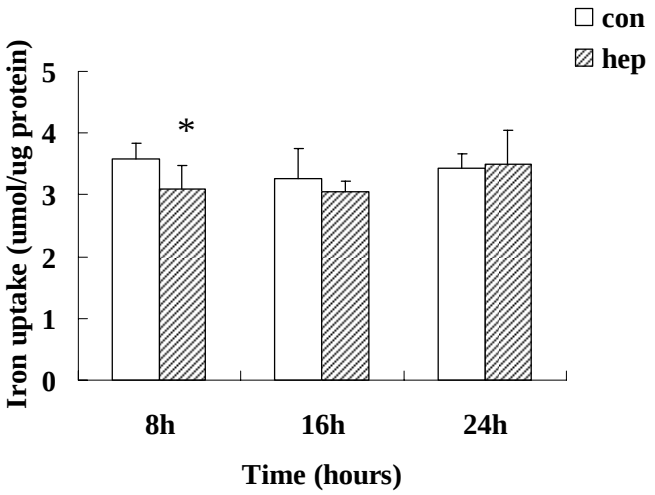


Figure 6-2. Effect of hepcidin on DMT1-IRE protein expression in C6 glioma cells. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~60kDa for DMT1-IRE and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the DMT1-IRE protein expression after various concentrations (B) of hepcidin treatment for different time (C) were normalized for β-actin and expressed as a percentage of the control level. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs controls; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other but incubated with hepcidin for the same time (**P* <0.05, ***P* <0.01).

A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8h
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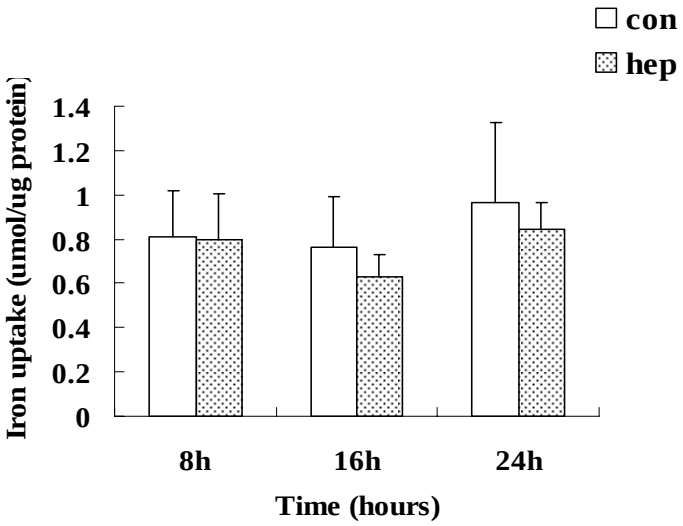
24h
Time (hours)

B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1.2
1.4
8h
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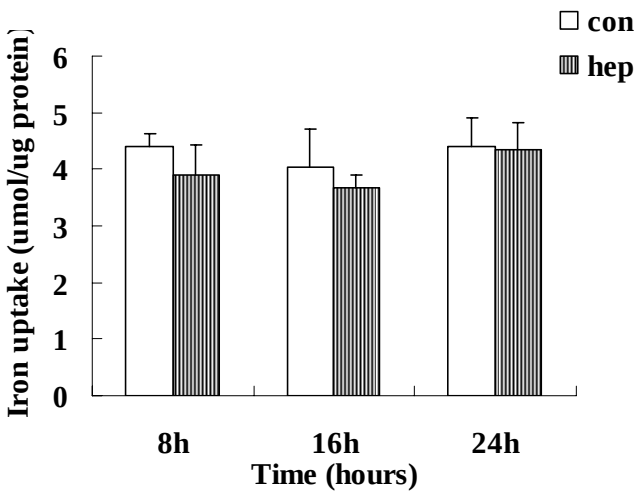
24h
Time (hours)

C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Iron uptake (umol/ug protein)
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8h
16h
24h
Time (hours)

Figure 6-3. Effect of hepcidin on the NTBI uptake in C6 glioma cells.

The C6 glioma cells were preincubated with or without 700nM hepcidin for 8 hours, 16 hours and 24 hours. At the indicated time points, the cells were washed and incubated with 2uM Fe(II) under the control conditions (0.27M sucrose, pH 6.5) at 37^{o}C for 30 minutes. The iron taken into cytosolic (A) and stromal (B) fractions were measured as described in Materials and Methods. The total of iron uptake (C) was the sum of the two parts. All these data were presented as mean$\pm$SD of 6 independent experiments performed in triplicate. Asterisks above the data bars indicated that these groups were statistically different from control (*P <0.05).

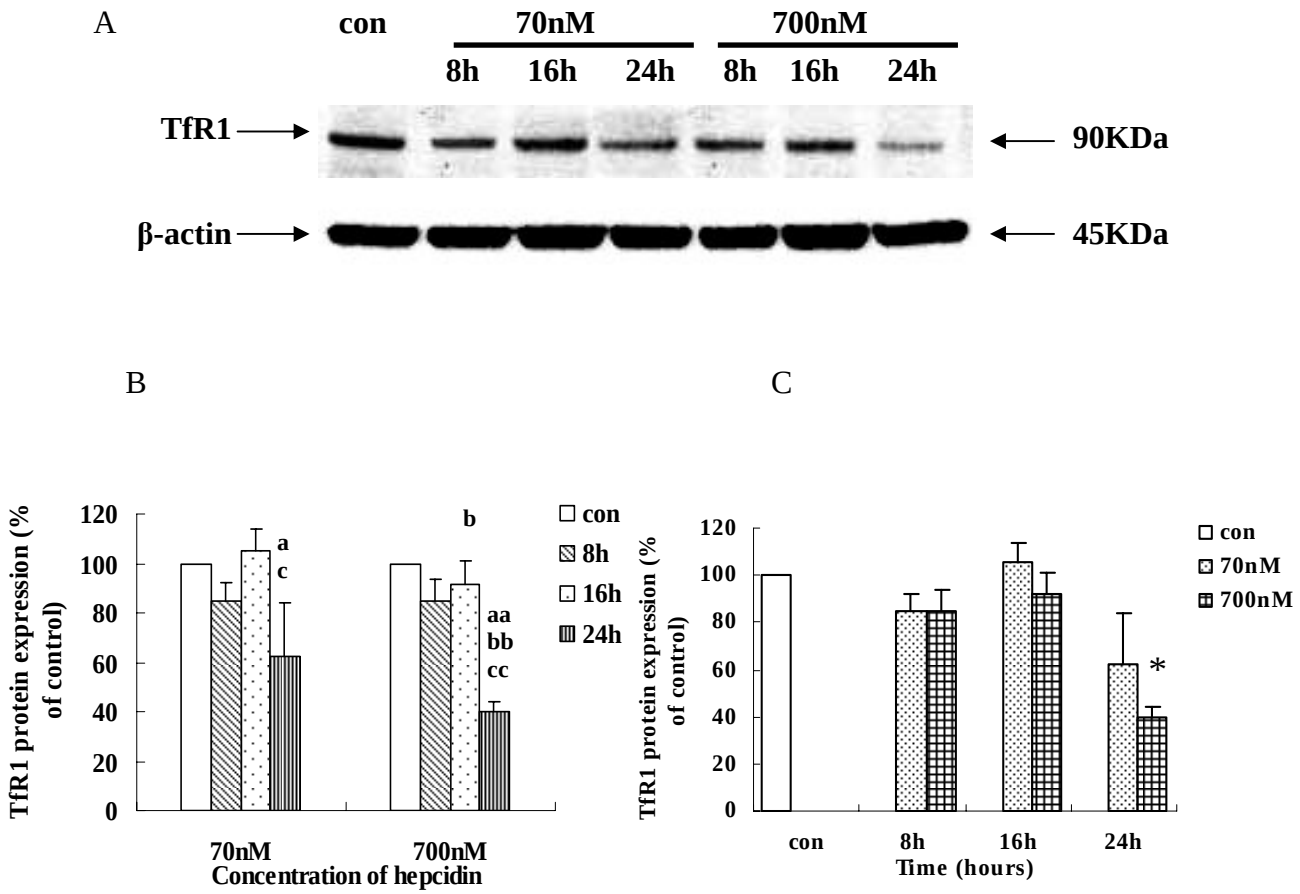
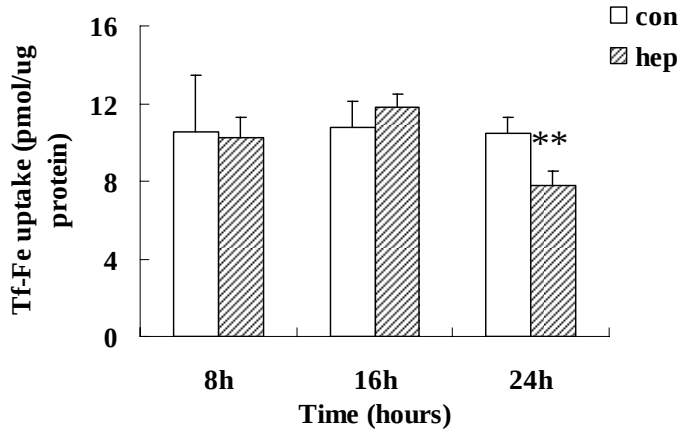


Figure 6-4. Effect of hepcidin on TfR1 protein expression in C6 glioma cells.

Western blot analysis was performed as described in Materials and Methods. The *M*r of ~90kDa for TfR1 and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The results of the TfR1 protein expression after various concentrations (B) of hepcidin treatment for different time (C) were normalized for β-actin and expressed as a percentage of the control level. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other but with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs controls; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other but incubated with hepcidin for the same time (**P* <0.05, ***P* <0.01).

A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Tf-Fe uptake (pmol/ug protein)
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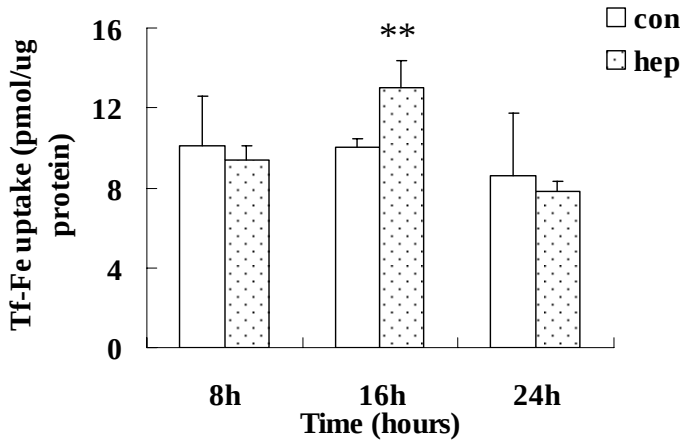
8h
16h
24h
Time (hours)

B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hep
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12
8
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Tf-Fe uptake (pmol/ug protein)
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8h
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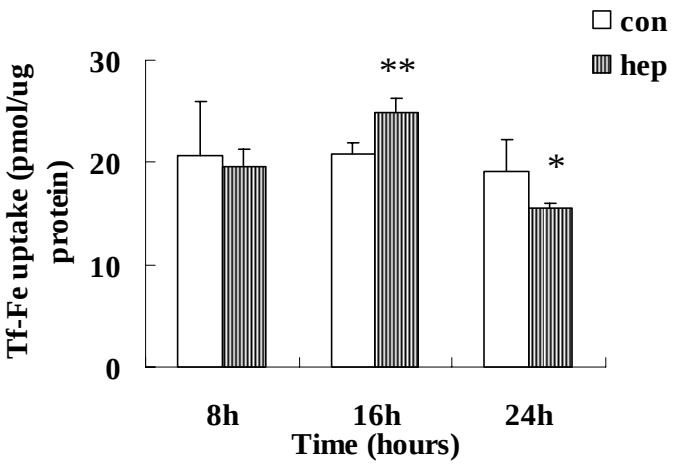
24h
Time (hours)

C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hep
30
20
10
0
Tf-Fe uptake (pmol/ug protein)
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*
8h
16h
24h
Time (hours)

Figure 6-5. Effect of hepcidin on the Tf-Fe uptake in C6 glioma cells.

The C6 glioma cells were preincubated with or without 700nM hepcidin for 8 hours, 16 hours and 24 hours. At the indicated time points, the cells were removed the endogenous transferrin and incubated with serum-free culture medium with 10ug/ml Tf-Fe at 37^{o}C for 60 minutes. The iron taken into cytosolic (A) and stromal (B) fractions was measured as described in Materials and Methods. The total of iron uptake (C) was the sum of the two parts. All these data were presented as mean$\pm$SD of 6 independent experiments performed in triplicate. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

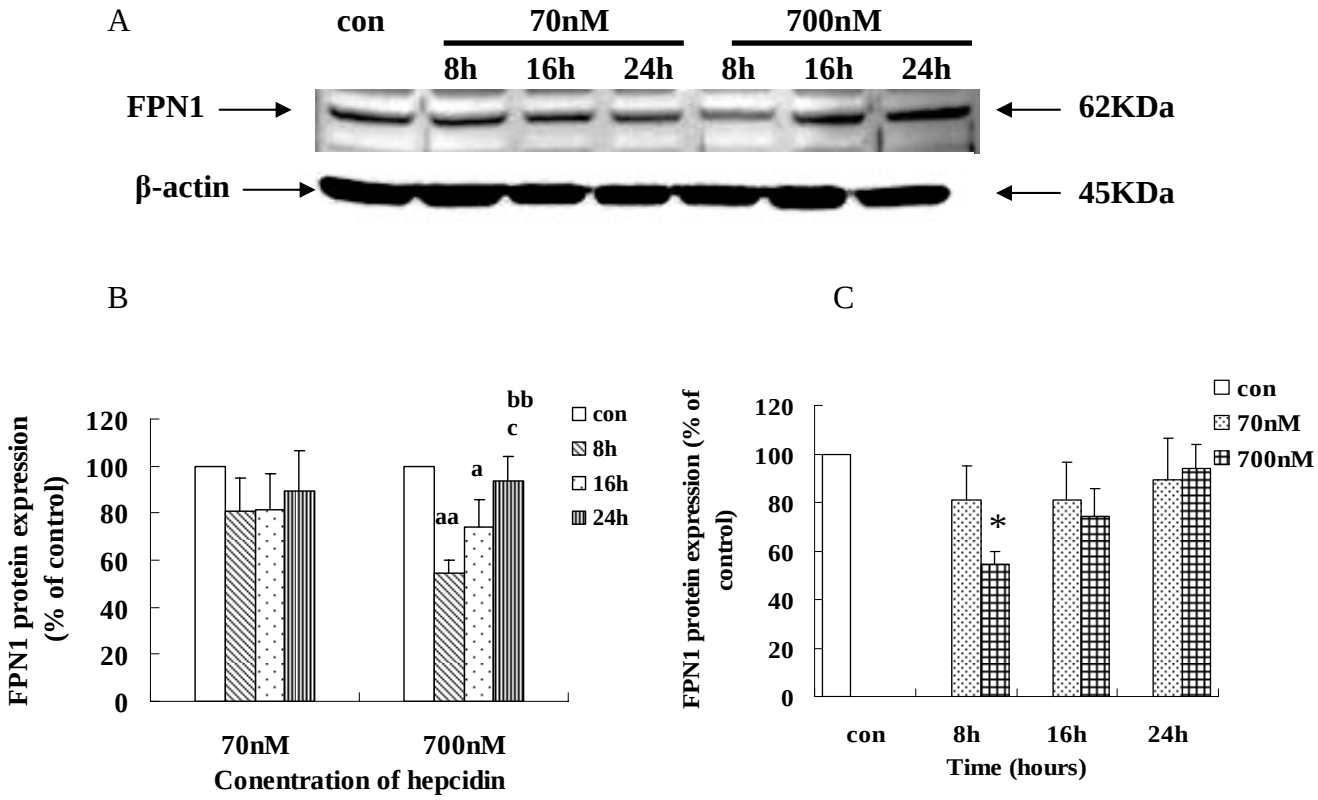


Figure 6-6. Effect of hepcidin on FPN1 protein expression in C6 glioma cells.

Western blot analysis was performed as described in Materials and Methods. The *M*r of ~62kDa for FPN1 and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The results of the FPN1 protein expression after various concentrations (B) of hepcidin treatment for different time (C) were normalized for β-actin and expressed as a percentage of the control levels. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other but with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs controls; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other but incubated with hepcidin for the same time (*P <0.05, **P <0.01).

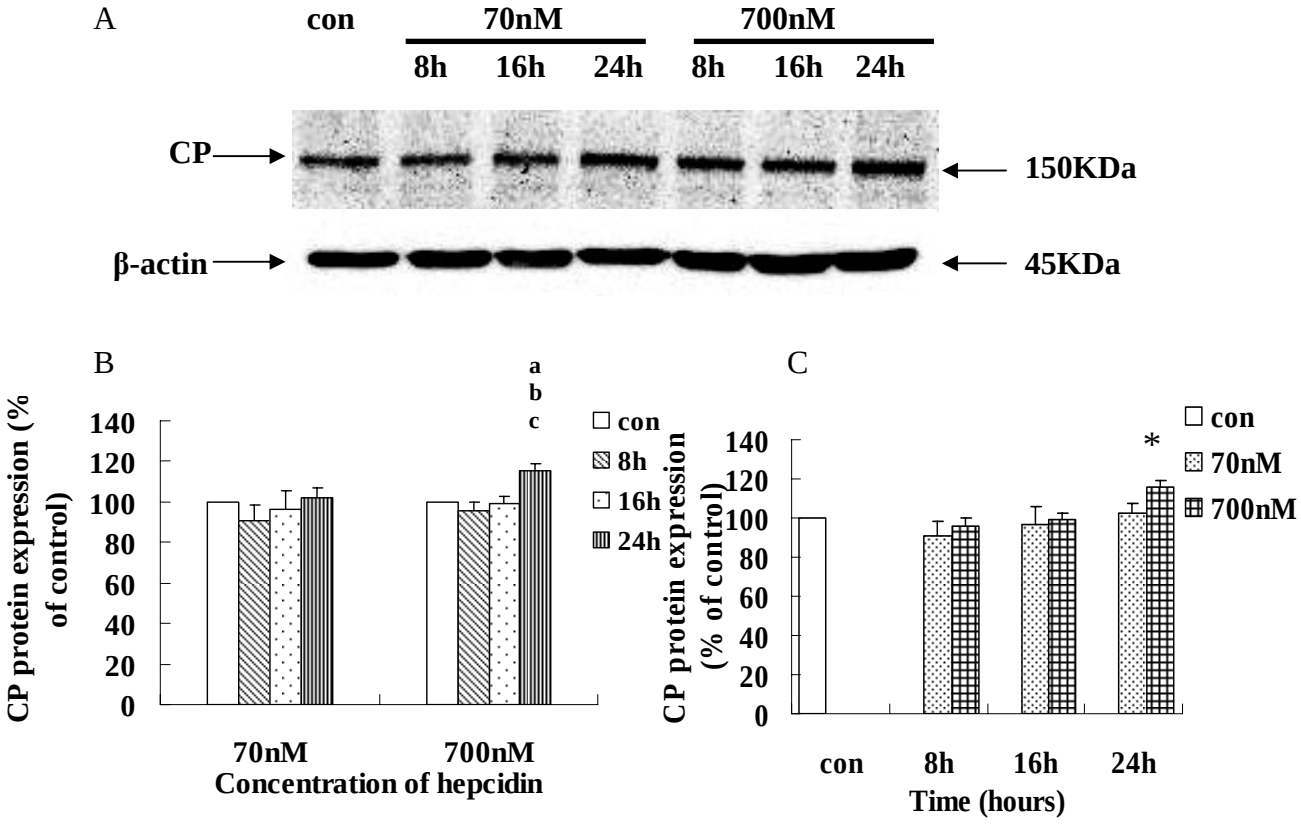


Figure 6-7. Effect of hepcidin on CP protein expression in C6 glioma cells.

Western blot analysis was performed as described in Materials and Methods. The *M*r of ~150kDa for CP and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The results of the CP protein expression after various concentrations (B) of hepcidin treatment for different time (C) were normalized for β-actin and expressed as a percentage of the control level. The data were presented as mean±SD of 4 independent experiments. Letters above data bars indicated that these groups were statistically different from each other but with the same concentration of hepcidin: a: $P<0.05$, aa: $P<0.01$ vs controls; b: $P<0.05$, bb: $P<0.01$ vs 8 hours group; c: $P<0.05$, cc: $P<0.01$ vs 16 hours group. Asterisks above data bars indicated that these groups were statistically different from each other but incubated with hepcidin for the same time (**P* <0.05, ***P* <0.01).

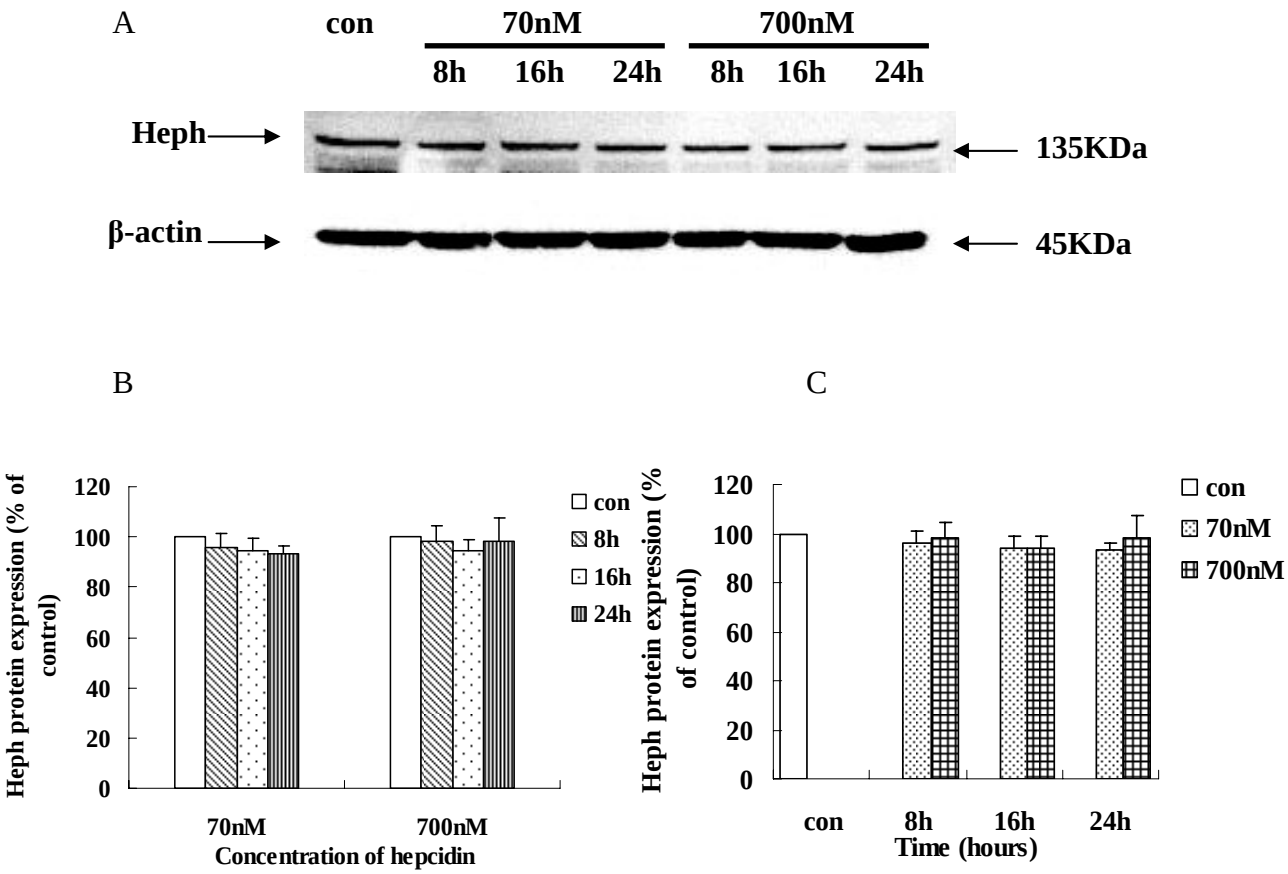
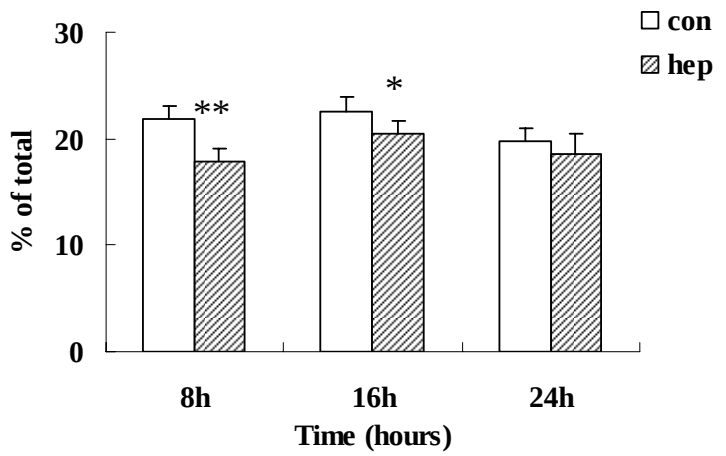
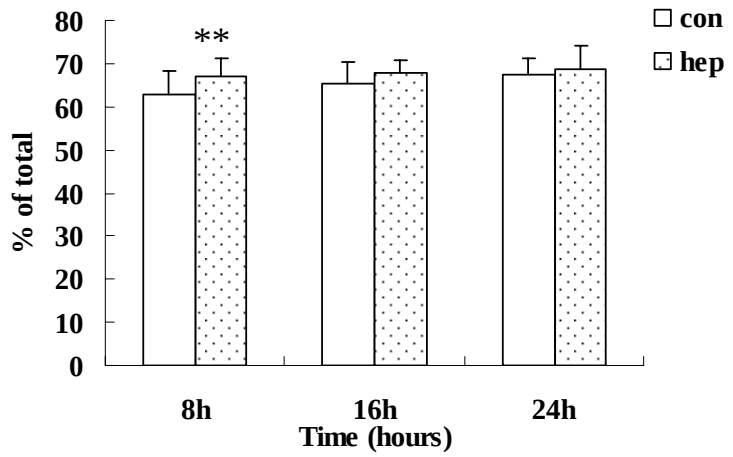


Figure 6-8. Effect of hepcidin on Heph protein expression in C6 glioma cells. Western blot analysis was performed as described in Materials and Methods. The *M*r of ~135 kDa for Heph and β-actin of ~45kDa (**A**) appeared as a single band on each gel. The result of the Heph protein expression after various concentrations (B) of hepcidin treatment for different time (C) were normalized for β-actin and expressed as a percentage of the control level. The data were presented as mean±SD of 4 independent experiments.

A


30
20
10
0
% of total
**
*
con
hep
8h
16h
24h
Time (hours)

B


80
70
60
50
40
30
20
10
0
% of total
**
con
hep
8h
16h
24h
Time (hours)

C

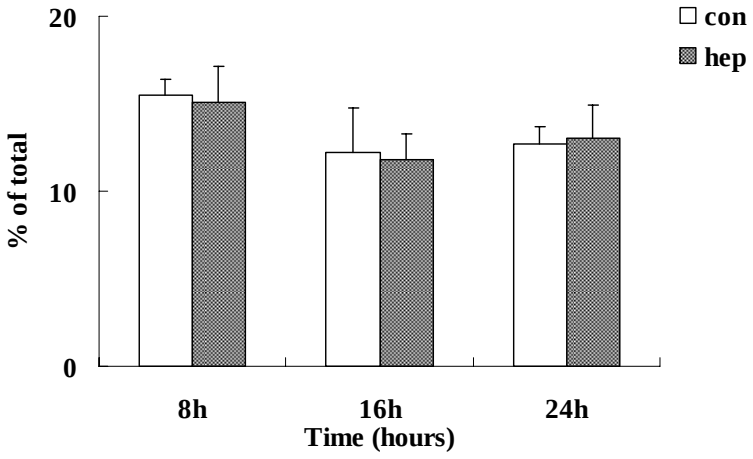

20
10
0
% of total
con
hep
8h
16h
24h
Time (hours)

Figure 6-9. Effect of hepcidin on the iron release by the C6 glioma cells.

The cells were preloaded with iron by incubation with 2uM ^{55}Fe(II) in sucrose (pH 6.5) for 30 minutes at 37^{o}C, and washed with ice-cold PBS. They were then incubated with or without 700nM hepcidin at 37^{o}C. At different time points during iron release, the medium was collected and the cells were lysed. Radio-labelled iron release into the media (A) and in the cytosolic (B) and stromal (C) fractions was measured as described in Materials and Methods. Results were expressed as a percentage of total iron present in the system (=media+cytosol+stromal). All these data were presented as mean$\pm$SD of 6 independent experiments performed in triplicate. Asterisks above data bars indicated that these groups were statistically different from the control (*P <0.05, **P <0.01).

CHAPTER 7

GENERAL DISCUSSION

Although iron is important for nearly all living organisms, there is a relatively small amount of daily exchange of iron between the body and the environment. This metal largely exchanges among the internal organs. The reticuloendothelial system (RES) is responsible for this process. The RES, which is comprised mainly of monocytes and tissue macrophages, plays two major roles in iron metabolism: it recycles iron from the senescent red blood cells and serves as a large storage depot for excess iron. In the body, about 80% of the circulating iron is en route between the RES and the bone marrow. A small amount of plasma iron is contributed by the hepatic iron stores and by the absorption of dietary iron from the duodenum, but most of the circulating iron is contributed by the RES through the release of iron from the catabolism of the senescent red cells. So the RES plays an important role in the system iron metabolism.

Although iron recycling by the RES represents the largest pathway of iron efflux in the body, the precise mechanisms involved and its regulation have remained elusive.

Recently our understanding of iron metabolism of RES has been greatly advanced by the identification and characterization of the transmembrane iron transport proteins. It was suggested that these iron transporters, including divalent metal ion transporter 1 (DMT1), transferrin receptor 1 (TfR1), ferroportin 1 (FPN1) (Gunshin et al., 1997) and CP (Gruenheid et al., 1999; Yang et al., 2002) discovered in the small intestine, seemed to play roles in the iron metabolism in RES. Another noteworthy advance made was the identification of hepcidin, a serum peptide that appeared to affect the iron storage in the RES (Nicolas et al., 2001).

Hepcidin, which is a newly discovered peptide, is mainly synthesized in the liver, secreted into the bloodstream and excreted through the kidney. Recently, several lines of evidence have suggested that the mature bioactive 25-amino-acid form of hepcidin acts as the principal iron-regulatory hormone to maintain iron homeostasis (Pigeon et al., 2001). This peptide hormone is prospective to regulate the iron absorption of intestine, iron recycling by macrophages and iron mobilization from the hepatic stores. At the molecular level, it is proposed that hepcidin presumably acts by regulating the expression or the activity of one or more of iron transporters on the cell membrane. However, at present little is known about the function of hepcidin on the expression of iron transporters and the iron uptake and release in the RES.

The studies described in this thesis focused on investigating the role of hepcidin on the iron metabolism in peritoneal macrophage cells. The results obtained were summarized in Chapter 3, 4, 5 and 6. The experiments in Chapter 3 demonstrated that hepcidin had little effect on the expression of the DMT1+IRE, but it induced a biphasic change of the expression of DMT1-IRE protein in vitro, which was suppressed by hepcidin incubation for 8 hours while promoted with high concentration hepcidin incubation for 16 and 24 hours. Accompanied with the increase of DMT1-IRE protein, the non-transferrin bound iron (NTBI) uptake by macrophages was also increased. This result was distinct from the observation in the $CaCO_2$ epithelial cells in which DMT1+IRE expression was decreased and DMT1-IRE was unresponsive after hepcidin incubation (Laftah et al., 2004). Based on these findings, it was proposed that DMT1 protein expression was different in organs, tissues or cells in responsed to hepcidin. To confirm this suggestion, the effects of hepcidin on the DMT1-IRE expression and the NTBI uptake in other peripheral cell (in cardiomyocytes) or in the CNS cell (in the brain glioma cell) were also studied and presented in Chapter 5 and 6. It was found that after 700nM hepcidin incubation for 8 hours, both the expression of DMT1-IRE and the NTBI uptake in the H9C2 cardiomyocytes and the C6 glioma cells were down-regulated. In good agreement with our results, there were strong inversely correlated data between

hepcidin expression and the duodenal level of DMT1 and Dcytb proteins in various pathophysiological situations including the Usf2 gene knockout (hepcidin-deficient) (Nicolas et al., 2001), low or high iron diet feeding (Frazer et al., 2002) and phenylhydrazine treatment (Frazer et al., 2004). At present, there is little knowledge about the mechanism involved in the regulation of DMT1-IRE protein. It is not known whether the decreased expression of DMT1 is a direct effect of hepcidin or due to the relative iron overload secondary to the decrease of FPN1 mediated iron export. According to the results of Frazer et al, the regulation of the brushborder iron transporters (DMT1 and Dctyb) was proposed to be modulated in a secondary step, in response to the changing enterocyte iron levels (Frazer and Anderson, 2003). In contrast, Wang et al (Wang et al., 2005a) in the study of the role of SMAD4 in iron metabolism, showed that the disruption of the SMAD4 in $Smad4^{Co/Co}$; Alb-Cre mice resulted in a diminished expression of hepcidin, followed resulting in a significantly increased expression of iron transporters (DMT1 and Dctyb) in the small intestine, which then led to iron overload. These observations strongly suggested that hepcidin acted directly on the DMT1 and Dctyb. In our studies, the form of DMT1-IRE was associated with the absence of IRE in the 3'UTR of the mRNA, so the regulation of DMT1-IRE expression by hepcidin might not be through the IRE-IRP regulatory theory, but some other unknown regulatory mechanisms might be involved in the

regulation of the effect of hepcidin on DMT1-IRE expression in these cells. Further study on the identification of IRE-IRP independent iron regulatory pathways is needed. Although the mechanism involved in the regulation of DMT1 protein expression by hepcidin is not yet understood, based on the observation that the distinctive response of DMT1-IRE expression and the NTBI uptake to the hepcidin in the peripheral cells (macrophages and H9C2 cardiomyocytes) and in the central nervous cell (C6 glioma cells), it is concluded that the regulation of hepcidin on the DMT1-IRE expression is tissue- or cell-specific.

In Chapter 4, we further investigated the relationship between the hepcidin and the iron transporters and iron uptake and release in peritoneal macrophages in vivo. As mentioned in Introduction, LPS induced a rapid increase in hepatic hepcidin mRNA expression, and it peaked within 4 h after LPS injection in our experiments. The western blot results showed that DMT1-IRE protein expression slightly decreased after LPS treatment for 2 hours, but with no significant changes in the NTBI uptake of macrophages. There seemed to be little relation between this decrease and hepcidin and such a decrease might be due to the direct effect of LPS. Similar results were also observed in the cultured alveolar macrophages that DMT1-IRE mRNA was down-regulated by LPS for 2 hours (Nguyen et al., 2006). The different patterns

of protein regulation and iron uptake in vitro and in vivo implied that multiple principles might be involved.

In addition, very interestingly, we found that whether in the macrophage or in the heart and brain cells, the effect of hepcidin was dose-dependent, and sometimes both dose- and time-dependent. The maximal effect was produced by hepcidin at the concentration of 700nM. The prevailing serum hepcidin concentration is not exactly clear. Extrapolations from urinary measurements suggested that circulating hepcidin was within the nanomolar range (Park et al., 2001). However, Dallalio's study indicated that serum levels could be much higher, being in the mid- to high-micromolar range (Dallalio et al., 2003). In 2005, Rivera et al, based on the blood levels of hepcidin after the administration of 50ug hepcidin intraperitoneally, estimated that hepcidin exerts its hypoferremic acitivity at blood concentrations in the 100nM to 1000nM range (Rivera et al., 2005b). It was also consistent with the concentration of hepcidin proposed by Nemeth (Nemeth et al., 2004b). The concentration of human synthetic 25 amino acids hepcidin used in our study (70nM, 700nM) sat between these 2 extremes.

Iron is delivered to most tissues via the endocytosis of transferrin bound to its cell

surface receptor. Isolated human monocytes express transferrin receptor (Bjorn-Rasmussen et al., 1985) and are able to take up iron from transferrin (Sizemore and Bassett, 1984). When cultured monocytes differentiate into macrophages, the expression of transferrin receptor increases greatly (Andreesen et al., 1984). Transferrin-binding activity has also been demonstrated in various macrophages from mice (Hamilton et al., 1984), rats (Nishisato and Aisen, 1982) and humans (Andreesen et al., 1984; Montosi et al., 2000; Testa et al., 1987). TfR1 plays an important role in the iron uptake of the RES. In order to study the regulation of hepcidin on the expression of TfR1, in Chapter 3, the TfR1 protein level and Tf-Fe uptake in peritoneal macrophage were detected after hepcidin treatment. Our results showed that a low concentration (70nM) of hepcidin had little effect on TfR1 protein expression. Only the hepcidin with a concentration of 700nM significantly increased the TfR1 protein level and Tf-Fe uptake in the peritoneal macrophages. Studying the macrophages from rats pre-treated with LPS in the Chapter 4, we found that the TfR1 protein expression was affected by the LPS and positively correlated to the hepcidin mRNA in liver. Both the TfR1 protein and the Tf-Fe uptake were increased after LPS injection for 6 hours. Although the exact mechanism involved in the effect of hepcidin and LPS on TfR1 expression is presently unknown, it can at least be concluded from our present study that the translational regulatory mechanism is

involved in this process. The increase of TfR1 protein expression and the iron uptake corresponding to hepcidin in vitro and in vivo implied that the iron retention in macrophages induced by hepcidin might be partly attributed to the increase of iron uptake into cells.

In Chapter 5 and 6, we examined the correspondence of TfR1 protein expression and Tf-Fe uptake to the hepcidin incubation in H9C2 cardiomyocytes and brain C6 glioma cells. The expression of TfR1 and Tf-Fe in the H9C2 cell and C6 glioma cell were both down-regulated by hepcidin in dose- and time-dependent manner, which was different from the observation in peritoneal macrophages that both TfR1 protein and Tf-Fe were increased by hepcidin. How to explain the different regulations of hepcidin on the TfR1 in the macrophages and in the heart or brain cells? It is not known whether this can be attributed to the different roles of tissue and cell in the system iron homeostasis, which is related to the functions of different kinds of cells. At present, there is no report on the direct or indirect effect of hepcidin on TfR1. However based on our results, and in view that TfR1 contains an IRE in its mRNA 3'-UTR, we proposed that the IRE motif of TfR1 might function as an iron regulatory element in the regulation of TfR1 expression through the IRE-IRP regulatory theory secondary to the intracellular iron change induced by hepcidin. A

block of iron export by hepcidin would result in a rise in intracellular iron and then a suppression of the synthesis of the TfR1, which could explain the decrease of TfR1 in H9C2 and C6 cells. In mammalian cells, the synthesis of many key molecules involved in iron metabolism is tightly controlled by intracellular iron. Iron binds directly to iron regulatory proteins and interferes with the binding of IRP to IRE, which in turn determines the expression levels of the IRE genes. The molecular mechanisms of how iron interacts with IRP and how IRE-IRP affects the expression of IRE-containing genes have been well determined. Under conditions of iron deficiency, the binding of iron regulatory proteins to iron responsive elements in the 5'–UTR of ferritin mRNA blocks the translation of ferritin. Concurrently, the binding of iron regulatory proteins to iron responsive elements in the 3'–UTR of TfR1 mRNA results in the increased stability of TfR1 mRNA and thereby the increased expression of TfR1 at the cell surface (Casey et al., 1988; Mullner and Kuhn, 1988). On the other hand, a high concentration of intracellular iron inhibits the binding of iron regulatory proteins to iron responsive elements, resulting in the efficient translation of ferritin and decrease in the stability of TfR1 mRNA. However, this theory could not explain the increase of TfR1 in the peritoneal macrophages. So the mechanism of hepcidin regulation in TfR1 needs to be further investigated.

As shown in Introduction, the mechanism of cellular iron homeostasis involves several stages, including cellular iron uptake, storage and release. FPN1, the only mammalian iron exporter identified to date, is highly expressed in duodenal enterocytes and in macrophages (Abboud and Haile, 2000; Donovan et al., 2000). The expression profile of FPN1 in macrophages suggests that this protein plays an important role in the iron recycling in the RES. In Chapter 3, 5 and 6, we investigated the FPN1 protein expression under hepcidin regulation in the peritoneal macrophages, in the H9C2 cells and in the C6 glioma cells respectively. Our results showed that there was same change in the three types of cells, that hepcidin rapidly induced a dramatic reduction in the FPN1 protein levels. The suppression of FPN1 expression might result from an increased degradation or impaired synthesis. Nemeth et al gave an explanation of the mechanisms involved in these findings, which demonstrate that hepcidin could decrease FPN1 expression through the direct protein-protein interaction, which led to the internalization and degradation of the protein in lysosomes (Nemeth et al., 2004b). The post-translational regulation of FPN1 by hepcidin might thus complete a homeostatic loop: Iron regulated the secretion of hepcidin, which in turn controlled the concentration of FPN1 on the cell surface. Importantly, our result showed that the loss of FPN1 was associated with a decrease in ^{55}Fe release after the loading of $^{55}Fe(II)$. Similar results were also found

in the macrophage cell line J774 (Knutson et al., 2003), in which hepcidin induced a rapid drop in ferroportin1 protein and significantly reduced the efflux of ^{59}Fe after erythrophagocytosis. In other cells, such as the THP1 monocytes (Andriopoulos and Pantopoulos, 2006) and bone marrow derived macrophages (Delaby et al., 2005), hepcidin displayed the same effect on FPN1.

In vivo studies of animal models of acute inflammation secondary to LPS administration have demonstrated that LPS could decrease the mRNA of FPN1 in rat liver and intestine (Yeh et al., 2004). In Chapter 4, we also observed a biphasic change in FPN1 protein expression after LPS injection. The first decrease happened at 2 hours after LPS administration, which might result from a direct action of LPS. Similar results were also observed in the alveolar macrophages treated with LPS *in vitro*, which decreased the FPN1 protein mRNA at 2 hours. However, we could not exclude the possibility that hepcidin might act as the effector, because Liu et al found that LPS could induce the hepcidin expression on the mouse peritoneal macrophages and splenic macrophages (Liu et al., 2005). Locally produced hepcidin might act on cells extracellularly at the plasma membrane, where concentration would be high, or result in the sorting of FPN1 directly from the secretory pathway to lysosomes. There are other reasons that locally produced hepcidin may be important in the regulation

of FPN1. The secondary decrease of FPN1 protein was perhaps regulated by hepcidin induced by LPS. We observed that the FPN1 protein expression was inversely correlated to the hepatic hepcidin mRNA level after LPS treatment, which suggested a causal relationship between hepcidin and FPN1.

Besides the FPN1 protein, the iron release from macrophages seemed to require ceruloplasmin, a multicopper ferroxidase, which appears to mobilize iron from storage sites by catalyzing the oxidation of ferrous iron to the ferric form (Harris et al., 1999). Interestingly, in our studies, we found that whatever in the cultured peritoneal macrophages or in the heart and brain cell lines, CP protein had a similar response to hepcidin. The CP protein synthesis was not significantly affected by hepcidin until after 700nM hepcidin treatment for 24 hours. The possible reason for the changes of CP was not consistent with the response of FPN1 and the mechanism involved is still elusive. On the other hand, *in vivo* study provided different results, in which the down-regulation of CP by LPS administration for 12 to 24 hours was different from the report of an up-regulation of CP mRNA in the LPS-treated rat liver (Gitlin et al., 1992).

At present, little is known about the cellular and molecular mechanisms involved in

the regulation of CP. Factors that have been reported to cause changes in the synthesis of CP include copper, hormones and leucocytic endogenous mediator (Weiner and Cousins, 1983). Mukhopadhyay et al.(Mukhopadhyay et al., 2000) reported that iron chelators also increase CP mRNA expression and protein synthesis in human hepatocarcinoma HepG2 cells. Our early data also showed that the post-transcriptional regulation by iron status involved in CP protein synthesis in brain substantia nigra *in vivo* and in C6 glioma cells *in vitro*. However, the precise mechanism involved in the regulation of CP expression by hepcidin needs to be further clarified.

Hephaestin is highly homologous to CP, which has a ferroxidase activity similar to ceruloplasmin. CP has a ferroxidase activity that is likely to facilitate iron export from the reticuloendothelial system to the plasma (Harris et al., 1999; Lee et al., 1968), while Heph plays a similar role in intestinal enterocytes. Our earlier data showed that Heph was also expressed in the rat brain and heart, and was proposed to have the same role in iron transport in brain and heart cells as it worked in enterocytes. However, in our study, it seemed that hepcidin had no effect on the Heph protein expression whether there was a low or high concentration for different time.

In addition, in Chapter 4, we also observed an inverse correlation between the hepatic hepcidin expression and the serum iron. The serum iron concentration fell to the lowest after LPS administration for 6 hours, two hours later than the peak of hepcidin expression. The time-course of the changes after LPS treatment presented here consisted of a causal relationship between the hepcidin mRNA and serum iron. *In vivo* studies of the animal models of acute inflammation secondary to LPS or turpentine administration have demonstrated several mechanisms for the decline in serum iron. LPS and turpentine induce an accelerated clearance of iron from blood, which is thought to be due to an increase in transferrin-dependent uptake of iron by hepatocytes and other cells (Alvarez-Hernandez et al., 1989; Beguin et al., 1989; Feldman et al., 1981a; Feldman et al., 1981c; Hershko et al., 1974; Moldawer et al., 1989). Suppressing the FPN1 synthesis and blocking the iron efflux from macrophages by LPS may also be a contributing factor to this acute decline, which results in accumulation of iron in the RES compartment (Beguin et al., 1989; Feldman et al., 1981b; Feldman et al., 1981c; Fillet et al., 1989; Hershko et al., 1974).

Hepcidin is a protein that seems to act as an important iron-regulator protein in peripheral organs (Nicolas et al., 2002b; Nicolas et al., 2002c). It is conceivable that

it may operate as a regulator of iron metabolism in the CNS as well. In 2006，Zechel et al (Zechel et al., 2006) first investigated the distribution of prohepcidin/hepcidin-1 in the CNS. Their results indicated that hepcidin is expressed at low levels, but is nevertheless widely distributed throughout the CNS. In our current study, we also detected mRNA expression in several brain areas, including the cortex, hippocampus, striatum and substantia nigra, which suggested that hepcidin might play an essential role in iron metabolism in these brain areas. Moreover, we found that the level of hepcidin mRNA expression was changed after LPS administration but it varied in different brain regions. LPS could markedly induce the expression of the hepcidin mRNA in cortex and substantia nigra, but had no effect on the hepcidin mRNA expression in the hippocampus and striatum. This suggested that the regulation of hepcidin mRNA expression in the brain might be region-dependent.

There are several factors, such as iron level, hypoxia and anemia, which regulate the expression of hepcidin in liver and other peripheral organs. In addition, the synthesis and release of hepcidin are also rapidly mediated by bacterial lipopolysaccharide and cytokine release, especially interleukin-6 (Lee et al., 2004). However, the brain possesses a specialized barrier system that excludes plasma proteins, metals and polar substances from gaining free access to the brain interior. LPS as a

macromolecule could not cross the blood-brain barrier (BBB) to directly regulate the hepcidin expression in the brain. Recently several studies have found that infection in the periphery stimulates immune cells to produce inflammatory cytokines such as interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor α (TNFα). These cytokines activate the neural and humoral communication pathways that induce glial cells in discrete brain regions to produce the same inflammatory cytokines (Buttini and Boddeke, 1995; Maier et al., 1998; Quan et al., 1998; Rivest, 2003). Within the CNS, cytokines provide a sensory representation of the conditions in the periphery (Blalock, 1994) and induce neurochemical changes that elicit an adaptive behavioral response (Dantzer, 2001; Dantzer, 2004; Kelley et al., 2003; Pugh et al., 1998). This may explain why the administration of LPS in the periphery could regulate the hepcidin mRNA expression in the CNS.

In summary, the studies described in this thesis focus on the role of hepcidin in iron metabolism and the related mechanisms in the macrophages, and compare the effect of hepcidin on other type cells, such as H9C2 cardiomyocyte and C6 brain glioma cell. Some findings are new discoveries that have not been reported by others. First, it was found that the expression of DMT1–IRE responding to hepcidin was tissue- or cell-specific. Second, it was demonstrated that hepcidin could regulate the expression

of TfR1 and Tf-Fe uptake in peritoneal macrophages, H9C2 cells and C6 glioma cells. Third, our data provided the first direct evidence that hepcidin could regulate the heart and brain cell iron uptake and release by interacting with the iron transporters. In addition, it was demonstrated that the rat brain had the ability to express hepcidin, which was significantly regulated by LPS administration. These findings provide fundamental insights into understanding the mechanisms involved in the regulation the RES iron metabolism. On the other hand, they also widen our knowledge of the biological role of hepcidin in body iron metabolism. However, a lot of questions in this area remain to be answered, and much more research is needed if we are to enter a new phase of investigation in the physiologic effect of hepcidin. Some suggestions on the future direction of relevant studies could be emerged from this Ph.D. study. First, more work needs to be done about the effect of hepcidin on the mRNA expression of these iron transporters, which will be helpful on understanding whether hepcidin affects the transcription of these genes. Second, it is necessary to explore whether the DMT1 and TfR1 protein expression is a direct interaction of hepcidin or a secondary effect to the intercellular iron change induced by hepcidin. Third, it still remains to be determined what are the molecular mechanisms and signaling pathway involved in the rapid hepcidin-induced internalization of FPN1 in macrophages, as well as in heart and brain cells. Fourth, in

light of the systemic signaling via hepcidin, it needs to address how the macrophage integrates both organismal and cellular information relaying cues about availability and overload to export the appropriate amount of iron. Fifth, the exact physiologic function of hepcidin in the normal subjects' heart and brain and the mechanism of its regulation involved remain to be clarified.

The discovery of hepcidin and its role as a negative regulator of macrophages iron release has significantly progressed our understanding of iron metabolism. It is highly likely that we will have a more detailed picture of the regulation of the system and cellular iron homeostasis. Further research towards understanding hepcidin's molecular control, cellular receptors, pharmacodynamics and analogues will be helpful in future therapeutic applications. The modulation of hepcidin activity using agonist or antagonists might therefore offer new treatment strategies for patients with anemia of chronic inflammation, hereditary hemochromatosis and even disorders apparently unrelated to iron homeostasis.

ABBREVIATIONS

ACD	Anemia of chronic disease
ACP	Acid phosphotase
AD	Alzheimer’s disease
AI	Anemia of inflammation
ApoTf	apotransferrin
BBB	Blood brain barrier
BMP	Bone morphogenetic protein
C/EBP	CCAAT/enhancer-binding protein
CNS	Central nervous system
CP	Ceruloplasmin
CPM	Counting per minute
Dcyt b	Duodenal cytochrome b
ddH_2O	Double distilled water
DMT1	Divalent metal transporter 1
EPO	Erythropoietin
FPN1	Ferroportin 1
GPI	Glycosylphosphatidylinositol

Hb	Hemoglobin
HBSS	Hank’s balanced salt solution
Hct	Hematocrit
HEPC	hepcidin
Heph	Hephaestin
HH	Hereditary haemochromatosis
HJV	Hemojuvelin
HNF4	Hepatocyte nuclear factor 4
IFN	Interferon
IL	Interleukin
IRE	Iron response element
IREG1	Iron-regulated transporter 1
IRP	Iron regulatory protein
JH	Juvenile hemochromatosis
LEAP-1	Liver-expressed antomicrobial peptide
Lf	Lactoferrin or lactotransferrin
LPS	Lipopolysaccharide
mk mouse	Microcytic anemia mouse
MRI	Magnetic resonance imaging

MTP1	Metal transport protein 1
MTT	3-(4,5-dimethylathiazlo-2-yl)-2,5-diphenyletertrazolium bromide
Nramp2	Natural resistance-associated macrophage protein 2
NDs	Neurodegenerative diseases
NTA	Nitrilotriacetic acid
NTBI	Non-transferrin-bound iron
PD	Parkinson’s disease
RBC	Red blood cell
RES	Reticuloendothelial system
RGD	Repulsive guidance domain
RGM	Repulsive guidance molecular
RPM	Rat peritoneal macrophage
Tf	Transferrin
Tf-Fe	Transferrin-bound iron
TfR (1,2)	Transferrin receptor (1 and 2)
TIBC	Total iron-binding capacity
TLR-4	Toll-like receptor 4
TNFα	Tumor necrosis factor α

UTR	Untranslated region
RT-PCR	Reverse transcriptase-mediated polymerase chain reaction
SD rats	Sprague-Dawley rats
SN	Substantia nigra
UIBC	Unsaturated iron-binding capacity

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