Journal of Cellular Biochemistry 104:629–641 (2008)

Decreased DMT1 and Increased Ferroportin 1 Expression Is the Mechanisms of Reduced Iron Retention in Macrophages by Erythropoietin in Rats

Wei-Na Kong,[1] Shu-E Zhao,[2] Xiang-Lin Duan,[1] Zhe Yang,[1] Zhong-Ming Qian,[1,3] and Yan-Zhong Chang[1]***

[1]Laboratory of Molecular Iron Metabolism, College of Life Science, Hebei Normal University, Shijiazhuang 050016, Hebei Province, P. R. China
[2]Department of Medicine, the 3rd Hospital of Hebei Medical University, Shijiazhuang 050051, Hebei Province, P. R. China
[3]Laboratory of Brain Iron Metabolism, Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University, Hong Kong

Abstract Recycled iron from reticuloendothelial macrophages to erythroid precursors is important to maintain the iron homeostasis. However, the molecular mechanisms underlying iron homeostasis in macrophages are poorly understood. In this study, male Sprague–Dawley rats were treated with recombinant human erythropoietin (rHuEpo, 500 IU/day, s.c.) for 3 days. At the fifth day, peritoneal exudate macrophages were harvested, and then ^{55}Fe uptake and release were measured by liquid scintillation counting method. The expression of divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1) in peritoneal exudate macrophages was detected by RT-PCR and Western blot. In order to exclude the direct effect of rHuEpo on macrophages, the parallel experiments were performed with incubation normal peritoneal exudate macrophages with rHuEpo (2 IU/ml). Our results showed rHuEpo injection reduced the peritoneal exudate macrophages iron retention. The uptake of Fe(II) was decreased via the suppression of DMT1 (+IRE) expression and the release of Fe(II) was increased with increasing the expression of FPN1 in macrophages. Moreover, the expression of *HAMP* mRNA was four times lower in rHuEpo-treated liver of rats than control group (CG). *HAMP* mRNA expression was increased; the synthesis of DMT1 had no significant change, whereas the FPN1 was decreased in normal peritoneal exudate macrophages after treatment with rHuEpo in vitro. We conclude that hepcidin may play a major, causative role in the change of FPN1 synthesis and that decreased the iron retention in macrophages of rHuEpo-treated rats. J. Cell. Biochem. 104: 629–641, 2008.

Key words: erythropoietin; macrophage; divalent metal-ion transporter 1; ferroportin 1; hepcidin

Iron plays an essential role in the synthesis of hemoglobin (Hb) and myoglobin, electron transfer, and serves as a cofactor in many enzyme systems. Excess or deficiency of iron is deleterious for the growth and survival of almost every organism. To maintain iron homeostasis, there is tight regulation of iron absorption by the duodenum and iron recycling by the

Abbreviations used: CG, control group; DMT1, divalent metal-ion transporter 1; EG, rHuEpo injected group; Epo, erythropoietin; FPN1, ferroportin 1; Hb, hemoglobin; Hct, hematocrit; IRE, iron response element; RBC, Red Blood Cell; rHuEpo, recombinant human erythropoietin; s.c., subcutaneous; SI, serum iron; TS, transferrin saturation; TIBC, total iron-binding capacity; UIBC, serum unsaturated iron-binding capacity.

Grant sponsor: National Natural Science Foundation of China; Grant number: 30570957; Grant sponsor: Natural Science Foundation of Hebei Province; Grant numbers: C2006000152, C2006000155; Grant sponsor: Science Foundation of Hebei Normal University; Grant numbers: L2003B15, B200209, B2002010.

*Correspondence to: Prof. Yan-Zhong Chang, PhD, Laboratory of Molecular Iron Metabolism, College of Life Science, Hebei Normal University, Shijiazhuang 050016, Hebei Province, P. R. China.
E-mail: frankyzchang@yahoo.com.hk

**Correspondence to: Xiang-Lin Duan, The Key Laboratory of Animal Physiology, Biochemistry and Molecular Biology of Hebei Province, Hebei Normal University, Shijiazhuang, 050016, Hebei Province, P. R. China.
E-mail: xlduan0311@163.com

Received 6 February 2007; Accepted 2 November 2007
DOI 10.1002/jcb.21654

macrophages. The daily absorption and loss are small and iron metabolism in human is characterized by a limited external exchange and by an efficient reutilization of iron from internal sources. Iron is mainly stored in the reticuloendothelial cells and parenchymal cells. Iron recycling is carried out by specialized reticuloendothelial macrophages, which are in the liver, spleen, bone marrow, and peritoneal exudate macrophages. Macrophages engulf aged erythrocytes, lyse them, and catabolize their Hb. The macrophages store part of the recovered iron and export the rest to the plasma, where iron is loaded into apo-transferrin in the circulation and transported to the iron-utilizing cells, including erythroid precursors.

Divalent metal-ion transporter 1 (DMT1), also known as *SLC11A2*, divalent cation transporter 1 (DCT1), and Nramp2, are associated with the plasma membrane and recycling endosomes. The function of this protein is a pH-dependent iron transporter that is involved in iron uptake from low molecular mass iron complexes at the plasma membrane and from transferrin-bound iron in recycling endosomes [Fleming et al., 1997; Gunshin et al., 1997; Tandy et al., 2000; Canonne-Hergaux et al., 2001; Bannon et al., 2003]. In the RAW264.7 and J774 macrophage cell lines, DMT1 is colocalized with transferrin and associated with erythrocyte-containing phagosomes [Gruenheid et al., 1999; Jabado et al., 2002] suggesting a role in recycling iron from transferrin-bound iron or from effete red blood cell (RBC) -containing phagosomes to the cytoplasm. *SLC11A2* gene generates two alternatively spliced mRNAs that differ at their 3′-untranslated region (UTR) by the presence or absence of an iron response element (IRE) [Tchernitchko et al., 2002]. Wardrop and Richardson [2000] demonstrate that there is a marked increase in the DMT1 (+IRE) transcript in RAW264.7 cells after treatment with lipopolysaccharide (LPS), while ^{59}Fe uptake from ^{59}Fe-nitrilotriacetic acid is also increased. Therefore, it is inferred that DMT1 (+IRE) can also transport iron from low molecular mass complexes in macrophages. Furthermore, *SLC11A2* (+IRE) mRNA has an IRE sequence in the 3′-UTR, suggesting that it may be regulated like transferrin receptor 1 (TfR 1) [Pantopoulos, 2004]. It is found that the DMT1 (+IRE) form that is responsive to iron treatment is cell type specific [Gunshin et al., 2001]. *SLC11A2* mRNA is stabilized under low iron condition and degraded under iron loading in intestinal cell line Caco-2 [Tandy et al., 2000]. However, in J774 cells, *SLC11A2* mRNA levels do not respond to changes in cellular iron status [Wardrop and Richardson, 2000]; the expression of DMT1 is not changed after erythrophagocytosis [Knutson et al., 2003]. Consequently, very little is known about the role and regulation of DMT1 in macrophage iron metabolism.

Ferroportin 1 (FPN1), also known as *SLC 40A1*, iron-regulated transporter 1 (IREG1), and metal transporter protein 1 (MTP1), is the first clarified cellular iron exporter [Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000]. In duodenal epithelial cells, FPN1 localizes to the basolateral membrane where it is believed to export iron from the duodenum into the blood circulation [McKie et al., 2000]. FPN1 is particularly abundant in reticuloendothelial macrophages of the liver, spleen, and bone marrow, and predominantly localized in intracellular vesicular compartment, FPN1 are also detected at the plasma membrane of macrophages. It suggests that FPN1 may be stored within the cell until it is needed for iron export, at which point it may be recruited to the membrane [Canonne-Hergaux et al., 2006]. Delaby et al. [2005] and Knutson et al. [2003, 2005] discover that FPN1 expression of macrophages is increased after erythrophagocytosis, overexpressing FPN1 release 70% more ^{59}Fe after erythrophagocytosis than control cells. They also suggest that FPN1 appears to specifically mediate the export of non-heme iron during erythrocyte–iron recycling. *SLC40A1* mRNA contains an IRE sequence in the 5′-UTR. The expression of FPN1 may be regulated by cellular iron in a manner similar to ferritin [Pantopoulos, 2004]. In human alveolar macrophages, ferric ammonium citrate (FAC) significantly increases *SLC40A1* mRNA level [Yang et al., 2002]. Similarly, FPN1 expression is increased with iron-dextran treated in mouse Kupffer cells [Abboud and Haile, 2000]. Knutson et al. [2003] also demonstrate that FP1 mRNA level was increased in murine J774 macrophages on loading with Fe-nitrilotriacetate (Fe-NTA) but decreased on iron depletion with desferrioxamine mesylate (DFO) or salicylaldehyde isonicotinoyl hydrazone (SIH).

The erythroid bone marrow requires a large, continuous supply of iron in quick erythropoiesis, which induces the store iron decrease in

reticuloendothelial macrophages. However, the molecular mechanisms are not fully understood. Erythropoietin (Epo) is the central regulator of RBC production and can stimulate proliferation and differentiation of erythroid progenitor cells [Krantz, 1991]. Now recombinant human erythropoietin (rHuEpo) is widely used in patients with cancer and anemia of chronic disease. In the present study, we detected the role and mechanisms of rHuEpo on iron retention in macrophages in rats to understand the iron metabolism of macrophages.

MATERIALS AND METHODS

Animals and Treatment

Male Sprague–Dawley (SD) rats (250 g) were purchased from Hebei Medical University and housed in stainless steel rust-free cages at 22–24°C temperature, 45–55% relative humidity. All animals were provided free access to food and distilled water. The animal Care and Use Committee of Hebei Science and Technical Bureau in PRC approved the experimental protocol.

Rats were injected daily with 500 IU rHuEpo dissolved in normal saline or with saline alone (controls) subcutaneously (s.c.) for 3 days. Experiments were carried out 48 h after the last injection.

Sampling of Blood and Tissue

At the end of the study, animals were anesthetized with 0.4% pentobarbital sodium (10 ml/kg body weight, i.p.). Blood samples were then collected into heparinized syringes and aliquots were taken immediately for the determination of RBC count, Hb concentration, and hematocrit (Hct). RBC was measured by haemocytometer. Hb concentration was determined by the cyanmethemoglobin method. Hct was measured using the microhematocrit centrifuge. The serum samples were analyzed for serum iron (SI), serum unsaturated iron-binding capacity (UIBC), total iron-binding capacity (TIBC), and transferrin saturation (TS), using Iron and Iron-binding Capacity Reagent (Sigma). These methods are all described previously [Ke et al., 2003, 2005; Liu et al., 2006]. The rats were perfused with ice-cold phosphate-buffered saline (PBS) through the left ventricle. Then the liver, spleen, bone marrow, heart, kidney, and muscle were excised, frozen in liquid nitrogen, and stored at −70°C for the measurement of non-heme iron.

Macrophages Harvest

Rat peritoneal exudate macrophages were obtained by lavage of the peritoneal cavity with 80 ml RPMI 1640 containing 1% fetal bovine serum. Cells were collected by centrifugation (500*g*, 10 min) at 4°C, then suspended in RPMI 1640 containing 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and maintained at 37°C in a humidified atmosphere containing 5% CO_2 for 2.5 h. We removed nonadherent cells by washing once with PBS at pH 7.2. Adherent cells consisted of more than 95% macrophages. To investigate the effect of rHuEpo on macrophages in vitro, the parallel experiments were performed with incubation normal peritoneal exudate macrophages with rHuEpo (0.2, 2, 20 IU/ml) for 24 h for the measurement of macrophage *HAMP* mRNA level, and incubation normal peritoneal exudate macrophages with rHuEpo (2 IU/ml) for 24 h for the measurement of macrophage DMT1, FPN1 proteins synthesis, and iron uptake or release.

Measurement of Tissue Non-Heme Iron

Non-heme iron concentrations of tissues were measured as described previously [Qian et al., 1999, 2001]. Acid mixture was prepared by 0.6 M trichloroacetic acid in hydrochloride, and added to tissue homogenates. After mixing and closing the tube, the content was heated in water bath at 65°C for 20 h. By centrifugation, a clear acid extract was obtained and then transferred into another set of iron-free tubes. Color was developed by the addition of 1 ml of chromagen solution containing 0.2% thioglycollic acid, and the absorbance of the solution was measured in a spectrophotometer at a wavelength of 535 nm against reagent blank. Chromagen solution was freshly prepared by dissolving 0.5 mM bathophenanthroline in sodium acetate. Absorbance of reagent blank at 540 nm against iron-free water was less than 0.010.

Measurement of Iron Uptake and Release

Iron-55 ($^{55}FeCl_3$, Perkin-Elmer Life Sciences Company, Wellesley City, MA) solution was prepared by mixing $^{55}FeCl_3$ with $FeSO_4$ in a molar ratio of 1:10 followed by a 50-fold molar excess of 2-mercaptoethanol and 0.27 M sucrose (pH 6.5) as described previously [Chen et al.,

2005]. After washing three times with PBS, the macrophages (about 1×10^6 cells) were incubated with $^{55}FeCl_3$ solution (1 μM) for 30 min at 37°C and then washed three times with PBS at 4°C. The macrophages were homogenized in buffer containing 1% sodium dodecyl sulphate (SDS), and aliquots of the total cell extract were assayed for released radioactivity with Liquid Scintillation Analyzer (Beckman) and protein concentrations by Lowry method. For measurement of iron release, the macrophages were incubated with $^{55}FeCl_3$ solution for 30 min and then washed three times with PBS at 4°C. The macrophages were then incubated with 1 ml PBS at 37°C for 30 min. The supernatant was collected. The macrophages were homogenized after washing three times with PBS. Both of the supernatant and cell extract were assayed for radioactivity. $\%^{55}Fe$ release = (cpm in supernatant)/(cpm in supernatant + cpm in cells) × 100%.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from peritoneal exudate macrophages or liver using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed in a 20 μl reaction system using Reverse Transcription System (Promega). After denaturation of RNA at 70°C for 10 min in a thermal cycler, the reaction was performed for 1 h at 42°C. As shown in Table I, the primers for PCR of *SLC11A2* (+IRE), *SLC11A2* (−IRE) [Ke et al., 2005], *SLC40A1*, and *HAMP* [Liu et al., 2006] were based on the cDNA sequence (GenBank® accession number AF008439, AF029757, AF394785, and AF344185). β-actin cDNA (GenBank® accession number V01217) [Liu et al., 2006] was amplified simultaneously as the internal control. The reaction mixture lacking RT was used as a negative control. A 20 μl PCR amplification reaction (TaKaRa Taq DNA Polymerase Kit) was prepared. Amplification was performed (Eppendorf Mastercycler gradient PCR System) with initial denaturation at 95°C for 4 min, followed by 22 cycles (β-actin) or 29 cycles (*SLC11A2* (+IRE, −IRE), *SLC40A1*) or 24 cycles (*HAMP*, in liver); 34 cycles (*HAMP*, in peritoneal exudate macrophages) at 94°C for 45 s, 60°C for 45 s, 72°C for 60 s, and a single final extension at 72°C for 10 min. The PCR products were analyzed on a 1% agarose gel using Gel-Pro Analyzer® Analysis software (Media Cybernetics). Gene expression values were normalized with β-actin.

Western Blot Analysis

The method of Western blot has been described previously by [Chang et al., 2005]. After washing three times with PBS, the peritoneal exudate macrophages were harvested and sonicated in Tris-buffered salt (TBS) buffer. TBS (1 ml; pH 8.0) buffer contained 1% NP-40, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 μg/ml PMSF. After centrifugation at 12,000*g* for 30 min at 4°C, the supernatant was collected. Protein concentrations in supernatant were assayed by Bradford method. The total cell extract containing 30 μg protein were loaded on a single track of 10% SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions and transferred by electroblotting onto NC membrane (Trans-Blot® Transfer Medium Pure Nitrocellulose Membrane, Bio-RAD, 0.45 μm) for 45 min at 4°C. Molecular weight standards (Amersham Pharmacia Biotech) were run in parallel. The blots were blocked by using 5% blocking reagent (Amersham Biosciences) in a solution of TBS with Tween-20 (TBS-T) (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h at room

TABLE I. Sequences and Accession Number of Primers Used to Amplify Rat mRNA by RT-PCR

Gene	Primer	5′ → 3′	Position	Size (bp)	Accession
β-actin	Forward	GGTCACCCACACTGTGCCCATCTA	2268–2291	263	V01217
	Reverse	GACCGTCAGGCAGCTCACATAGCTCT	2507–2530		
SLC11A2	Forward	TATCTAGATGACCAACAGCC	1697–1716	335	AF029757
−IRE	Reverse	ATCTTACCCAAACTGGCACG	2012–2031		
SLC11A2	Forward	CTGAGCGAAGATACCAGCG	1755–1773	838	AF008439
+IRE	Reverse	GGAGCCATCACTTGACCACAC	2572–2592		
SLC40A1	Forward	AATGGGAACTGTGGCCTTCA	1298–1317	436	AF394785
	Reverse	AGTTCATGGAGTTCTGCACAC	1714–1733		
HAMP	Forward	CAAGATGGCACTAAGCACTCG	21–41	337	AF344185
	Reverse	AATAAGGACAGGATGGGGTCG	337–357		

temperature, then separately incubated with rabbit anti-rat DMT1 (+IRE) antiserum (1:5,000, ADI, San Antonio, TX), rabbit anti-rat DMT1 (−IRE) antiserum (1:5,000, ADI), FPN1 antiserum (1:5,000, ADI), and TfR1 antiserum (1:2,000, AbD, Oxford, UK) overnight at 4°C. After washed with TBS-T, the blots were incubated in goat-anti-rabbit secondary antibody-conjugated horseradish peroxide (1:5,000, Amersham Biosciences) for 1.5 h at room temperature. Immunoreactive proteins were detected by using enhanced chemiluminescence method (ECL, Amersham Biosciences). The blots were detected using Chemiluminescence Imaging System (Ω MEGA12ic) and quantified by transmittance densitometry using volume integration with Gel-Pro Analyzer® Analysis software (Media Cybernetics) to determine the enrichment of the DMT1 and FPN1 proteins in macrophages. To ensure even loading of the samples, the same membrane was probed with rabbit anti-human β-actin polyclonal antibody (Sigma-Aldrich, MO) at 1:5,000 dilutions. DMT1 and FPN1 protein concentrations in each specimen were normalized for β-actin in that specimen.

Statistics

The results were expressed as means ± SEM. Statistical analysis was carried out using the SPSS statistics package (SPSS Inc., Chicago, IL). Independent-Samples *T*-Test was utilized where appropriate. A probability value of $P < 0.05$ was taken to be statistically significant.

RESULTS

Effect of rHuEpo (s.c.) on Blood Parameters, SI Status, and Tissue Non-Heme Iron Concentrations

Administration of rHuEpo induced abundant erythropoiesis with RBC ($P = 0.002$, Table II), Hb ($P = 0.004$, Table II), and Hct ($P < 0.001$, Table II) levels marked increasing, whereas the SI and TS decreased dramatically (all $P < 0.001$, Table II). Non-heme iron levels in the peritoneal exudate macrophages ($P = 0.002$, Table III), liver ($P = 0.001$, Table III), spleen ($P < 0.001$, Table III), and bone marrow ($P < 0.001$, Table III) were remarkably decreased after rHuEpo treatment. The non-heme iron of the heart, kidney, and muscle was constant ($P > 0.05$, Table III). The data suggested that rats did develop increased erythropoiesis and body iron deficiency characterized by decreased iron contents of transport and storage after injected with rHuEpo. Iron was transferred from the storage to bone marrow for the synthesis of Hb. In addition, iron levels in the kidney, muscle, and heart would not be altered before complete depletion of storage iron.

TABLE II. Effect of rHuEpo (s.c.) on Blood Parameters, Serum Iron Status in Rats

	CG	EG
RBC (10^6/mm^3)	7.85 ± 0.14	9.08 ± 0.26**
Hb (g/L)	142.30 ± 2.64	161.09 ± 4.36**
HCT (%)	44.19 ± 0.60	54.23 ± 1.03**
SI (μg/dl)	169.21 ± 10.88	69.44 ± 9.52**
UIBC (μg/dl)	236.87 ± 26.09	403.80 ± 9.46**
TIBC (μg/dl)	406.08 ± 19.61	473.25 ± 11.99*
TS (%)	42.55 ± 4.31	14.57 ± 1.77**

Results are expressed as mean ± SEM, n = 6.
*$P < 0.05$ versus control group.
**$P < 0.01$ versus control group.

TABLE III. Effect of rHuEPO (s.c.) on Non-Heme Iron Concentrations (μg/g) in Tissues

	CG	EG
Liver	295.16 ± 15.74	176.39 ± 9.94*
Spleen	581.49 ± 44.30	338.66 ± 17.23*
Bone marrow	378.50 ± 26.09	195.64 ± 13.54*
Macrophages	50.23 ± 3.31	33.16 ± 2.36*
Heart	208.14 ± 9.42	215.19 ± 6.63
Kidney	88.23 ± 7.48	79.28 ± 5.57
Muscle	52.33 ± 2.12	51.74 ± 1.18

Dry weight (μg/g) in the liver, spleen, bone marrow, heart, kidney, and muscle; wet weight (μg/g) in peritoneal exudate macrophages. Results are expressed as mean ± SEM, n = 6.
*$P < 0.01$ versus control group.

Effect of rHuEpo (s.c.) on Iron Uptake and Release by Macrophages

Since treatment of rats with rHuEpo is known to result in the decrease in iron peritoneal exudate macrophages as shown in Table III, we examined whether the rHuEpo injection may regulate the iron uptake and release of macrophages. After incubating peritoneal exudate macrophages with ^{55}Fe solution for 30 min, we found that the iron uptake of macrophages of rHuEpo injection rats was significantly decreased than that of control (Fig. 1A, $P < 0.001$); the iron release of macrophages was increased in rHuEpo-treated rats ($P = 0.011$), as shown in Figure 1B. These data suggested that the store iron of macrophages, as an internal reserve, could be mobilized during increased erythropoiesis via suppression of iron uptake and enhancement of iron release.

In order to eliminate the influence of non-specific ^{55}Fe-binding, further experiment was

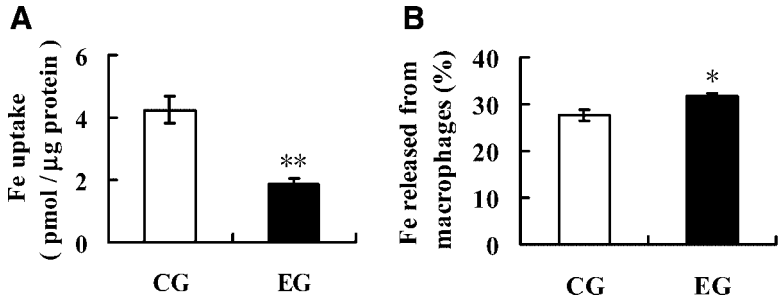

Fig. 1. Effect of rHuEpo (s.c.) on iron uptake and release by peritoneal exudate macrophages. Rats were injected subcutaneously daily with 500 IU rHuEpo dissolved in normal saline or with saline alone (controls) for 3 days, and the peritoneal exudate macrophages were harvested after 48 h of the last injection. The macrophages were incubated with $^{55}FeCl_3$ solution (1 μM) for 30 min at 37°C and then aliquots of the total cell extract were assayed for released radioactivity with liquid scintillation counting method after washing with PBS. For measurement of iron release, the macrophages were incubated with $^{55}FeCl_3$ solution for 30 min and then incubated with 1 ml PBS at 37°C for 30 min after washing with PBS. Both of the supernatant and cell extract were assayed for radioactivity. Results are expressed as mean ± SEM, n = 6. $^{**}P < 0.01$ versus control group, $^{*}P < 0.05$ versus control group.

further done. We used another washing solution (ice-cold PBS containing EDTA) to detect the degree of non-specific binding according to the previous publications [Han and Wessling-Resnick, 2002; Priwitzerova et al., 2005]. After incubating peritoneal exudate macrophages with ^{55}Fe solution for 30 min, the cells were washed three times with ice-cold PBS with or without 5 mM EDTA. We have not found the significant difference either in the control group (CG) (PBS without EDTA: 3.9167 ± 0.0717 pmol Fe/μg protein; PBS with EDTA: 3.5590 ± 0.1303 pmol Fe/μg protein; n = 3, $P > 0.05$) or EG (PBS without EDTA: 2.1587 ± 0.0583 pmol Fe/μg protein; PBS with EDTA: 2.3071 ± 0.0275 pmol Fe/μg protein; n = 3, $P > 0.05$). The significant decrease of iron uptake by macrophages in EG than that of CG was also observed.

DMT1 activity is pH-dependent. In order to make sure DMT1 is responsible for iron uptake across the macrophage surface, iron uptake studies at pH 5.5, 6.5, 7.5 were performed in macrophages. Our results exhibited that ^{55}Fe uptake was inversely proportional to pH, with the highest uptake at pH 5.5 (Fig. 2, pH 5.5 vs. pH 6.5, $^{**}P = 0.001$; pH 6.5 vs. pH 7.5, ▲ $P = 0.036$). The results were similar to previous study [Han and Wessling-Resnick, 2002]. This result suggested that the DMT1 may be involved in the process of ^{55}Fe uptake in macrophage.

Effect of rHuEpo (s.c.) on DMT1 and FPN1 Expression in Macrophages

In order to understand the mechanisms of change of iron homeostasis in peritoneal exudate macrophages by rHuEpo injection, the levels of *SLC11A2* and *SLC40A1* mRNA were analyzed by RT-PCR. As shown in Figure 3, the expression of *SLC11A2* (+IRE) mRNA of macrophages was significantly decreased (Fig. 3A, $P = 0.039$), in contrast, there was no significant change of *SLC11A2* (−IRE) mRNA levels in macrophages following rHuEpo treatment (Fig. 3B, $P > 0.05$). Furthermore, the expression of *SLC40A1* mRNA was enhanced in macrophages collected from rHuEpo-treated rats (Fig. 3C, $P = 0.001$). The synthesis of DMT1

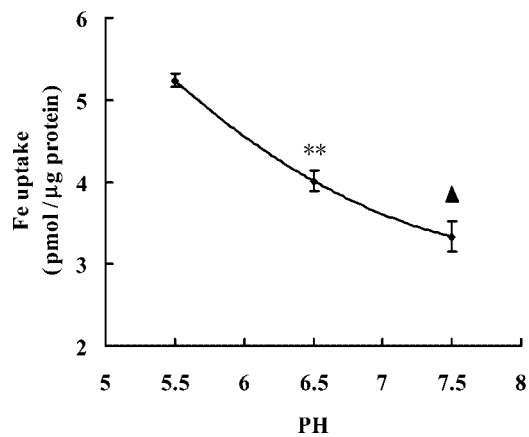

Fig. 2. Effect of pH on iron uptake of peritoneal exudate macrophage. The normal macrophages were incubated with $^{55}FeCl_3$ solution (1 μM) at pH 5.5, 6.5, 7.5 for 30 min at 37°C and then aliquots of the total cell extract were assayed for released radioactivity with liquid scintillation counting method after washing with PBS. Results are expressed as mean ± SEM, n = 3. $^{**}P < 0.01$, pH 5.5 versus pH 6.5; ▲ $P < 0.05$, pH 6.5 versus pH 7.5.

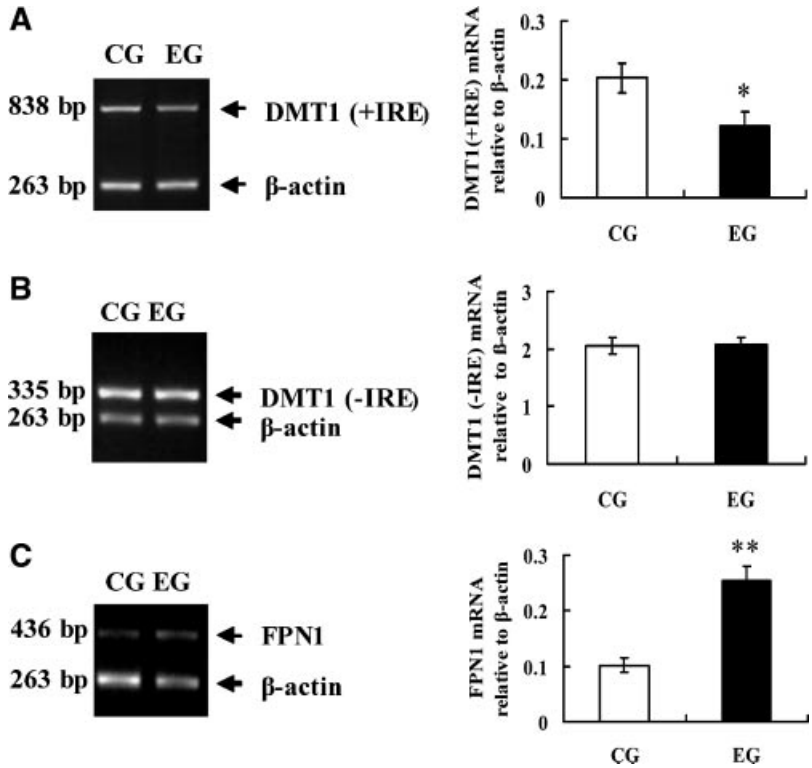

Fig. 3. Effect of rHuEpo (s.c.) on *SLC11A2* and *SLC40A1* mRNA expression in peritoneal exudate macrophages. Rats were injected subcutaneously daily with 500 IU rHuEpo dissolved in normal saline or with saline alone (controls) for 3 days, and then the peritoneal exudate macrophages were harvested after 48 h of the last injection. The expression of *SLC11A2* and *SLC40A1* mRNA were investigated by RT-PCR analysis as described in Materials and Methods. PCR amplification was performed with β-actin (22 cycles) primers, and *SLC11A2* (+IRE), *SLC11A2* (−IRE), *SLC40A1* (29 cycles) primers. Gene expression values were normalized with β-actin. The expression of *SLC11A2* (+IRE) mRNA (**A**), *SLC11A2* (−IRE) mRNA (**B**), and *SLC40A1* mRNA (**C**) was quantified, results are expressed as mean ± SEM, n = 6. $^{**}P < 0.01$ versus control group, $^{*}P < 0.05$ versus control group.

and FPN1 proteins in peritoneal exudate macrophages following rHuEpo injection were also determined by Western blot analysis. In parallel to changes in *SLC11A2* (+IRE) and *SLC40A1* mRNA levels, rHuEpo injection significantly decreased DMT1 (+IRE) protein synthesis (Fig. 4A, $P = 0.040$) and increased FPN1 synthesis compared to the controls (Fig. 4C, $P = 0.041$). The change of DMT1 (−IRE) protein synthesis was not observed (Fig. 4B, $P > 0.05$) in macrophages of rHuEpo injection rats. These results imply that the reduced iron uptake in macrophages may be a consequence of down-expression of DMT1 (+IRE) and the increased cellular iron release may be a consequence of up-expression of FPN1.

In addition, another iron uptake protein TfR1 expression in macrophages and bone marrow was also measured by Western blot. It was found that the immunoactivity bands of TfR1 in peritoneal exudate macrophages were very weak in both groups (Fig. 5). In contrast, high level of TfR1 expression in bone marrow was observed. rHuEpo injection increased TfR1 synthesis in EG (Fig. 5). Therefore, TfR1-dependent iron uptake may not be the important route in peritoneal exudate macrophages.

Effect of rHuEpo (s.c.) on *HAMP* mRNA Expression in the Liver of Rats

To test the hypothesis that hepcidin might regulate the DMT1 and FPN1 expression in peritoneal exudate macrophages, hepatic *HAMP* mRNA levels were analyzed by RT-PCR. A 337-bp fragment of *HAMP* and a 263-bp fragment of β-actin were amplified from hepatic cDNA, which were consistent with the expected size of PCR products. Expression of *HAMP* mRNA was quantified by normalizing to β-actin mRNA. As shown in Figure 6, rHuEpo treatment induced a significant decrease in *HAMP*

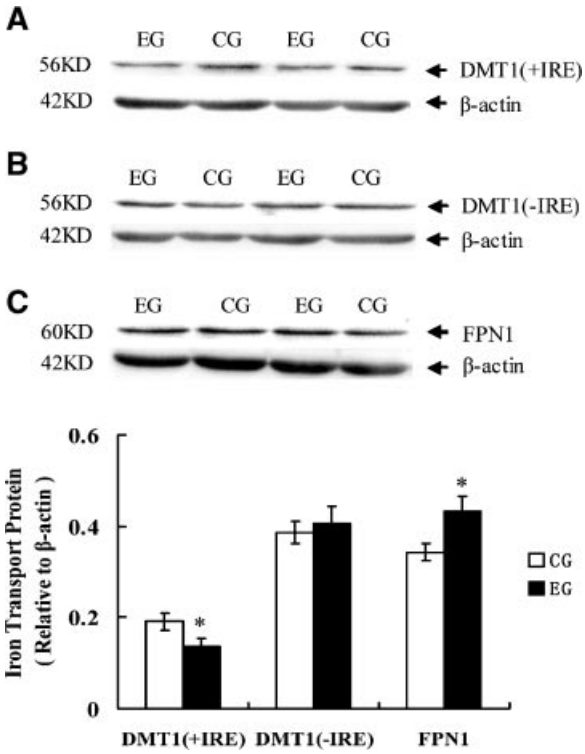

Fig. 4. Effect of rHuEpo (s.c.) on the synthesis of DMT1 and FPN1 proteins in peritoneal exudate macrophages. Rats were injected subcutaneously daily with 500 IU rHuEpo dissolved in normal saline or with saline alone (controls) for 3 days, and then the peritoneal exudate macrophages were harvested after 48 h of the last injection. The syntheses of DMT1 and FPN1 proteins were investigated by Western blot analysis as described in Materials and Methods. DMT1 (+IRE) (**A**), DMT1 (−IRE) (**B**), and FPN1 (**C**) protein concentrations in each specimen were normalized for β-actin in that specimen. Results are expressed as mean ± SEM, n = 6. *$P < 0.05$ versus control group.

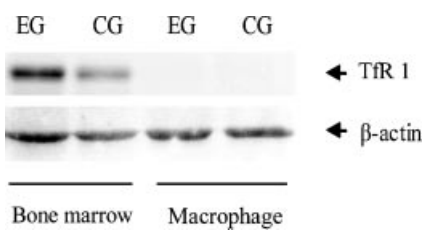

Fig. 5. The expression of TfR1 in peritoneal exudate macrophages and bone marrow. Rats were injected subcutaneously daily with 500 IU rHuEpo dissolved in normal saline or with saline alone (controls) for 3 days, and then the peritoneal exudate macrophages and bone marrow were harvested after 48 h of the last injection. The synthesis of TfR1 protein was investigated by Western blot analysis as described in Materials and Methods.

mRNA expression, reaching almost undetectable levels ($P < 0.001$).

Effect of rHuEpo on Macrophage Iron Metabolism In Vitro

In order to eliminate the direct effect of rHuEpo on peritoneal exudate macrophages iron metabolism during rHuEpo injection, the parallel experiments were performed. Normal peritoneal exudate macrophages were incubated with rHuEpo (2 IU/ml) for 24 h. As shown in Figure 7A, the effect of 2 IU/ml rHuEpo on iron uptake of macrophages was not detected ($P > 0.05$); iron release from macrophages was inhibited in rHuEpo treatment group (Fig. 7B, $P = 0.030$).

The synthesis of DMT1 and FPN1 proteins were also determined in peritoneal exudate macrophages treated with rHuEpo by Western blot analysis. rHuEpo did not alter the synthesis of DMT1 (either +IRE or −IRE) in macrophages compared to untreated control cells (Fig. 8A,B, $P > 0.05$). FPN1 synthesis was reduced in macrophages incubated with rHuEpo (Fig. 8C, $P = 0.027$). This result indicated that the role of rHuEpo was different on FPN1 synthesis of macrophages in vivo and in vitro.

In order to study the mechanisms of rHuEpo influencing FPN1 expression of peritoneal exudate macrophages in vitro, normal peritoneal exudate macrophages were incubated with rHuEpo (0.2, 2, 20 IU/ml) for 24 h, and the *HAMP* mRNA expression was detected by RT-PCR. The data showed that 0.2 IU/ml rHuEpo did not change *HAMP* mRNA level of peritoneal exudate macrophages (Fig. 9, $P > 0.05$), 2, 20 IU/ml rHuEpo treatments, respectively, led to a significant increase in macrophage *HAMP* mRNA expression (Fig. 9, 2 IU/ml, $P = 0.006$; 20 IU/ml, $P = 0.008$). Those data suggested that the decrease in FPN1 synthesis of peritoneal macrophages by rHuEpo treatment is conducted by the increase in *HAMP* mRNA expression.

DISCUSSION

In this study, we addressed the mechanisms of reduced iron retention in macrophages by Epo in rats. Rats were injected with rHuEpo for 3 days, the iron storage of peritoneal exudate macrophages, liver, spleen, and bone marrow was significantly reduced, the decrease in SI

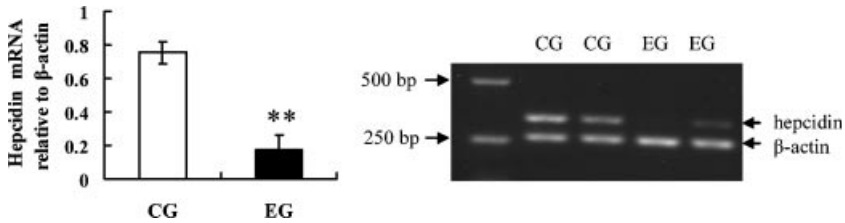

Fig. 6. Effect of rHuEpo (s.c.) on *HAMP* mRNA expression in the liver. Rats were injected subcutaneously daily with 500 IU rHuEpo dissolved in normal saline or with saline alone (controls) for 3 days, and then the total RNA was isolated from the liver using TRIzol Reagent after 48 h of the last injection. PCR amplification was performed with β-actin (22 cycles) primers, and *HAMP* (24 cycles) primers. All PCR products had the respective predicted size (337 bp for *HAMP* and 263 bp for β-actin). The reported quantifications for mRNA levels were normalized with respect to β-actin mRNA. Results are expressed as mean ± SEM, n = 5. $^{**}P < 0.01$ versus control group.

and TS was also detected at the fifth day. Reduced iron retention was a consequence of inhibited iron uptake with down-regulation of the DMT1 (+IRE) expression and increased iron release with up-regulation of FPN1 expression; in addition, continual accumulation of inhibited iron uptake and increased iron release for 4 days was an important reason to reduce iron retention in macrophages. Furthermore, we cannot exclude the possibility that other factors affect the process.

In order to eliminate whether there is the direct effect of rHuEpo on macrophages, normal peritoneal exudate macrophages were incubated with rHuEpo in vitro. It was notable that no changes of DMT1 expression but decrease in FPN1 synthesis were observed. It suggested that the changes of DMT1 or FPN1 expression in peritoneal exudate macrophages in rHuEpo injection rats (s.c.) were not due to the direct functional role of rHuEpo. Consequently, it remains to be answered how rHuEpo injection (s.c.) regulated DMT1 and FPN1 protein synthesis in peritoneal exudate macrophages?

The recently identified hepatic peptide hepcidin (also known as *HAMP*, liver-expressed antimicrobial peptide, LEAP-1) has been proposed to act as the principal iron-regulatory hormone to maintain iron homeostasis. Hepcidin is synthesized by the liver in the form of an 84 amino acid propeptide and is detected in the plasma as a peptide of 25 amino acids [Krause et al., 2000; Park et al., 2001]. The most important cellular targets for hepcidin appear to be the villus enterocyte, the hepatocyte, and the reticuloendothelial macrophage. In the enterocyte, hepcidin would be expected to decrease basolateral iron transfer and thus reduce dietary iron absorption. In the reticuloendothelial macrophage and the hepatocyte, hepcidin would lead to a decrease in iron export and thus increase iron store [Nicolas et al., 2001; Pigeon et al., 2001]. Using RT-PCR, we found that hepatic *HAMP* expression was decreased

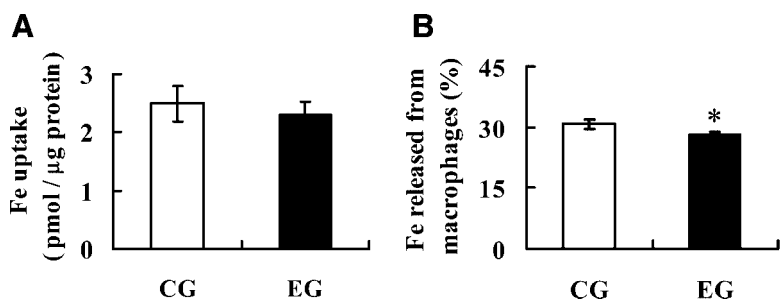

Fig. 7. Effect of rHuEpo on peritoneal exudate macrophage iron uptake and release in vitro. Normal peritoneal exudate macrophages were incubated with rHuEpo (2 IU/ml) for 24 h in order to obviate the direct effect of rHuEpo on iron uptake and release by macrophages following rHuEpo injection (s.c.). After treatment with rHuEpo, the macrophages were incubated with $^{55}FeCl_3$ solution (1 μM) for 30 min at 37°C, and then homogenized in buffer containing 1% SDS after washing with PBS. Aliquots of the total cell extract were assayed for released radioactivity with liquid scintillation counting method. For measurement of iron release, the macrophages were incubated with $^{55}FeCl_3$ solution for 30 min and then incubated with 1 ml PBS at 37°C for 30 min after washing with PBS. Both of the supernatant and cell extract were assayed for radioactivity. Results are expressed as mean ± SEM, n = 6. $^{*}P < 0.05$ versus control group.

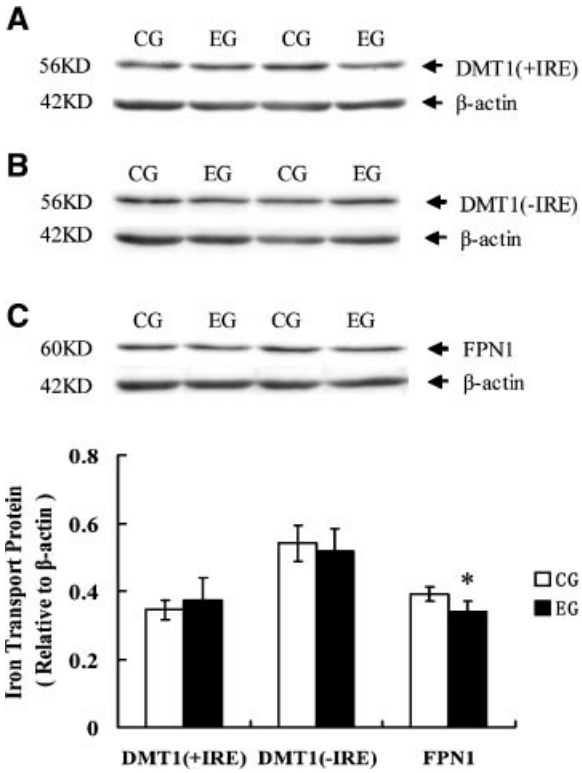

Fig. 8. Effect of rHuEpo on peritoneal exudate macrophage DMT1 and FPN1 proteins expression in vitro. Normal peritoneal exudate macrophages were incubated with rHuEpo (2 IU/ml) for 24 h as the parallel experiments to eliminate the direct effect of rHuEpo on the expression of DMT1 and FPN1 in macrophages. The syntheses of DMT1 and FPN1 proteins were investigated by Western blot analysis as described in Materials and Methods. DMT1 (+IRE) (**A**), DMT1 (−IRE) (**B**), and FPN1 (**C**) protein concentrations in each specimen were normalized for β-actin in that specimen. Results are expressed as mean ± SEM, n = 6. $^{*}P < 0.05$ versus control group.

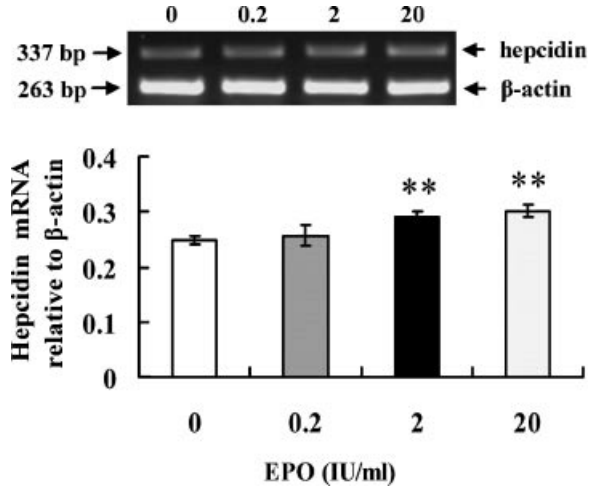

Fig. 9. Effect of rHuEpo on peritoneal exudate macrophage *HAMP* mRNA expression in vitro. Normal peritoneal exudate macrophages were incubated with rHuEpo (0.2, 2, 20 IU/ml) for 24 h for measurement of macrophage *HAMP* mRNA level by RT-PCR analysis. PCR amplification was performed with β-actin (22 cycles) primers, and *HAMP* (34 cycles) primers. All PCR products had the respective predicted size (337 bp for *HAMP* and 263 bp for β-actin). The reported quantifications for mRNA levels were normalized with respect to β-actin mRNA. Results are expressed as mean ± SEM, n = 4. $^{**}P < 0.01$ versus control group.

markedly after rHuEpo injection, which was consistent with previous reports [Nicolas et al., 2002; Vokurka et al., 2006]. Therefore, hepcidin may helpfully illuminate the regulatory mechanisms of iron homeostasis in peritoneal exudate macrophages following rHuEpo injection (s.c.).

Nemeth et al. [2004] have reported that hepcidin decreases the functional activity of FPN1 by directly binding to it and causing it to be internalized from the cell surface and degraded, thereby blocking cellular iron efflux. Then Rivera et al. [2005] have further demonstrated that radiolabeled hepcidin is accumulated in the FPN1-rich organs, such as liver, spleen, and proximal duodenum. Moreover, FPN1 expression is up-regulated in the liver (predominantly in the Kupffer cells) in the hepcidin-deficient mice [Viatte et al., 2005]. In the present study, we showed that the expression of FPN1 was increased in peritoneal exudate macrophages following rHuEpo injection, while hepatic *HAMP* mRNA level was dramatically decreased. Thus, we infer that FPN1 expression in macrophages was regulated by the suppression of hepcidin expression induced by rHuEpo injection. This idea is strongly supported by Knutson et al. [2005]. After the J774 cells were incubated with hepcidin, they found that the expression of FPN1 was decreased dramatically, while the ^{59}Fe release from macrophages was significantly reduced after erythrophagocytosis.

Does hepcidin influence the expression of DMT1? In Caco-2 cells (human intestinal epithelial cell line), hepcidin significantly reduces apical iron uptake accompanied by a decrease in the expression of DMT1 (+IRE) [Yamaji et al., 2004]. In our study, DMT1 expression was down-regulated by rHuEpo treatment (s.c.) in peritoneal exudate macrophages, while hepatic *HAMP* mRNA level was also decreased. That was contrary to the results

of Caco-2 cells. Probably, the interaction of hepcidn and DMT1 was also cell type specific; however, we did not have direct evidence that detected relationship between hepcidin and DMT1 in this study.

What factor(s) down-regulate DMT1 (+IRE) expression in macrophages after rHuEpo injection in rats? Previous studies showed that the low intracellular iron levels could stimulate the expression of DMT1 and increase cellular iron uptake, which, in turn, decrease cellular iron uptake in the duodenum [Tandy et al., 2000; Gunshin et al., 2001; Thomas and Oates, 2002]. Other studies revealed that the regulation of DMT1 expression mediated by the IRP–IRE system is cell type specific [Gunshin et al., 2001]. In the macrophages, DMT1 expression is not modulated in response to Fe-depletion or Fe-loading like TfR mRNA. In our study, iron levels of peritoneal exudate macrophages were decreased in rHuEpo injection rats associated with suppression of DMT1 expression. Similar results have been obtained in the liver and HepG2 hepatoma cell line [Trinder et al., 2000; Scheiber-Mojdehkar et al., 2003]. The liver, as the major storage site for iron, plays a central role in iron metabolism. DMT1 protein expression was increased in iron-loaded liver, and almost not detected in iron-deficient liver. Furthermore, in RAW264.7 cells, DFO had no effect on SLC11A2 mRNA levels, whereas the Fe donor FAC slightly increased the levels of SLC11A2 mRNA transcripts [Wardrop and Richardson, 2000]. These findings are seemingly all inconsistent with the IRP–IRE regulation system. The 5′-promoter/exon 1A region and the IRE-containing terminal exon participate in iron regulation of DMT1 expression. It is possible that the 5′-UTR of the transcript or the N-terminal domain of the protein may modify the regulatory effects of the IRE in a tissue-specific manner [Hubert and Hentze, 2002]. These findings may help to illuminate our study and other seemingly contradictory results [Trinder et al., 2000; Wardrop and Richardson, 2000; Scheiber-Mojdehkar et al., 2003]. Moreover, the SLC11A2 gene contains metal responsive elements in its 5′-regulatory region [Lee et al., 1998]. Therefore, we speculate that iron levels maybe regulate the DMT1 expression both at the transcriptional and at the posttranscriptional level. However, at present, the regulatory mechanism here is uncertain.

In addition, our study reveals that treatment with rHuEpo induces a dramatic reduction in iron release of peritoneal exudate macrophages and further demonstrates that the suppression of FPN1 synthesis maybe involved in this process. To our knowledge, this is the first report about the regulation of FPN1 protein synthesis induced by rHuEpo treatment in macrophages. Further study revealed that the peritoneal exudate macrophages can express *HAMP* mRNA, which is supported by Nguyen et al. [2006] report. RT-PCR analysis result further showed that the expression of macrophage *HAMP* mRNA was increased following rHuEpo treatment. Therefore, increased macrophage HAMP mRNA expression may result in the decrease of FPN1 synthesis after rHuEpo treatment in vitro.

rHuEpo injection decreased hepatic HAMP expression in vivo. Contrarily, rHuEpo treatment increased macrophage HAMP mRNA expression in vitro. What induced the discrepant results between in vivo and in vitro experiments? rHuEpo injection can stimulate TfR1 expression in the bone marrow, and increase uptake of iron into cells resulting in reduced diferric transferrin levels in vivo [Weiss et al., 1997]. Hepcidin synthesis in the liver may be regulated by altering TS [Frazer and Anderson, 2003]. In our study, we demonstrated that serum TS was decreased dramatically following rHuEpo injection, which may induce a decrease in hepatic hepcidin mRNA expression. The specific receptors of Epo exist on the cell surface of peritoneal exudate macrophages [Van Zant and Chen, 1983]. After Epo binds to its receptor, the intracellular domain of the Epo receptor transmits signals leading to the activation of transcription factor NF-κB [Bittorf et al., 2001]. Another report showed that *HAMP* gene expression may be regulated by NF-κB [Liao et al., 2006]. It is a possibility that NF-κB may be activated by rHuEpo, and then enhanced hepcidin synthesis in vitro.

We also found the different role of rHuEpo on the regulation of macrophages FPN1 expression between in vivo and in vivo. The expression of hepcidin may be involved in the regulated mechanisms. In vivo, FPN1 expression in macrophages was increased by the suppression of hepatic hepcidin expression. In vitro, rHuEpo decreased FPN1 synthesis through increasing macrophage HAMP mRNA level. In addition, consideration of the synthesis of hepcidin in

macrophage itself is little, inversely; in vivo the decrease of hepatic hepcidin expression was significant, we infer that the role of hepatic hepcidin may cover the direct function of rHuEpo on the regulation of macrophages hepcidin mRNA expression.

Taken together, in the present study, we demonstrated that the decreased levels of DMT1 induced by the injection of rHuEpo could prevent iron transfer into the macrophages and the increased levels of FPN1 could transfer more iron from macrophages into circulation to meet the needs of erythropoiesis. As a result, the iron level of macrophages is decreased. The expression of *HAMP* mRNA was significantly decreased by erythropoietic activity following rHuEpo injection, which may control the expression FPN1 directly in peritoneal exudate macrophages.

ACKNOWLEDGMENTS

We thank Dr. Guangjun Nie (Physiology Department, McGill University) for helping us to correct this manuscript.

REFERENCES

Abboud S, Haile DJ. 2000. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J Biol Chem 275(26):19906–19912.

Bannon DI, Abounader R, Lees PS, Bressler JP. 2003. Effect of DMT1 knockdown on iron, cadmium, and lead uptake in Caco-2 cells. Am J Physiol Cell Physiol 284(1): C44–C50.

Bittorf T, Buchse T, Sasse T, Jaster R, Brock J. 2001. Activation of the transcription factor NF-kappaB by the erythropoietin receptor: Structural requirements and biological significance. Cell Signal 13(9):673–681.

Canonne-Hergaux F, Zhang AS, Ponka P, Gros P. 2001. Characterization of the iron transporter DMT1 (NRAMP2/DCT1) in red blood cells of normal and anemic mk/mk mice. Blood 98:3823–3830.

Canonne-Hergaux F, Donovan A, Delaby C, Wang HJ, Gros P. 2006. Comparative studies of duodenal and macrophage ferroportin proteins. Am J Physiol Gastrointest Liver Physiol 290(1):G156–G163.

Chang YZ, Qian ZM, Wang K, Zhu L, Yang XD, Du JR, Jiang L, Ho KP, Wang Q, Ke Y. 2005. Effects of development and iron status on ceruloplasmin expression in rat brain. J Cell Physiol 204(2):623–631.

Chen Y, Qian ZM, Du J, Duan X, Chang Y, Wang Q, Wang C, Ma YM, Xu Y, Li L, Ke Y. 2005. Iron loading inhibits ferroportin1 expression in PC12 cells. Neurochem Int 47(7):507–513.

Delaby C, Pilard N, Goncalves AS, Beaumont C, Canonne-Hergaux F. 2005. Presence of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and down-regulated by hepcidin. Blood 106(12):3979–3984.

Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, Law TC, Brugnara C, Lux SE, Pinkus GS, Pinkus JL, Kingsley PD, Palis J, Fleming MD, Andrews NC, Zon LI. 2000. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature 403 (6771):776–781.

Fleming MD, Trenor CC, Su MA, Foernzler D, Beier DR, Dietrich WF, Andrews NC. 1997. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nat Genet 16:383–386.

Frazer DM, Anderson GJ. 2003. The orchestration of body iron intake: How and where do enterocytes receive their cues? Blood Cells Mol Dis 30:288–297.

Gruenheid S, Canonne-Hergaux F, Gauthier S, Hackam DJ, Grinstein S, Gros P. 1999. The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. J Exp Med 189(5):831–841.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA. 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388(6641): 482–488.

Gunshin H, Allerson CR, Polycarpou-Schwarz M, Rofts A, Rogers JT, Kishi F, Hentze MW, Rouault TA, Andrews NC, Hediger MA. 2001. Iron-dependent regulation of the divalent metal ion transporter. FEBS Lett 509(2):309–316.

Han O, Wessling-Resnick M. 2002. Copper repletion enhances apical iron uptake and transepithelial iron transport by Caco-2 cells. Am J Physiol Gastrointest Liver Physiol 282(3):G527–533.

Hubert N, Hentze MW. 2002. Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: Implications for regulation and cellular function. Proc Natl Acad Sci USA 99:12345–12350.

Jabado N, Canonne-Hergaux F, Gruenheid S, Picard V, Gros P. 2002. Iron transporter Nramp2/DMT-1 is associated with the membrane of phagosomes in macrophages and Sertoli cells. Blood 100(7):2617–2622.

Ke Y, Chen YY, Chang YZ, Duan XL, Ho KP, Jiang de H, Wang K, Qian ZM. 2003. Post-transcriptional expression of DMT1 in the heart of rat. J Cell Physiol 196(1):124–130.

Ke Y, Chang YZ, Duan XL, Du JR, Zhu L, Wang K, Yang XD, Ho KP, Qian ZM. 2005. Age-dependent and iron-independent expression of two mRNA isoforms of divalent metal transporter 1 in rat brain. Neurobiol Aging 26(5):739–748.

Knutson MD, Vafa MR, Haile DJ, Wessling-Resnick M. 2003. Iron loading and erythrophagocytosis increase ferroportin 1 (FPN1) expression in J774 macrophages. Blood 102(12):4191–4197.

Knutson MD, Oukka M, Koss LM, Aydemir F, Wessling-Resnick M. 2005. Iron release from macrophages after erythrophagocytosis is up-regulated by ferroportin 1 overexpression and down-regulated by hepcidin. Proc Natl Acad Sci USA 102(5):1324–1328.

Krantz SB. 1991. Erythropoietin. Blood 77(3):419–434. Review.

Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K. 2000. LEAP-1, a novel highly disulfide-bonded human peptide,

exhibits antimicrobial activity. FEBS Lett 480(2–3): 147–150.

Lee PL, Gelbart T, West C, Halloran C, Beutler E. 1998. The human Nramp2 gene: Characterization of the gene structure, alternative splicing, promoter region and polymorphisms. Blood Cells Mol Dis 24:199–215.

Liao RX, Sun JG, Zhong XL, Zhou JX, Wang YZ. 2006. Experimental study on transcription regulation of mouse hepcidin gene by NF-kB. Zhonghua Gan Zang Bing Za Zhi 14(2):118–123.

Liu YQ, Duan XL, Chang YZ, Wang HT, Qian ZM. 2006. Molecular analysis of increased iron status in moderately exercised rats. Mol Cell Biochem 282(1–2):117–123.

McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW, Simpson RJ. 2000. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 5(2):299–309.

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. 2004. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306(5704): 2090–2093.

Nguyen NB, Callaghan KD, Ghio AJ, Haile DJ, Yang F. 2006. Hepcidin expression and iron transport in alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 291(3):L417–L425.

Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A, Vaulont S. 2001. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proc Natl Acad Sci USA 98(15):8780–8785.

Nicolas G, Viatte L, Bennoun M, Beaumont C, Kahn A, Vaulont S. 2002. Hepcidin, a new iron regulatory peptide. Blood Cells Mol Dis 29(3):327–335.

Pantopoulos K. 2004. Iron metabolism and the IRE/IRP regulatory system: An update. Ann N Y Acad Sci 1012: 1–13.

Park CH, Valore EV, Waring AJ, Ganz T. 2001. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276(11):7806–7810.

Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O. 2001. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 276(11):7811–7819.

Priwitzerova M, Nie G, Sheftel AD, Pospisilova D, Divoky V, Ponka P. 2005. Functional consequences of the human DMT1 (SLC11A2) mutation on protein expression and iron uptake. Blood 106(12):3985–3987.

Qian ZM, Xiao DS, Tang PL, Yao FYD, Liao QK. 1999. The increased expression of transferrin receptor on the membrane of erythroblast in strenuous exercised rats. J Appl Physiol 87:523–529.

Qian ZM, Xiao DS, Ke Y, Liao QK. 2001. Increased nitric oxide is one of the causes of changes of iron metabolism in strenuously exercised rats. Am J Physiol 280:R739–R743.

Rivera S, Nemeth E, Gabayan V, Lopez MA, Farshidi D, Ganz T. 2005. Synthetic hepcidin causes rapid dose-dependent hypoferremia and is concentrated in ferroportin-containing organs. Blood 106(6):2196–2199.

Scheiber-Mojdehkar B, Sturm B, Plank L, Kryzer I, Goldenberg H. 2003. Influence of parenteral iron preparations on non-transferrin bound iron uptake, the iron regulatory protein and the expression of ferritin and the divalent metal transporter DMT-1 in HepG2 human hepatoma cells. Biochem Pharmacol 65:1973–1978.

Tandy S, Williams M, Leggett A, Lopez-Jimenez M, Dedes M, Ramesh B, Srai SK, Sharp P. 2000. Nramp2 expression is associated with pH-dependent iron uptake across the apical membrane of human intestinal Caco-2 cells. J Biol Chem 275(2):1023–1029.

Tchernitchko D, Bourgeois M, Martin ME, Beaumont C. 2002. Expression of the two mRNA isoforms of the iron transporter Nramp2/DMTI in mice and function of the iron responsive element. Biochem J 363(Pt 3):449–455.

Thomas C, Oates PS. 2002. IEC-6 cells are an appropriate model of intestinal iron absorption in rats. J Nutr 132(4): 680–687.

Trinder D, Oates PS, Thomas C, Sadleir J, Morgan EH. 2000. Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. Gut 46:270–276.

Van Zant G, Chen BD. 1983. Erythropoietin causes down regulation of colony-stimulating factor (CSF-1) receptors on peritoneal exudate macrophages of the mouse. J Cell Biol 97(6):1945–1949.

Viatte L, Lesbordes-Brion JC, Lou DQ, Bennoun M, Nicolas G, Kahn A, Canonne-Hergaux F, Vaulont S. 2005. Deregulation of proteins involved in iron metabolism in hepcidin-deficient mice. Blood 105(12):4861–4864.

Vokurka M, Krijt J, Sulc K, Necas E. 2006. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. Physiol Res 55(6):667–674.

Wardrop SL, Richardson DR. 2000. Interferon-gamma and lipopolysaccharide regulate the expression of Nramp2 and increase the uptake of iron from low relative molecular mass complexes by macrophages. Eur J Biochem 267(22):6586–6593.

Weiss G, Houston T, Kastner S, Johrer K, Grunewald K, Brock JH. 1997. Regulation of cellular iron metabolism by erythropoietin: Activation of iron-regulatory protein and upregulation of transferrin receptor expression in erythroid cells. Blood 89(2):680–687.

Yamaji S, Sharp P, Ramesh B, Srai SK. 2004. Inhibition of iron transport across human intestinal epithelial cells by hepcidin. Blood 104(7):2178–2180.

Yang F, Wang X, Haile DJ, Piantadosi CA, Ghio AJ. 2002. Iron increases expression of iron-export protein MTP1 in lung cells. Am J Physiol Lung Cell Mol Physiol 283: L932–L939.