

## Hepcidin suppression and defective iron recycling account for dysregulation of iron homeostasis in heme oxygenase-1 deficiency

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### Abstract

Heme oxygenase-1 (HO-1) contribution to iron homeostasis has been postulated, because it facilitates iron recycling by liberating iron mostly from heme catabolism. This enzyme also appears to be responsible for the resolution of inflammatory conditions. In a patient with HO-1 deficiency, inflammation and dysregulation of body iron homeostasis, including anemia and liver and kidney hemosiderosis, are evidenced. Here we postulated that HO-1 is critical in the regulation of ferroportin, the major cellular iron exporter, and hepcidin, the key regulator of iron homeostasis central in the pathogenesis of anemia of inflammation. Our current experiments in human THP-1 monocytic cells indicate a HO-1-induced iron-mediated surface-ferroportin expression, consistent with the role of HO-1 in iron recycling. Surprisingly, we observed low hepcidin levels in the HO-1-deficient patient, despite the presence of inflammation and hemosiderosis, both inducers of hepcidin. Instead, we observed highly increased soluble transferrin receptor levels. This suggests that the decreased hepcidin levels in HO-1 deficiency reflect the increased need for iron in the bone marrow due to the anaemia. Using human hepatoma cells, we demonstrate that HO-activity did not have a direct modulating effect on expression of *HAMP*, the gene that encodes for hepcidin. Therefore, we argue that the decreased iron recycling may, in part, have contributed to the low hepcidin levels. These findings indicate that dysregulation of iron homeostasis in HO-1 deficiency is the result of both defective iron recycling and erythroid activity-associated inhibition of hepcidin expression. This study therefore shows a crucial role for HO-1 in maintaining body iron balance.

**Keywords:** heme oxygenase-1 • inflammation • iron • hepcidin and hepaidin • ferroportin

### Introduction

Heme oxygenase (HO) catalyses the break-down of haem into carbon monoxide (CO), free iron, and biliverdin that is rapidly converted into bilirubin by biliverdin reductase. To date, two functional isoforms of HO have been described in human beings [1]. HO-1 is inducible by diverse stimuli, including mediators of oxidative

stress and inflammation, whereas HO-2 is thought to be mainly constitutively expressed [2]. HO-1 is important as part of the defense mechanism against oxidative stress and inflammation. Moreover, because HO-1 is largely responsible for iron recycling by liberating iron mostly from haem catabolism after phagocytosis of senescent erythrocytes by tissue macrophages, its contribution to iron homeostasis has been postulated.

Recently, hepcidin, a peptide mostly produced in hepatocytes that is encoded by the *HAMP* gene in human beings, has been demonstrated as a key regulator of body iron homeostasis [3]. This peptide inhibits iron efflux from enterocytes and macrophages by inactivating the major cellular iron exporter, ferroportin [4, 5]. Hepcidin action may explain several genetic causes of iron overload syndrome [6]. Additionally, hepcidin levels are

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**Table 1** Comparison of some conditions between HO-1-deficient mice and man

	HO-1-deficient mice [13]	HO-1-deficient patient [11, 12]
Anemia	+	+
Fragmentation	?	+
RBC morphology	Microcytic, anisocytosis	Microcytic, anisocytosis
Blood transfusion	–	Regularly
Leukocytosis	+	+
Ferritin	Elevated	Elevated
Serum iron	Low	Low normal
Transferrin saturation	Low	Low
Hemosiderosis	Hepatic parenchymal and Kupffer cells, renal proximal tubules	Hepatic parenchymal and Kupffer cells, renal proximal tubules
Hepatomegaly	+	+
Splenomegaly	Enlarged spleen	Asplenia
Glomerulonephritis	+	+
Chronic inflammation	Progressive	Steady, receiving anti-inflammatory medications

HO-1, heme oxygenase-1; RBC, red blood cells.

elevated during inflammation [3]. This acts on the one hand as a crucial causative factor in pathogenesis of anaemia of inflammation, by retention of heme-derived iron in the iron storage sites, thereby withholding it from the erythroid compartment [7–9]. On the other hand, hepcidin suppression is observed in anemias, necessary to increase iron supply needed for the accelerated erythropoietic activity [10].

It has been reported that HO-1 deficiency in human exhibits both signs of inflammation and dysregulation of body iron homeostasis, such as iron accumulation in the kidneys and the liver (Table 1) [11, 12]. Similarly, *Ho-1* knock-out mice (*Ho-1* KO mice) developed severe inflammation and anemia associated with low transferrin iron, and also hepatic and renal iron accumulation (Table 1) [13]. Therefore, it has been proposed that HO-1 facilitates iron efflux from hepatic and renal cells. Another *in vitro* study suggested a function for HO-1 in regulating cellular iron by inducing iron efflux [14]. Indeed, in 2000, the same group proposed the presence of an iron ATPase transporter that in association with HO-1 acts as an iron exporter [15].

In the present study, we hypothesized that dysregulation of iron homeostasis present in HO-1 deficiency was mediated by modulation of hepcidin expression. We therefore investigated HO-1 cross-talk with hepcidin and ferroportin, in order to delineate the

mechanistic basis of body iron regulation, and furthermore to help explain the dysregulation of iron homeostasis in HO-1 deficiency. Direct links between HO-1 and *HAMP* expression were assessed in human hepatoma cell lines, whereas effects of HO-1 on ferroportin expression were examined in human THP-1 monocytic cells. Furthermore, we evaluated these results with the measured levels of serum hepcidin, inflammatory markers, hematological measurements and iron parameters, in blood or serum samples of the HO-1-deficient patient.

## Materials, methods and patient

### Cell culture

Both human hepatoma cell lines, HepG2 and Hep3B, were cultured in a humidified 37°C incubator with 5% CO<sub>2</sub> using PC-1 medium (Cambrex, Walkersville, MD, USA). Human THP-1 monocytic cells were cultured in RPMI1640 (Gibco, Breda, The Netherlands) supplemented with 2 mmol/L L-glutamine and 10% heat-inactivated foetal calf serum (Gibco).

### Viability test

Cell viability after various treatments was monitored using a standard trypan blue exclusion test. In all experiments, both hepatoma and THP-1 cells were >95% viable after indicated treatments.

### Quantitative polymerase chain reaction (Q-PCR)

Confluent hepatoma cells were treated for 6 hrs with different agents, including tin mesoporphyrin (SnMP, Frontier Scientific, UT, USA), cobalt protoporphyrin (CoPP, Frontier Scientific), hemin (Frontier Scientific) as the source of heme, and interleukin-6 (IL-6, R&D systems, Minneapolis, MN, USA). Two mmol/L stock solutions of SnMP, CoPP and hemin were freshly prepared as previously described [16]. Isolation of total RNA and subsequent cDNA synthesis were performed [17], followed by Q-PCR of the following human cDNAs: *HAMP*, *HO-1* and, for normalization of expression, the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT*). Q-PCR was performed in a 25- $\mu$ l total reaction, using TaqMan Universal Master Mix (PE Applied Biosystems, Foster City, CA, USA). TaqMan enzyme was activated for 10 min. at 95°C before 40 two-step cycles were performed (30 sec., 95°C; 1 min., 60°C) in a ABI Prism 7700 (PE Applied Biosystems). All primers and probes were purchased from PE Applied Biosystems (*HAMP*, assay no. Hs00221783\_m1; *HO-1*, assay no. Hs00157965\_m1; *HPRT*, assay no. 4310890E). Relative expression of the specific target gene in comparison to the reference housekeeping gene was calculated based on a mathematical model as previously described [18].

### Flow cytometry

After treatment with *HO-1* inducers, HO-activity inhibitor, Fe(III)ammonium-citrate (Sigma, MO, USA) or deferoxamine (DF, Novartis, The Netherlands), THP-1 cells were fixed using phosphate-buffered saline (PBS, Gibco) containing 4% paraformaldehyde (Sigma) for 5 min. Blocking of Fc

receptors was carried out by incubating the cells in PBS containing 20% normal human serum (NHS) for 30 min. at 4°C. Incubation with 1:25 ferroportin antibody (a generous gift from Dr. A.T. Mckie and Dr. G.O. Latundedada, King's College, London, UK), 1:50 HO-1 antibody (SPA895, Stressgen Sanbio, Uden, The Netherlands) or 5 µg/ml rabbit IgG isotype control (Rockland Immunochemicals, Gilbertsville, PA, USA) was done for 3 hrs in PBS containing 10% NHS at 4°C. After washing, labelling with 5 µg/ml Alexa-fluor-488-labelled secondary antibody (Molecular Probes, Eugene, OR, USA) was performed for 30 min. in PBS containing 10% NHS at 4°C. For HO-1 labelling, 0.05% tween-20 (Sigma) was included during incubation for permeabilization. Each flow cytometric measurement was performed using a Becton Dickinson FACScan (Oxnard, CA, USA) and  $1 \times 10^4$  cells were analysed.

## HO-activity assay

After treatment as described in the previous section,  $1 \times 10^5$  cells were resuspended in 89.9 µl 0.1 mol/l phosphate buffer, 2 mmol/l MgCl<sub>2</sub> (pH 7.4) and subsequently lysed by three freeze-thaw cycles. HO-activity was measured as described previously [55]. In brief: to the cell lysate, 10.1 µl master mix was added that consisted of 2.5 µl 80 mmol/l glucose-6-phosphate, 0.1 µl 0.5 U/µl glucose-6-phosphate dehydrogenase in 5 mmol/l glycine (both Sigma-Aldrich), 5 µl 32 mmol/l NADPH tetrasodium salt (Roche, Basel, Switzerland) and 2.5 µl 2 mmol/l hemin. The samples were incubated for 60 min. at 37°C in the dark. Next, 100 µl methanol was added and the samples were centrifuged at  $13,000 \times g$ . A 50-µl aliquot of this sample was run on a HPLC (Spectrasystem SCM400, Spectra-Physics Analytical, Minneapolis, MN, USA) equipped with a 5 µm discovery C18 column and a discovery C18 super guard cartridge 5 µm particle size precolumn (both Sigma-Aldrich). Separation was done by performing a gradient of 80% solvent A (40% 100 mmol/l ammoniumacetate pH 5.5, 5% methoxy ethanol and 50% methanol (all Sigma-Aldrich) to 20% solvent B (5% methoxy ethanol and 95% methanol) over a 20-min. time interval at a flow rate of 1 ml/min. Biliverdin and bilirubin were detected at a wavelength of 377 and 450 nm, respectively. Standards of biliverdin and bilirubin (both Frontier Scientific) were also run in order to quantify the amount of biliverdin/bilirubin found in the samples.

## Fluorescence microscopy

After treatment, THP-1 cells were spun at  $500 \times g$  for 10 min. in 100 µl PBS onto a slide, air dried and fixed in acetone. Incubation with 1:25 ferroportin antibody was done for 1 hr in PBS containing 10% NHS at room temperature. After careful washing, labelling with 5 µg/ml Alexa-fluor-488-labelled secondary antibody was performed for 30 min. in PBS containing 10% NHS at room temperature. The slide was covered with fluorescence-preserving mounting media (Invitrogen Lifesciences, Breda, The Netherlands) and a glass cover slip, and further visualized using a Leica fluorescence microscope (Leica Microsystems, GmbH, Heidelberg, Germany).

## Calcein assay for measurement of intracellular labile iron

A calcein assay was used to monitor intracellular labile iron accumulation as described elsewhere [19, 20]. In this assay, after treatment and washing, hepatoma or THP-1 cells were incubated with 0.1 µmol/l calcein-AM (Molecular Probes, OR, USA) for 30 min. at 37°C, in the presence of

5 µmol/l PSC-833, a P-glycoprotein inhibitor (Novartis), to avoid efflux of calcein-AM [21]. The cells were then washed twice to remove the remaining extracellular calcein-AM. The fluorescence signals of calcein (excitation 485 nm, emission 530 nm) were read in the Cytofluor II microplate reader (Perspective Biosystems, Framingham, MA, USA). Fractional decreases in fluorescence intensity correlates with intracellular labile iron level [19, 20]. Fe(III)ammoniumcitrate was used as a positive control to illustrate sensitivity of the assay.

## HO-1-deficient patient and laboratory measurements

A patient with no functional *HO-1* expression because of a two-nucleotide deletion within exon3 and a complete loss of exon2 of the *HO-1* genes has been described previously [11, 12]. Informed consent was obtained and the study was approved by the Ethics Committee of Kanazawa University [11, 12]. The sera of the patient taken at two different time points exhibited low transferrin saturation, but elevated levels of ferritin and heme (Table 2). Several laboratory parameters of the sera, as shown in Table 2, were measured in previous studies [11, 12], and remeasured in the present study. The two samples were taken 5 months apart when the patient was 6 years old. The boy died several months after the last blood sample was taken. Both samples were stored at  $-80^\circ\text{C}$ . Remeasurement of laboratory parameters of the stored samples in the present study was carried out using routine laboratory methods [8] (Department of Clinical Chemistry, Radboud University Nijmegen Medical Centre, The Netherlands). Serum hepcidin was measured using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) as described elsewhere [22]. As reference values, hepcidin levels were measured in sera of healthy volunteers that had been stored at  $-80^\circ\text{C}$  for a similar period of time as the patient sera, *i.e.* 8 years. Soluble transferrin receptor (sTfR) was measured using the Dade Behring sTfR assay (Marburg, Germany). Ferric iron deposition has been reported in the renal and hepatic tissues, following a standard Prussian-blue staining method (Table 1) [11, 12]. The patient also suffered from persistent haemolytic anaemia, characterized by erythrocyte fragmentation and elevated LDH and aspartate aminotransferase (AST) levels in the presence of only slightly elevated alanine aminotransferase (ALT) levels (Table 2). The patient was asplenic and required regular transfusions of erythrocytes (Table 1).

## Statistical analysis

Results are expressed as means  $\pm$  standard errors of the means (S.E.). Differences in quantitative measures were tested for significance using one-way ANOVA. All statistics were performed using the GraphPad Prism 4 statistical program (San Diego, CA, USA).

## Results

### HO-activity increases intracellular labile iron levels in THP-1 cells

Previous studies [13–15] have suggested a role for HO-1 in maintaining intracellular iron levels by promoting iron efflux. This may imply a central role for HO-1 in maintaining intracellular iron homeostasis.

**Table 2** Hematological, iron and inflammatory parameters of the HO-1-deficient patient

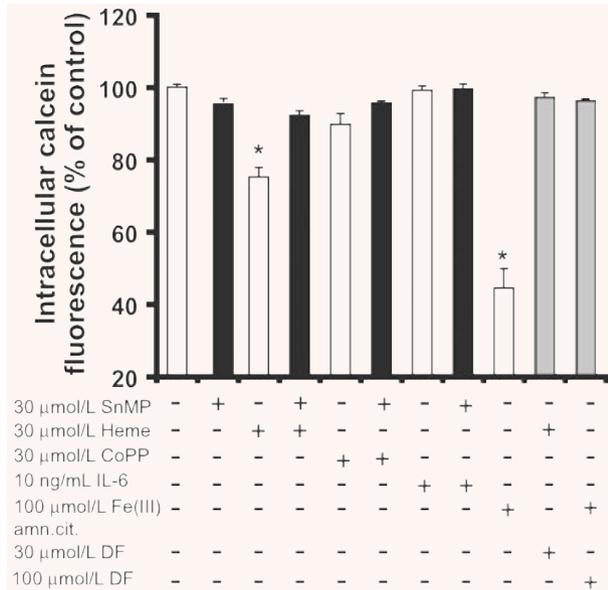
	First blood sampling	Second blood sampling	Reference values
Hematological parameters			
WBC (/ $\mu$ l)	27,300	19,300	3300–8800
RBC ( $\times 10^4$ / $\mu$ l)	221	195	430–550
Hb (g/dl)	7.1	5.6	13.5–17.0
Hct (%)	19.1	15.3	39.7–51.0
MCV (fL)	86.4	78.5	83–102
MCHC (%)	37.2	36.6	32–36
Iron parameters			
Ferritin (ng/ml)	965 (2146)	595 (2065)	6–280 (26–360)
TIBC ( $\mu$ mol/l)	98 (94)	76 (82)	48–71 (45–75)
Serum iron ( $\mu$ mol/l)	7.5 (12)	12 (10)	8.9–30 (7–25)
Transferrin saturation (%)	(12.8)	(12.2)	(17–42)
Transferrin (mg/dl)	424	n.d.	214–390
Serum heme ( $\mu$ mol/l)	> 400	> 400	<1
Inflammatory parameters and organ injury			
CRP (mg/l)	12.4 (17)	6.1(9)	<0.5 (<10)
IL-6 (pg/ml)	(20)	(28)	(<5)
Creatinine ( $\mu$ mol/l)	(97)	(74)	(44–71)
ALT (IU/l)	40 (49)	40 (54)	2–39 (10–25)
AST (IU/l)	408	369	15–50
LDH (IU/l)	18,010	19,620	196–355
TP (g/dl)	10.1	9.1	6.6–8.1
Haptoglobin (mg/dl)	878	n.d.	30–252
Serum hepcidin (MInt/l)	(<0.1)	(<0.1)	(0.6–9.9) <sup>‡</sup> , (1.02–6.26) <sup>*</sup>
sTfR (mg/l)	(19.4)	(34.1)	(0.76 – 1.76)

Laboratory parameters were measured in previous studies [11, 12] and the present study. The presented values measured in the present study are in brackets. Reference values were based on the laboratory standards for males age 4–9 years old (Department of Clinical Chemistry, Radboud University Nijmegen Medical Centre, The Netherlands) or previous studies [11, 12]. <sup>‡</sup>Based on a range of values obtained from 20 fresh sera of normal controls [22]. <sup>\*</sup>Based on values obtained from 6- to 8-year stored sera of normal controls (Mint/l). HO-1, heme oxygenase-1; WBC, white blood cells; RBC, red blood cells; Hb, haemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; TIBC, total iron-binding capacity; CRP, C-reactive protein; IL-6, interleukin-6; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; TP, total protein; sTfR, soluble transferrin receptor; IU, international unit; Mint/l, Mega intensity/litre; <sup>\*</sup>as previously described [22]; n.d., not determined.

Here, we analysed intracellular labile iron pool in THP-1 cells after induction or inhibition of HO-activity, utilizing a calcein assay.

In THP-1 cells, 24 hrs treatment with Fe(III)ammonium-citrate quenched the calcein signal (Fig. 1). The quenched

signal was returned to basal levels when DF, the specific iron chelator, was added. This result indicates that iron-rich conditions promote intracellular labile iron accumulation. In these cells, 24-hr induction of HO-1 by heme also promoted calcein quenching



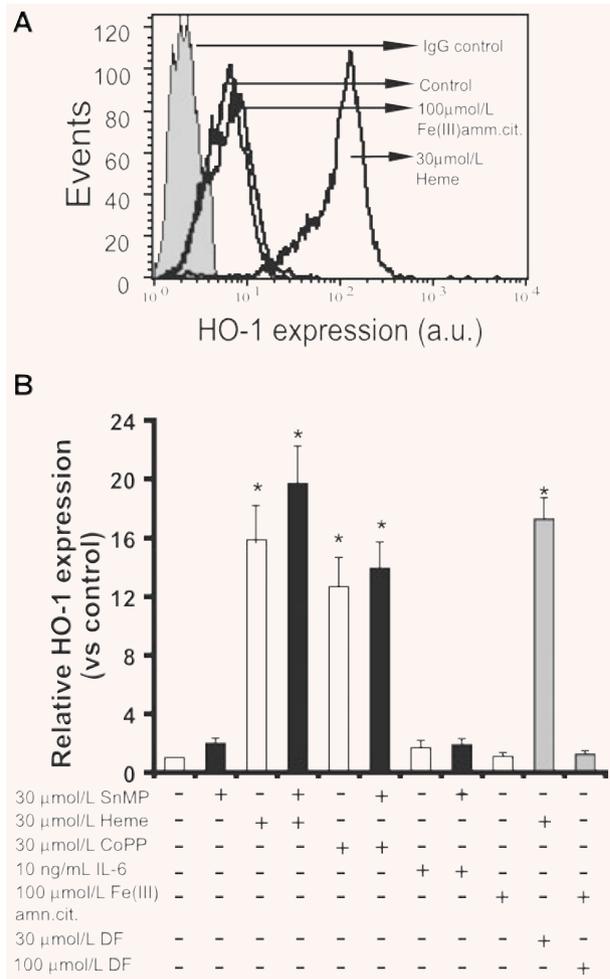
**Fig. 1** Detection of intracellular labile iron in THP-1 cells. Calcein assay was performed in THP-1 cells after indicated treatments for 24 hrs. Quenching of calcein fluorescence indicates increased intracellular labile iron. Each measurement is the mean value ± S.E., *n* = 3, \* lower than the corresponding controls (*P* < 0.05).

(Fig. 1). This effect could be counteracted using SnMP, the specific HO-activity inhibitor. Thus, this result indicates that iron derived from intracellular heme degradation by HO-activity contributes to the increased levels of intracellular labile iron. However, the specific HO-1 inducer CoPP [23] did not promote accumulation of intracellular labile iron in THP-1 cells. This is likely due to a shortage of heme as the substrate of HO in the cells after 24 hrs of CoPP incubation [24, 25]. IL-6 treatment moreover did not affect the levels of intracellular labile iron. Taken together, our data demonstrate that increased HO-activity, in the presence of sufficient heme substrate, promotes accumulation of intracellular labile iron. Thus although HO-1 promotes cellular iron efflux [13–15], the level of intracellular labile iron is still increased.

The calcein assay was carried out in the presence or absence of PSC-833, an inhibitor of the calcein-AM transporter, P-glycoprotein, to avoid efflux of calcein-AM [21]. However, no differences in calcein quenching levels were observed when the results were compared with the assays performed without PSC-833 (data not shown).

### Ferroportin induction by intracellular labile iron in human macrophage/monocyte-like cells

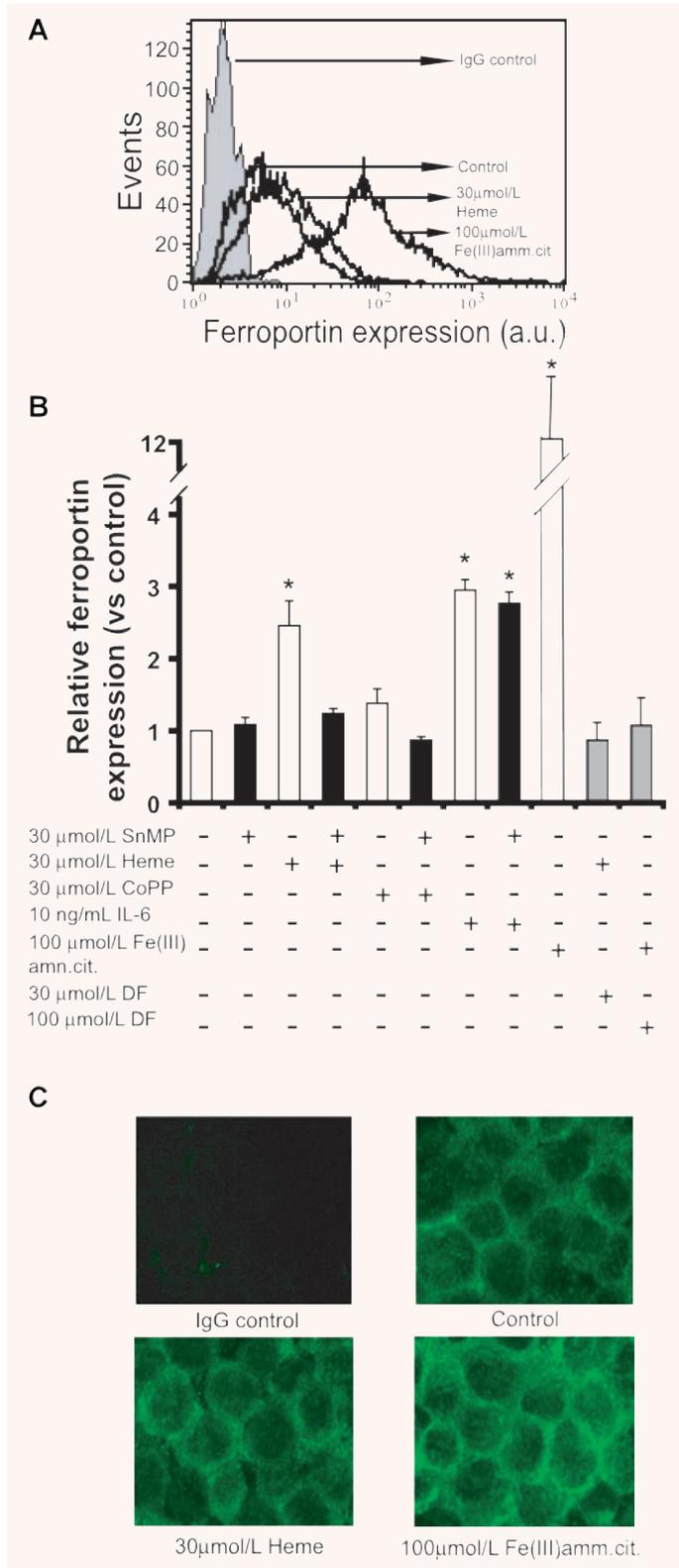
To examine whether HO-1 could directly regulate the iron exporter ferroportin, expression of ferroportin in THP-1 cells treated with



**Fig. 2** Detection of HO-1 protein expression in THP-1 cells. HO-1 expression was analysed by FACS analysis in THP-1 cells after indicated treatments for 24 hrs. Prior to FACS analysis, cells were permeabilized and stained with anti-HO-1 IgG antibody, followed by the labelled secondary antibody. (A) The histogram illustrates several read-outs of FACS analysis, y-axis = events or cell numbers, x-axis = protein expression based on fluorescence intensity, a.u. = arbitrary units. IgG control = cells stained with IgG isotype control. (B) shows HO-1 expression levels in THP-1 cells after indicated treatments. Each FACS measurement is the mean value ± S.E., *n* = 3–4, \* lower than the corresponding controls (*P* < 0.05).

HO-activity modifying agents was analysed. This study was also aimed to examine direct involvement of HO-1 in cellular iron efflux as has been postulated [13–15].

Increased HO-1 synthesis in THP-1 by heme (Fig. 2A and B) correlated with elevated expression of surface ferroportin (Fig. 3A–C). Here, an incubation period of 24 hrs was chosen to allow stable surface expression of ferroportin on THP-1 cells. Addition of the specific HO-activity inhibitor SnMP [16, 26] did not affect heme-induced HO-1 expression (Fig. 2B), however, it counteracted



**Fig. 3** Detection of ferroportin protein expression on THP-1 cells. Ferroportin expression was analysed by FACS analysis on THP-1 cells after indicated treatments for 24 hrs. Prior to FACS analysis, cells were fluorescently stained with anti-ferroportin antibody, followed by the labelled secondary antibody. **(A)** The histogram illustrates several read-outs of FACS analysis, y-axis = events or cell numbers, x-axis = protein expression based on fluorescence intensity, a.u. = arbitrary units. IgG control = cells stained with IgG isotype control. **(B)** shows ferroportin expression levels in THP-1 cells after indicated treatments. Each FACS measurement is the mean value  $\pm$  S.E.,  $n = 3-4$ , lower than the corresponding controls ( $P < 0.05$ ). **(C)** After 24 hrs of specified treatments, THP-1 cells were stained using anti-ferroportin antibody, followed by the labelled secondary antibody. Expression of surface ferroportin in green was visualized using fluorescence microscopy. Enhanced green colour indicates increased ferroportin expression. The images are representative of two independent experiments.

HO-induced ferroportin expression (Fig. 3B), confirming that HO-activity modulates surface ferroportin expression.

THP-1 incubation with CoPP for 24 hrs, however, although promoting HO-1 expression (Fig. 2B), did not stimulate ferroportin expression (Fig. 3B). This is consistent with the absence of intracellular iron accumulation after CoPP stimulation (Fig. 1). Thus, increased intracellular labile iron levels are necessary for induction of ferroportin expression. This was supported by our observation that iron chelator DF was able to counteract both heme-induced and iron-induced ferroportin expression (Fig. 3B). DF appeared to also counteract the increased levels of intracellular labile iron (Fig. 1).

In addition, we observed that ferroportin expression was induced by IL-6 (Fig. 3B). Such induction of ferroportin by a proinflammatory cytokine has been previously reported [27]. This induction however is likely independent of HO-activity because SnMP did not counteract the effect of IL-6.

Taken together, the results indicate that surface ferroportin expression is induced by intracellular labile iron. These data therefore suggest that the previously described HO-1-induced iron efflux [13–15] is likely mediated by a heme-derived iron-induced ferroportin expression.

### **Despite chronic inflammation and tissue iron loading in the HO-1-deficient patient there is lack of hepcidin synthesis**

Because HO-1 deficiency results in tissue iron loading (Table 1) and chronic inflammation shown by enhanced levels of IL-6, CRP and haptoglobin (Table 2), we questioned whether HO-1 modulates the synthesis of liver hepcidin. We measured hepcidin levels using SELDI-TOF-MS in serum samples of the HO-1-deficient patient and from six healthy volunteers that were stored in  $-80^{\circ}\text{C}$  for a similar time period and thus served as reference values (Table 2). Hepcidin levels from two serum samples of the patient from different periods of collection were measured to monitor the consistency of the values. Because of the presence of pathological chronic inflammation and tissue iron loading conditions, together with low transferrin saturation, but highly elevated ferritin levels (Table 2), hepcidin levels in the sera were expected to be increased. However, on the contrary, we found that hepcidin levels, in the two serum samples of the HO-1-deficient patient were very low (both values were  $<0.1$  Mint/l, whereas normal range of fresh sera =  $0.6$ – $9.9$  Mint/l [22], and normal range of stored sera =  $1.02$ – $6.26$  Mint/l, Table 2). These measured low levels of hepcidin in the stored patient sera are likely reflecting actual levels because (1) based on our methodology of hepcidin measurement, serum hepcidin preserves its stability even after several freeze-thaw cycles during storage [22], (2) similarly, stored samples from healthy volunteers showed significantly higher levels of hepcidin, (3) additionally, values of both iron and serum parameters that had been measured previously in the fresh samples of the HO-1 patient [11] were similar as measured in the present study (Table 2), indicating that the stored serum samples were well preserved, and

(4) moreover, both patient serum samples collected at different sampling points consistently showed low hepcidin levels. Together, these observations provide a strong argument that the HO-1 deficiency led to decreased hepcidin synthesis. The ferritin values however were higher in the current measurements compared to the previous ones [11], which is likely due to different equipments used in the two laboratories, as well as to the worldwide variability and a lack of standardization in ferritin assays. Thus despite increased iron stores and inflammation, the patient has very low hepcidin levels.

### **HO-1-activity was inducible in human hepatoma cells, and promoted intracellular labile iron accumulation**

Because HO-1 deficiency exhibited hepcidin suppression, we questioned whether HO-1 could directly modulate *HAMP* expression in liver cells. We first tested whether HO-1-activity could be induced in human hepatoma cells, and promote intracellular labile iron accumulation.

In HepG2 cells, treatment with  $10\ \mu\text{M}$  heme for 6 hrs promoted substantial induction of HO-activity when compared to basal activity levels, whereas exposure to  $10\ \mu\text{M}$  SnMP, completely abrogated HO-activity in these cells (data not shown). This confirms that in our *in vitro* systems, heme activated while SnMP inhibited HO-activity, as previously reported [16, 26].

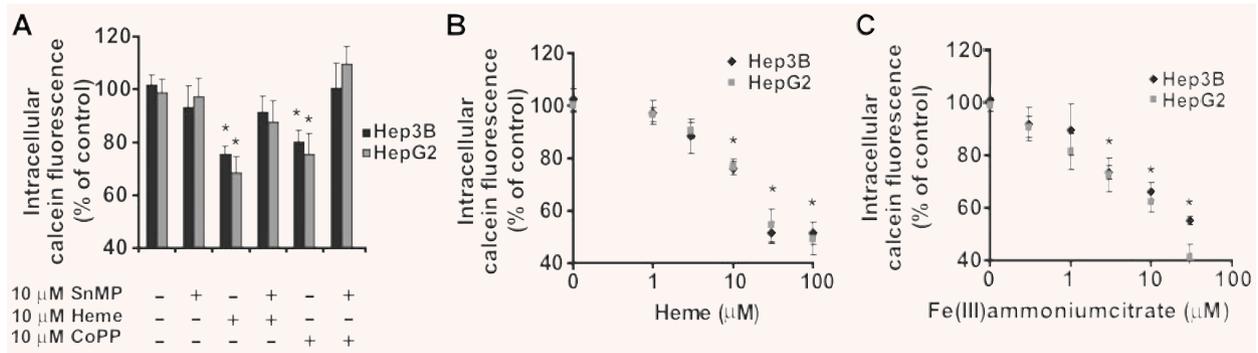
In HepG2 and Hep3B cells (Fig. 4A), after 6 hrs, induction of HO-1 with heme or CoPP quenched the calcein signal, indicating accumulation of intracellular labile iron. The quenched calcein signal could be returned to basal levels when SnMP was added, demonstrating that HO-activity in human hepatoma cell lines also promotes intracellular iron accumulation. Cells treated with either increasing concentrations of heme (Fig. 4B) or Fe(III) ammonium-citrate (Fig. 4C) also showed increased levels of intracellular labile iron. In agreement with previous observations using THP-1 cells, our data indicate that HO-1-activity promotes labile iron accumulation in the cells.

### **HO-1 does not modulate *HAMP* expression in human hepatoma cells**

We next examined whether HO-1 could directly down-modulate *HAMP* expression in liver cells. To this end, we incubated HepG2 and Hep3B cells with HO-1 modulating agents, and measured *HAMP* transcript levels.

Incubation with the specific HO-activity inhibitor [16, 26], SnMP, for 6 hrs modulated neither the amount of *HO-1* nor *HAMP* transcripts (Table 3). Likewise, prolonged incubation (*i.e.* 24 and 48 hrs, data not shown) did not affect transcript levels of both genes.

CoPP increased *HO-1* transcripts in both cell lines, however *HAMP* expression was not changed. Moreover, inhibition of HO-activity using SnMP in the presence of CoPP did not alter *HAMP*



**Fig. 4** Detection of intracellular labile iron in human hepatoma cells, HepG2 and Hep3B. (A–C) show relative levels of intracellular iron after 6 hrs of indicated treatments. Quenching of calcein fluorescence indicates increased intracellular labile iron in the cells. Each measurement is the mean value  $\pm$  S.E.,  $n = 3-4$ , \*lower than the corresponding controls ( $P < 0.05$ ).

**Table 3** Transcript levels of *HAMP* and *HO-1* in human hepatoma cells after 6 hrs of specified treatments

Treatment	Relative <i>HAMP</i> expression		Relative <i>HO-1</i> expression	
	Hep3B	HepG2	Hep3B	HepG2
Control	1	1	1	1
10 μmol/l SnMP	1.2 $\pm$ 0.2	1.1 $\pm$ 0.2	1.7 $\pm$ 0.2	1.3 $\pm$ 0.3
20 μmol/l SnMP	0.9 $\pm$ 0.3	1.8 $\pm$ 0.5	1.6 $\pm$ 0.8	1.9 $\pm$ 0.2
10 μmol/l CoPP	0.8 $\pm$ 0.2	1.2 $\pm$ 0.4	65 $\pm$ 24*	62 $\pm$ 15*
10 μmol/l CoPP + 10 μmol/l SnMP	0.7 $\pm$ 0.2	0.8 $\pm$ 0.3	62 $\pm$ 13*	74 $\pm$ 14*
10 ng/ml IL-6	72 $\pm$ 21*	22 $\pm$ 7.2*	3.4 $\pm$ 0.9	5.4 $\pm$ 2.1*
10 ng/ml IL-6 + 10 μmol/l SnMP	65 $\pm$ 11*	20.4 $\pm$ 5.9*	2.2 $\pm$ 1.1	5.2 $\pm$ 1.1*
10 μmol/l heme	0.4 $\pm$ 0.2	0.6 $\pm$ 0.3	75 $\pm$ 12*	69 $\pm$ 23*
10 μmol/l heme + 10 μmol/l SnMP	6.8 $\pm$ 2.1*	6.4 $\pm$ 4.1	89 $\pm$ 22*	87 $\pm$ 14*
10 μmol/l Fe(III)amn.cit.	0.8 $\pm$ 0.2	n.d.	14.3 $\pm$ 1.5*	n.d.
10 μmol/l Fe(III)amn.cit. + 10 μmol/l SnMP	1.6 $\pm$ 0.4	n.d.	18.6 $\pm$ 3.9*	n.d.

HO-1, heme oxygenase-1; SnMP, tin mesoporphyrin; CoPP, cobalt protoporphyrin; IL-6, interleukin-6; Fe(III)amn.cit., Fe(III)ammoniumcitrate. Each measurement is the mean value  $\pm$  S.E.,  $n = 3-4$ , n.d. = not determined, \*Higher than the corresponding controls ( $P < 0.05$ ).

transcription (Table 3). These results indicate no direct modulatory effects of *HO-1* levels and HO-activity on *HAMP* expression.

In the presence of IL-6, both *HO-1* and *HAMP* transcripts were up-regulated (Table 3). Because IL-6 could regulate both genes directly at transcriptional level [28–30], the observed up-regulation is likely independent from each other. Furthermore, when HO-activity was inhibited, IL-6-induced *HAMP* expression was not affected. This further confirms the absence of direct modulatory effects of HO-activity on *HAMP* expression in hepatoma cells.

In contrast, it was observed that upon *HO-1* induction by heme, *HAMP* was slightly decreased, although this was not significant (Table 3). This effect may be attributed to increased levels

of intracellular labile iron [31, 32] from heme degradation by HO-1 in hepatoma cells (Fig. 4). Similarly, after iron treatment (Table 3), although *HO-1* is up-regulated, *HAMP* expression was not changed. In this iron-enriched condition, when HO-activity was inhibited by co-exposure to SnMP, *HAMP* expression stayed low (Table 3). On the other hand, in the presence of both heme and SnMP, the unmetabolized heme up-regulated *HO-1*, and to a lesser extent, enhanced *HAMP* expression. The latter observation however may not be relevant *in vivo*, because this requires complete inhibition of HO-activity, whereas *in vivo*, other pathways of heme degradation including the HO-2 pathway [2] are present. Taken together, these cellular data show

lack of direct modulatory effects of HO-1 on cellular *HAMP* expression *in vitro*.

### Anemia in HO-1 deficiency increases erythropoietic activity that attenuates hepcidin synthesis

Because no direct modulation by HO-activity on *HAMP* expression was found *in vitro*, we questioned why hepcidin levels were so low despite the presence of chronic inflammation and iron accumulation in the HO-1-deficient patient. Because hepcidin expression can be down-regulated when an elevated erythropoietic drive is present [10, 33–35], we postulated that this hepcidin suppression in the HO-1-deficient patient could be the result of increased erythropoietic activity. To verify this, we measured sTfR, a measure of erythropoietic activity in the bone marrow [36], in the patient sera. sTfR levels were exceptionally high (19.4 mg/l from the first taken serum, and 34.1 mg/l from the second drawn serum, reference values are 0.76–1.76 mg/l) (Table 2). Increased sTfR levels in the two samples correlated well with decreased Hb levels, erythrocyte counts and the increased levels of the hemolytic indices AST and LDH (Table 2).

## Discussion

HO-1 is not only vital for iron recycling by liberating iron from heme and haemoproteins, it is also crucial for the resolution of oxidative stress and inflammatory conditions [1, 16, 37, 38]. HO-1 deficiency *in vivo* resulted in anaemia and hepatic and renal haemosiderosis [11, 16, 38–41]. The deficiency also promotes severe chronic inflammation [11, 16, 38–41] as shown by increased CRP, IL-6 and haptoglobin (Table 2). The serum levels of haptoglobin are usually very low during intravascular haemolysis, because haptoglobin binds to the free haemoglobin in the circulation which then will be rapidly cleared out by the reticuloendothelial system. In the HO-1 deficiency, markedly elevated haptoglobin levels are likely to be originated from the more dominant effect of inflammation than the observed haemolysis. Moreover, it was observed that the Kupffer cells of the HO-1-deficient patient had diminished haptoglobin-receptor levels (A. Yachie, unpublished observation). Finally, due to the asplenic condition, the clearance of haptoglobin-haemoglobin complex may also be prolonged in this patient. The circulating toxic unmetabolized heme may further enhance oxidant-mediated cellular damage [42, 43], inflammation [16, 37, 38] and haemolysis [11]. Similarly to the HO-1-deficient patient, accelerated hepatic iron loading has also been documented when *Ho-1* KO mice were challenged with stress stimuli [39–41]. Together these data suggest that HO-1 is crucial for both cell protection against oxidative and inflammatory injury as well as for maintaining iron homeostasis.

Our experiments were aimed at resolving whether distortion of iron homeostasis in HO-1 deficiency is mediated by hepcidin. As

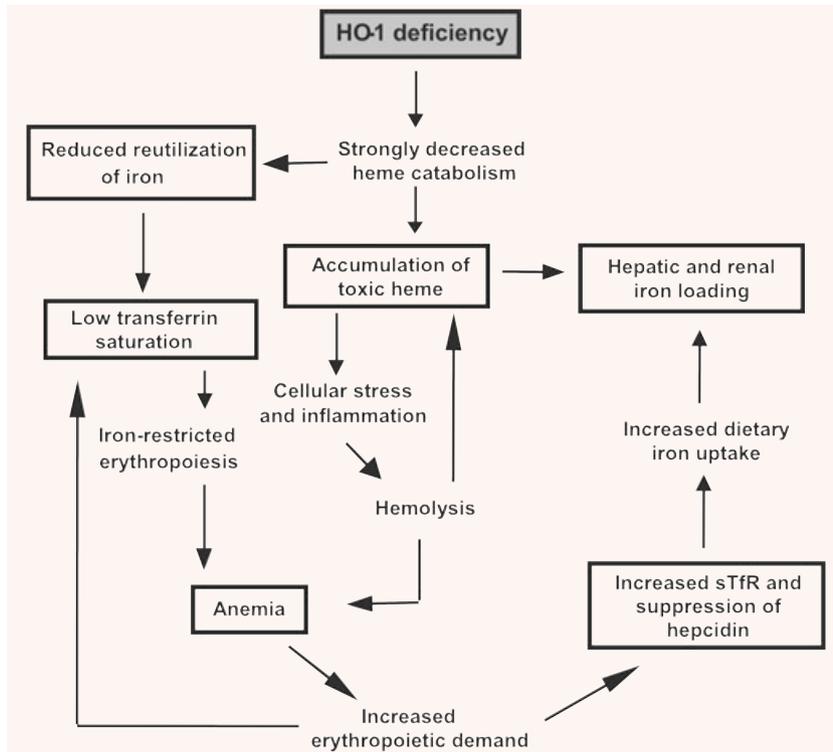
demonstrated in hepatoma cells, direct modulation of *HO-1* expression levels and HO-activity did not affect hepcidin expression, suggesting that HO-1 is not a direct upstream regulator of hepcidin expression.

To our surprise we observed very low serum hepcidin levels in the HO-1-deficient patient despite the presence of chronic inflammation and iron accumulation, both strong inducers of hepcidin. However, the observed hepcidin suppression is likely crucial as it provides bioavailable iron for erythropoiesis to compensate for the hemolysis and anemia (Fig. 5). Indeed, extremely high sTfR levels were measured, confirming the presence of increased erythropoietic activity. In agreement with our findings, in mice models of hemolysis, iron loading and inflammation are sustained, whereas production of hepcidin is suppressed [44]. This is further corroborated in a rat model of hemolysis [45]. The patient's decreased serum hepcidin levels are thus likely to be the result of increased erythroid expansion that, by analogy with growth differentiation factor-15 exclusively present in  $\beta$ -thalassaemia [35] through the systemic release of a thus far unknown factor, down-regulates hepatocyte hepcidin production. In addition, a down-regulatory effect of the anemia-associated tissue hypoxia on liver hepcidin expression may also contribute to the low hepcidin levels [46, 47].

We further investigated whether HO-1 could directly modulate ferroportin expression. Surface expression of ferroportin [5] was increased in THP-1 monocytic cells when HO-1 expression was induced in the presence of sufficient heme substrate. When HO-activity was inhibited by SnMP, HO-mediated ferroportin expression was abrogated. This induction of ferroportin by HO-activity may explain previously-reported HO-1-induced iron efflux [14, 15]. However, HO-mediated ferroportin expression appeared to be regulated by the HO-derived effector molecule, iron, because deferoxamine, an iron chelator, could inhibit the HO-activity-induced ferroportin. Thus, these findings indicate that iron reutilization is affected in HO-1 deficiency [13], due to failure in heme catabolism, and not to the absence of HO-1 as a regulator of cellular iron homeostasis [14] nor as being part of a putative iron exporter [15] as previously suggested. The HO-derived iron-induced ferroportin protein expression is possibly regulated at the transcriptional level *via* its iron responsive element [48].

Under normal circumstances, HO-activity promotes heme catabolism and thereby facilitates iron recycling back to the plasma as iron-bound transferrin to exert its regulatory function *via* the hepatocyte transferrin receptors [49]. The decreased iron recycling, because of HO-1 deficiency, and subsequently, lower serum iron-transferrin levels may, in part, have contributed to the observed low hepcidin levels. Both decreased recycling of iron from heme and fast consumption of bioavailable iron from the circulation by the bone marrow for the increased erythropoiesis activity may therefore have promoted low levels of transferrin saturation in this patient (Fig. 5).

During hemolysis, clearance of plasma heme and heme-containing proteins is carried out by the proximal tubular cells of the kidneys. This event is deleterious for the kidneys as exemplified by the extent of iron deposition in the proximal tubuli [11] and the tubular atrophy [12] of the HO-1-deficient patient. Iron also accumulated in the liver,



**Fig. 5** Proposed pathogenesis of heme oxygenase-1 deficiency. The figure illustrates the reported findings (in boxes) combined with current knowledge showing a hypothetical sequence of events initiated by HO-1 deficiency leading to severe dysregulation of iron metabolism. HO-1 deficiency promotes a substantial decrease in heme catabolism. This leads to iron-restricted erythropoiesis and stress-induced hemolysis, and both result in anemia. The anemia activates the body machinery of erythropoiesis, leading to induction of sTfR and suppression of hepcidin, necessary to fulfil iron demand for the accelerated marrow erythropoietic activity. The erythropoietic drive subsequently lowered serum iron and transferrin saturation. Suppression of hepcidin however promotes further distortion of iron homeostasis.

because this organ is capable of effectively incorporating the vast majority of plasma heme [50]. The loosely bound Fe(III) accumulation detected in the liver and the kidneys of this patient by Prussian blue (Tables 1 and 3) could be originated from heme that was degraded to Fe(III) by other pathways than HO-1, including the HO-2 pathway, which is constitutively present at a low level [2] and the non-CO-forming pathways [50], or from the oxidized free heme [51].

Besides having defective macrophage iron recycling, HO-1 deficiency also exhibited hepatocyte iron loading in the liver (Table 1) [11, 12]. Similarly, *Ho-1* KO mice show both hepatocyte and Kupffer iron overload [13], whereas stress stimuli further induce iron loading exclusively in hepatocytes [39]. In this study, we have observed low hepcidin levels in the patient with HO-1 deficiency, which are similar to that of patients with juvenile forms of hereditary hemochromatosis [52–54]. These low concentrations of hepcidin may help explain the presence of hepatocyte iron loading [11, 12]. Interestingly, notwithstanding the low hepcidin levels, and, therefore likely high surface ferroportin expression, in the HO-1-deficient patient, circulating iron levels are still low and iron accumulation is still present in the kidneys and the liver, suggesting that the export of cellular iron for the expanded erythropoiesis demand is still hampered in the patient. This further illustrates the crucial role of HO-1 to provide enough bioavailable iron for erythropoiesis. Further studies however are warranted to unravel mechanisms involved during accelerated erythropoiesis.

In this manuscript, we have examined the links between HO-1 and the recently found iron regulatory hormone hepcidin and its target molecule, ferroportin, in order to (i) delineate the mechanistic

basis of iron regulation and furthermore to (ii) help explain the dysregulation of iron homeostasis in HO-1 deficiency. We provided evidence that HO-1 increases intracellular labile iron levels, which in monocytic cells induces expression of the iron exporter ferroportin. We observed very low hepcidin levels in the HO-1-deficient patient, despite the presence of chronic inflammation and iron accumulation, both inducers of hepcidin. This condition may be relevant to many iron-loading anemia disorders, in which erythroid regulators together with hypoxic conditions dominantly suppress synthesis of the iron hormone hepcidin, despite the presence of stimuli from both inflammatory and iron regulators. Summarizing, our data suggest that the imbalance of iron metabolism in the HO-1-deficient patient is a result of both defective iron recycling of the reticulo-endothelial-system after erythrophagocytosis, and reduced hepatocyte hepcidin expression secondary to anemia-induced expansion of erythropoiesis. These findings support crucial roles of HO-1 and hepcidin in maintaining systemic iron homeostasis.

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