

Hepcidin mRNA Levels in Mouse Liver Respond to Inhibition of Erythropoiesis

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Summary

Hepcidin, a key regulator of iron metabolism, decreases intestinal absorption of iron and its release from macrophages. Iron, anemia, hypoxia, and inflammation were reported to influence hepcidin expression. To investigate regulation of the expression of hepcidin and other iron-related genes, we manipulated erythropoietic activity in mice. Erythropoiesis was inhibited by irradiation or posttransfusion polycythemia and stimulated by phenylhydrazine administration and erythropoietin. Gene expression of hepcidin and other iron-related genes (hemojuvelin, DMT1, ferroportin, transferrin receptors, ferritin) in the liver was measured by the real-time polymerase chain reaction. Hepcidin expression increased despite severe anemia when hematopoiesis was inhibited by irradiation. Suppression of erythropoiesis by posttransfusion polycythemia or irradiation also increased hepcidin mRNA levels. Compensated hemolysis induced by repeated phenylhydrazine administration did not change hepcidin expression. The decrease caused by exogenous erythropoietin was blocked by postirradiation bone marrow suppression. The hemolysis and anemia decrease hepcidin expression only when erythropoiesis is functional; on the other hand, if erythropoiesis is blocked, even severe anemia does not lead to a decrease of hepcidin expression, which is indeed increased. We propose that hepcidin is exclusively sensitive to iron utilization for erythropoiesis and hepatocyte iron balance, and these changes are not sensed by other genes involved in the control of iron metabolism in the liver.

Key words

Iron • Hepcidin • Liver • Erythropoiesis • Gene expression

Introduction

Iron is an essential element playing an important role in many biological processes (e.g. electron and oxygen transport, DNA synthesis) and consequently is essential for every cell in the organism (Ponka 1999, Aisen *et al.* 2001). However, excess iron poses a threat to cells and tissues because of its ability to catalyze the generation of reactive radicals (Papanikolaou and

Pantopoulos 2005).

Systemic iron metabolism is essentially a closed system because mammals lack a regulated physiological excretory pathway. Iron is absorbed in the duodenum to compensate the small losses due to cell desquamation or bleeding while the most of iron required by the bone marrow for erythropoiesis is provided by recycling iron from senescent red blood cells *via* macrophages.

Hepcidin is a key regulator of iron metabolism

controlling both iron absorption and recycling (Pigeon *et al.* 2001, Nicolas *et al.* 2001). It is a 25 amino acid antimicrobial disulfide-bonded peptide (Krause *et al.* 2000) synthesized by hepatocytes and secreted into the plasma. It can be detected in the urine (Park *et al.* 2001). Heparin decreases intestinal iron absorption and increases iron retention in reticuloendothelial cells. The target of hepcidin action is the iron exporter ferroportin (Nemeth *et al.* 2004) present mainly in basolateral membrane of enterocytes, and cell membranes of macrophages and hepatocytes.

Hepcidin synthesis in hepatocytes is induced by iron loading, and it is suppressed by anemia and hypoxia. A further important stimulus for hepcidin expression is inflammation (Pigeon *et al.* 2001, Nicolas *et al.* 2002a); hepcidin is also an acute phase reactant induced by IL-6 (Nemeth *et al.* 2003).

However, the molecular mechanisms underlying hepcidin regulation are still unclear (Beutler 2004, Ganz 2005), though abnormalities of this regulation have been implicated in two important clinical disorders – hereditary hemochromatosis (HH) (Nicolas *et al.* 2001) and anemia of inflammation (Weinstein *et al.* 2002).

The liver plays a central role in maintaining body iron homeostasis (Sharma *et al.* 2005) not only as a storage tissue and a site of hepcidin production, but also by a relatively specific expression of several other iron-related genes including HFE, hemojuvelin (HJV) and transferrin receptor 2 (TfR2). Mutations of these genes are responsible for hereditary hemochromatosis (Pietrangelo 2004). Heparin production was found to be inappropriately low for the degree of iron loading in various types of human HH and in their experimental models (Ahmad *et al.* 2002, Nemeth *et al.* 2005, Papanikolaou *et al.* 2004, Kawabata *et al.* 2005). Therefore, it is hypothesized that these genes could modify hepcidin production.

Apart from these genes with a relatively specific expression, the liver expresses other genes and molecules involved in cellular iron transport which are also present in other tissues. They include transferrin receptor 1 (TfR1), divalent metal transporter 1 (DMT1, Nramp2) and ferroportin (FPN).

The aim of the present study was to examine mRNA expression of hepcidin and several other iron-related genes in the liver of mice in situations with stimulated and inhibited erythropoiesis. Since hepcidin mRNA levels are known to be decreased in anemia, caused by bleeding or phenylhydrazine, we wanted to

study the effects of changes of erythropoietic activity on hepcidin expression. We observed an increase in hepcidin expression despite severe anemia induced by phenylhydrazine hemolysis if hematopoiesis had been suppressed by irradiation. Suppression of erythropoiesis by posttransfusion polycythemia or irradiation also increased hepcidin mRNA levels. Stimulation of erythropoiesis by exogenous erythropoietin decreased hepcidin mRNA levels but suppression of hematopoiesis by irradiation blocked the effect of erythropoietin. Compared to hepcidin, changes of mRNA of other genes studied were considerably less pronounced. We suggest that erythropoiesis driven changes in iron balance are important for modulating hepcidin expression.

Methods

Animals

All studies were performed in male mice C57BL/6N (Charles River), aged 2 to 3 months. The animals were maintained in a temperature- and light-controlled environment. They had free access to tap water and standard laboratory food. Control animals were subjected to experimental manipulations similar to those of treated mice. The animals were sacrificed and a part of the liver tissue was removed and placed in a RNAlater solution (Sigma Aldrich).

The Animal Care Committee of the First Faculty of Medicine approved the experiments.

Animal treatment

Erythropoietin administration

Human recombinant erythropoietin (EPO, Eprex®, Cilag AG) diluted in saline (50 U/mouse) was administered subcutaneously on four consecutive days prior to liver removal.

Polycythemia induction

Polycythemia was induced in mice by intravenous administration of 0.8 ml of 70 % red blood cells, washed and diluted in saline, on two consecutive days. The polycythemic mice were sacrificed for blood and liver collection on day 7 after the second dose of red blood cells.

Total body irradiation

Irradiation (4 Gy) was performed with ⁶⁰Co. In experiments combining hemolysis induced by phenylhydrazine (PHZ) with irradiation, PHZ was

injected 24 hours after irradiation and mice were sacrificed 16 and 48 h later. In experiments combining EPO administration with irradiation, first EPO injection was given to half of irradiated mice after 24 h and then repeatedly for four consecutive days. The animals of both groups were killed five days after irradiation.

Phenylhydrazine

Phenylhydrazine (Carlo Erba, Italy) diluted in saline was injected intraperitoneally to induce acute hemolysis. A single dose of 50 mg/kg was given 24 h after irradiation and the animals were sacrificed 16 and 48 h afterwards. In chronic hemolysis experiments, PHZ was given twice a week in a dose 30 mg/kg during four consecutive weeks (8 doses total) and the liver was collected 64 h after the last dose.

RNA isolation and reverse transcription

Total RNA was extracted from the liver using RNABlue (Top-Bio, Czech Republic). The tissue was homogenized in RNABlue and RNA was extracted using chloroform/isopropanol/ethanol. Its concentration and purity was tested by electrophoresis and spectrophotometrically at 260 and 280 nm. RNA was treated with Dnase I (Gibco, Life Technologies, USA). First-strand cDNA synthesis was performed in a total volume of 20 μ l containing 200 U of M-MuLV reverse transcriptase, 4 μ l 5 \times Reaction Buffer, 20 U of RNase inhibitor, 2 μ l of Deoxynucleotide Mix (final 1 mM), 1 μ l of oligo(dT)18 primer (0.5 μ g), and 1 μ g of total RNA, as recommended by the manufacturer (RevertAidTM First Strand cDNA Synthesis Kit, Fermentas). Template that was prepared from RNA incubated without reverse transcription was used as a negative control.

Real-time PCR

Gene expression studies were performed on a Roche LightCyclerTM real-time PCR instrument, using LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Germany) as described previously (Krijt *et al.* 2004a). To correct for the different amounts of cDNA present in the sample at the start of the LightCycler run, the obtained crossing points for the target mRNAs were normalized to β -actin mRNA: for each sample, the difference between target mRNA crossing point and β -actin mRNA crossing point was calculated, resulting in a β -actin normalized crossing point. The normalized crossing point for the control sample was then subtracted from the normalized crossing

point for the treated sample, giving the final difference (n) in cycle numbers between the control and treated samples. The values were obtained using 2n formula and represented as the amount of target mRNA relative to β -actin. Primer sequences were: β -actin forward 5'-GAC ATG GAG AAG ATC TGG CA-3', reverse 5'-GGT CTT TAC GGA TGT CAA CG-3'; hepcidin forward 5'-CTG AGC AGC ACC ACC TAT CTC-3', reverse 5'-TGG CTC TAG GCT ATG TTT TGC-3'; hemojuvelin forward 5'-CCC AGA TCC CTG TGA CTA TGA-3', reverse 5'-CAG GAA GAT TGT CCA CCT CAG-3'; DMT1-IRE forward 5'-CAA TGG AAT AGG CTG GAG GAT-3', reverse 5'-ACA GAC CCA ATG CAA TCA AAC-3'; ferroportin forward 5'-TCG GTT CCT CTC ACT CCT GT-3', reverse 5'-GTG GAG AGA GAG TGG CCA AG-3'; TfR1 forward 5'-TGG GTC TAA GTC TAC AGT GGC-3', reverse 5'-AGA TAC ATA GGG CGA CAG GAA-3'; TfR2 forward 5'-ATT CTC CTT TCT CCC TCT TT-3', reverse 5'-GCT GTC CAT CTC ACT CTC TA-3' ferritin forward 5'-GCC AGA ACT ACC ACC AGG AC-3', reverse 5'-TGG TTC TGC AGC TTC ATC AG-3'.

Hematological analysis

Mice were exsanguinated under halothane anesthesia from axillary blood vessels. Hematological parameters were measured using AdviaTM 60, Hematology Systems, Bayer.

Tissue iron determination

Liver non-heme iron was determined according to the method of Torrance and Bothwell (1980), and expressed per wet weight of tissue.

Statistical analysis

Multiple comparisons were evaluated by the one-way analysis of variance (ANOVA) followed by Tukey test. Student's test was used for comparison between two groups. Values in the figures are expressed as means \pm S.E.M. Significance was accepted at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 4.

Results

Chronic hemolysis in mice with functional erythropoiesis did not change hepcidin mRNA

Phenylhydrazine causes hemolysis and its administration leads to the stimulation of erythropoiesis.

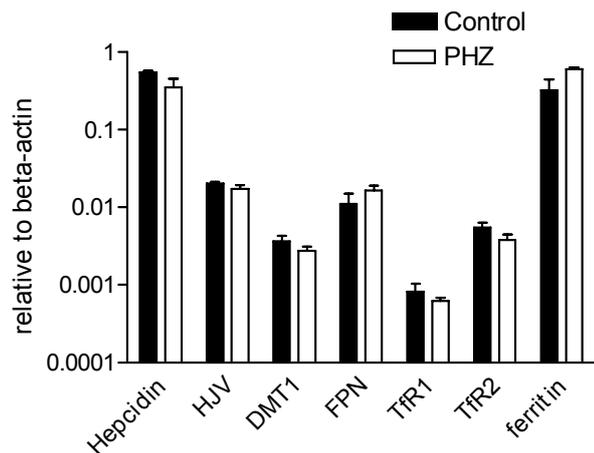


Fig. 1. Effect of repeated administration of phenylhydrazine (PHZ) on hepatic expression of hepcidin and other iron-related genes. PHZ was given twice a week in a dose 30 mg/kg during four consecutive weeks (a total of 8 doses). Values represent the relative amount of target mRNA compared to β -actin mRNA and are expressed as means \pm S.E.M. (n=4). Differences are not statistically significant.

To test the effect of long-term hemolysis on hepcidin mRNA, mice were treated with PHZ for 4 weeks in a model of chronic compensated hemolysis (Nečas and Neuwirt 1969). Control mice were injected with saline. The spleen size increased from 82.7 ± 5.9 in the control group to 364.7 ± 38.1 mg in the PHZ group ($p=0.0002$). Non-heme liver iron content increased from 46.0 ± 2.4 to 75.5 ± 1.4 mg/g wet tissue ($p<0.0001$). Hematocrit values decreased from 48.4 ± 0.4 to 41.7 ± 1.5 % ($p<0.005$). Hepcidin mRNA levels in the liver did not change significantly, as well as other genes examined (Fig. 1).

Suppression of erythropoiesis by polycythemia increased mRNA for hepcidin

Transfusion-induced polycythemia suppresses erythropoiesis and represents an extra iron load to the organism. In polycythemic mice the hematocrit increased to 70.0 ± 3.0 from 48.0 ± 1.0 % in control mice ($p<0.001$). Hepcidin mRNA level increased 5-6 fold ($p<0.05$), while expression of other genes did not change significantly (Fig. 2). Liver non-heme iron content increased from 32.1 ± 3.2 to 46.2 ± 0.6 μ g/g wet tissue ($p<0.01$).

Hepcidin mRNA level increases in mice with inhibited erythropoiesis despite anemia

Mice were first irradiated with 4 Gy to suppress their hematopoiesis and 24 h later were injected with PHZ. They were sacrificed 16 and 48 h later. PHZ-untreated mice were injected with saline. Three experimental groups were studied: a) control (non-

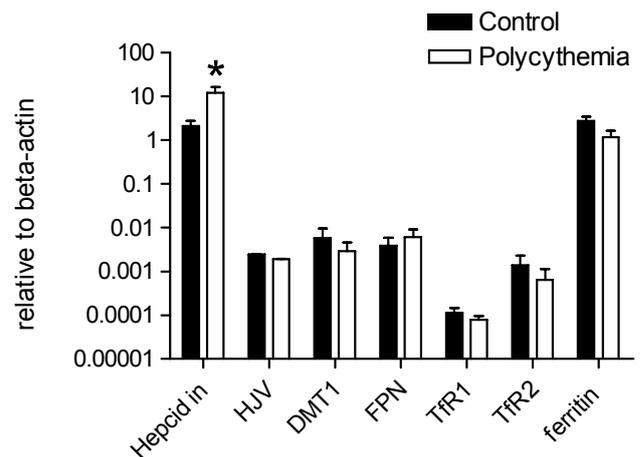


Fig. 2. Changes in mRNA levels of hepcidin and other iron-related genes in the liver of mice with posttransfusional polycythemia. Values represent the relative amount of target mRNA compared to β -actin mRNA and are expressed as means \pm S.E.M. (n=3). *) Statistically significant difference between control and polycythemic mice ($p<0.05$).

irradiated), b) control irradiated and c) irradiated + PHZ treatment.

Hepcidin expression was significantly increased in all treated animals in 16-h group. The increase was highest (9 fold) in irradiated mice, in PHZ-treated group the increase was 5-6 fold, and in the group with combined treatment it was 6-7 fold. In 48-h group, the hepcidin expression decreased significantly in PHZ-treated group while the levels in the irradiated groups were still elevated despite severe anemic condition (Fig. 3A). The changes in other genes studied were inconsistently small and negligible (Fig. 3B).

Hematocrit and liver non-heme iron concentrations are summarized in Table 1. After a 16-h interval, the combined treatment (irradiation and PHZ) led to a largest decrease of hematocrit. In a 48-h interval, all treated groups became distinctly anemic, the most severe anemia was in the group with combined treatment. Liver non-heme iron content increased in all the treated groups.

Exogenous erythropoietin suppression of hepcidin mRNA could be prevented by irradiation

Four daily injections of EPO (50 U each) stimulated erythropoiesis, the hematocrit increased from 48 ± 1 to 57 ± 2 % ($p<0.01$). Hepcidin expression decreased by almost two orders of magnitude ($p<0.05$), whereas TfR1 mRNA expression increased threefold after EPO treatment ($p<0.05$). However, this effect was prevented by previous whole-body irradiation (Fig. 4). The

Table 1. Hematocrit and non-heme iron concentration in the liver ($\mu\text{g/g}$ wet tissue) after red blood cell hemolysis induced by phenylhydrazine administration (P), irradiation (R), and their combination (RP) 16 and 48 h after the treatment ($N=3$).

	Control	P16	R16	RP16	P48	R48	RP48
HTK	47.65 \pm 0.1	38.75 \pm 0.8	44.20 \pm 1.2	25.50 \pm 2.3	30.50 \pm 1.1	30.27 \pm 4.1	22.10 \pm 22.1
Non-heme iron in the liver	54.5 \pm 5.0	87.5 \pm 3.2***	73.2 \pm 6.3*	89.7 \pm 0.4*	91.0 \pm 1.8*	84.6 \pm 3.4**	96.3 \pm 1.3*

Results expressed as mean \pm S.E.M. Statistical significance in comparison with the control group: * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$

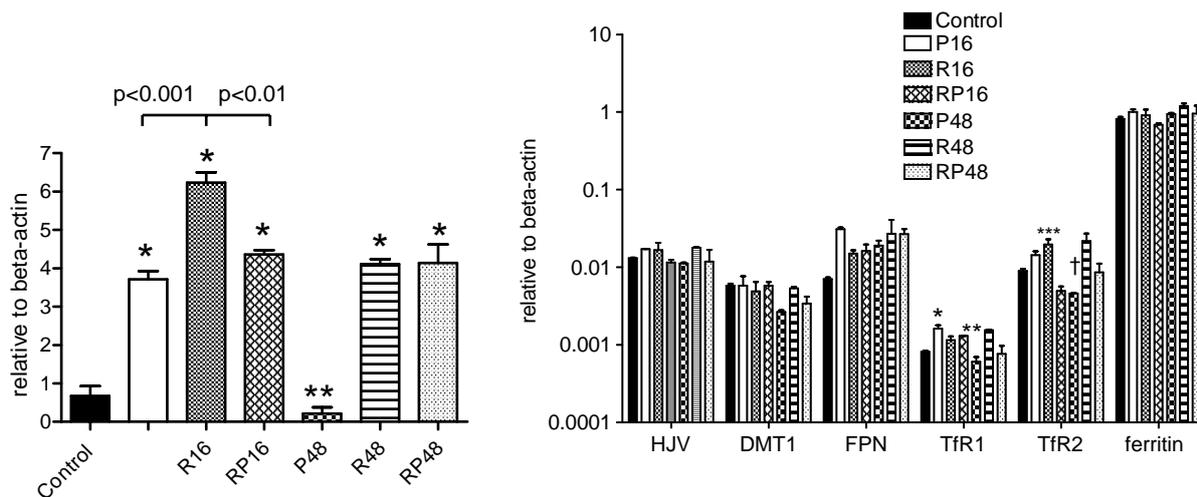


Fig. 3. Changes in mRNA levels of hepcidin (left panel) and other iron-related genes (right panel) in the liver of mice after red blood cell hemolysis induced by phenylhydrazine administration (P), irradiation (R), and their combination (RP) 16 and 48 h after the treatment. Values represent the relative amount of target mRNA compared to β -actin mRNA and are expressed as means \pm S.E.M. ($n=3$). Left panel – * significant in comparison with control group ($p < 0.001$); ** significant in comparison with P16, R16, RP16, R48 and RP48 groups ($p < 0.001$). Right panel – * Tfr1 mRNA in P16 group was significantly increased in comparison with P48 and RP48 groups ($p < 0.05$); ** Tfr1 mRNA in P48 group was significantly decreased in comparison with P16, RP16 and R48 groups ($p < 0.05$); *** Tfr2 mRNA in RP16 group was significantly decreased in comparison with P16, R16 and R48 groups ($p < 0.05$); † Tfr2 mRNA in P48 group was significantly decreased in comparison with P16, R16 and R48 groups ($p < 0.05$).

hematocrit values in the irradiated group did not change despite EPO administration and the 24-h incorporation of ^{59}Fe into blood decreased from 57.2 % (33.4-85.6) in control EPO-treated mice to 1.8 % (1.2-2.3) in irradiated EPO treated mice ($p=0.037$). Non-heme iron content in the liver increased from 51.1 \pm 4.5 $\mu\text{g/g}$ wet tissue in control animals to 72.8 \pm 0.3 in the irradiated group without EPO treatment and to 74.1 \pm 3.6 in the irradiated group treated with EPO ($p < 0.05$). EPO administration itself did not significantly change the iron content (48.4 \pm 7.1 $\mu\text{g/g}$ wet tissue).

Discussion

Hepcidin is a key hormone involved in iron regulation but the precise mechanism of hepcidin expression remains largely unknown. In the present study

we examined the amount of mRNA of hepcidin and other iron-related genes in the mouse liver to explore the influence of enhanced or inhibited erythropoiesis. We induced profound perturbations in recycling of iron between macrophages and erythropoiesis, as well as in the transfer of iron between storage tissues, bone marrow and macrophages.

Anemia, resulting either from blood loss or phenylhydrazine-induced hemolysis, had been previously shown to decrease hepcidin expression (Nicolas *et al.* 2002a, Latunde-Dada *et al.* 2004, Frazer *et al.* 2004). However, we have observed an increase in hepcidin mRNA levels after phenylhydrazine-induced anemia when the bone marrow was suppressed by previous irradiation. This increase occurred despite pronounced anemia. Occurrence of anemia and tissue iron overload is also encountered in mice with transferrin deficiency

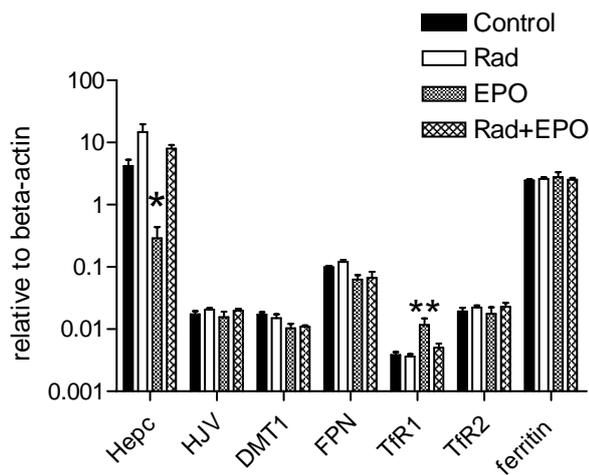


Fig. 4. Changes in mRNA levels of hepcidin and other iron-related genes in the liver of mice after irradiation (*Rad*), erythropoietin (*EPO*) and irradiation followed by *EPO* administration (*Rad+EPO*). Values represent the relative amount of target mRNA compared to β -actin mRNA and are expressed as means \pm S.E.M. ($N=3$). *) Statistically significant in comparison with all other groups ($p<0.05$); **) Statistically significant in comparison with control and irradiated groups ($p<0.05$).

(*hpx/hpx*) and in thalassemic mice which are both characterized by decreased hepcidin mRNA expression (Weinstein et al. 2002, Adamsky et al. 2004). However, erythropoiesis in such mice is functional, though iron-restricted or ineffective.

The importance of erythropoietic activity in PHZ-induced anemia can also be demonstrated on the time-course of hepcidin mRNA levels after PHZ injection. Frazer et al. (2004) observed delayed downregulation of hepcidin after PHZ; no change was observed until three days after administration of PHZ with the lowest level on the fifth day. In other studies the mRNA level was measured three (Latunde-Dada et al. 2004) or four (Nicolas et al. 2002a) days after PHZ administration. In all these situations erythropoiesis had already been stimulated. Accordingly, we have observed increased hepcidin mRNA expression 16 h after PHZ administration and a significant decrease after 48 h. The liver non-heme iron concentration increased in both cases and did not differ significantly. It is known from hypotransferrinemic mice that hepcidin expression can be low despite significant iron loading of the hepatocytes (Weinstein et al. 2002). These results show that requirements of erythropoiesis for iron influence hepcidin expression in the liver more than anemia or non-heme iron content of the liver.

This is further documented by an increase of hepcidin mRNA level after irradiation alone. Blocking

erythropoiesis by irradiation causes a significant increase in hepcidin mRNA expression after 16 h. Red blood cell production is quantitatively the largest iron consuming process in the body which abolishes or ablates a profound effect on iron body distribution and metabolism. Similarly, a less pronounced increase of hepcidin expression was observed in mice when erythropoiesis was selectively inhibited by transfusion-induced polycythemia. Presumably, continuing iron absorption in the gut and its release from macrophages would be highly undesirable in a situation of suppressed erythropoiesis. Raja et al. (1988) observed that obliteration of erythropoiesis in animals was accompanied by a marked decrease in the transfer of iron from the mucosa. In accordance with our observation, this could probably be mediated by increased hepcidin expression which regulates cellular iron efflux by binding and internalization of ferroportin on basolateral membrane of enterocytes (Nemeth et al. 2004).

Since hepcidin is also an acute phase reactant, a nonspecific increase due to irradiation is a possibility. Irradiation itself did not increase mRNA of interleukin-6, an alternative stimulus for hepcidin production (data not shown).

On the other hand, compensated hemolysis induced by repeated PHZ administration (Nečas and Neuwirt 1969) did not significantly change hepcidin expression despite an increase of the non-heme iron content in the liver and mild anemia. The unchanged hepcidin expression could be in agreement with the fact that hemolytic anemias with effective erythropoiesis do not have increased iron absorption and do not usually have an iron overload (Erlandson et al. 1962). However, the differences between acute and chronic hemolysis and the role of ineffective erythropoiesis on hepcidin expression require further investigation.

Potent downregulation of hepcidin expression in the liver after erythropoietin administration has previously been described by Nicolas et al. (2002b). Inhibition of erythropoiesis by irradiation abolished the effect of EPO on hepcidin expression. This demonstrates that the action of erythropoietin in down-regulating hepcidin was indirect.

Compared to hepcidin, less attention has been paid to the expression of other iron-related genes in the liver in connection with disturbed iron metabolism. Latunde-Dada et al. (2004) measured the amounts of mRNA of ferroportin, DMT1-IRE and TfR1 after PHZ administration. The TfR1 expression significantly

increased, DMT1-IRE was downregulated. The expression of TfR1 was decreased in thalassemic mice (Adamsky *et al.* 2004), while the mRNAs for ferroportin, HFE and TfR2 were unchanged. In our experiments, changes of mRNAs for all other genes examined were less pronounced and inconsistent in comparison with hepcidin. TfR1 expression increased after EPO treatment and decreased 16 h after PHZ administration in comparison with the expression after 48 h. TfR2, DMT1 and ferritin expression varied slightly and were inconsistent in different experiments. Hemojuvelin mRNA levels did not change at all and this agrees with our previous observations (Krijt *et al.* 2004b).

Our results contribute to the search for a hierarchy of regulators of hepcidin expression. Anemia and probably hypoxia themselves do not seem to be of primary importance. Insignificant changes of the hepcidin mRNA levels in the model of chronic compensated hemolysis and pronounced decrease of hepcidin after

EPO administration which is absent if the bone marrow is suppressed, suggest that it is erythropoietic activity and subsequent changes in iron metabolism that are important for hepcidin expression. The saturation of transferrin as proposed by Frazer *et al.* (2002) could mediate such changes.

In conclusion, our data document a wide range of acute hepcidin mRNA responses to changes of erythropoiesis and iron mobilization or liver iron loading. Hepcidin can be viewed as a molecule that sensitively reacts to changes of erythropoiesis and its requirements for iron.

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