The effects of erythropoetic activity and iron burden on hepcidin expression in patients with thalassemia major

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Hepcidin production is homeostatically regulated by iron stores, anemia and hypoxia. We evaluated the effect of iron overload and of ineffective erythropoeisis on hepcidin expression in patients with thalassemia major. Liver hepcidin mRNA levels correlated with hemoglobin concentration and inversely correlated with serum transferrin receptor, erythropoietin and non-transferrin-bound iron. They did not correlate with indices of iron load. Urinary hepcidin levels were disproportionably suppressed in regards to iron burden. We conclude that hepcidin expression is regulated mainly by increased erythropoietic activity rather than by iron load and that hepcidin plays a central regulatory role in iron circulation and iron toxicity in patients with thalassemia.

Key words: thalassemia, hepcidin, erythropoiesis, hemosiderosis.

Haematologica 2006; 91:809-812

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Hepcidin, an iron-regulating hormone, is a 25-amino acid peptide produced by hepatocytes.[1,2] Increased hepcidin production inhibits intestinal iron absorption and iron release from macrophages, while decreased production has the opposite effects.[2] Hepcidin deficiency leads to iron overload and hereditary hemochromatosis.[3] Iron absorption in humans, is mainly regulated by iron stores (*stores* regulator) and the rate of erythropoiesis (*erythroid* regulator).[4] Erythroid stimulation of iron absorption occurs regardless of body iron stores, aiming to provide an adequate iron supply for red blood cell production.[4] In thalassemia, massive erythroid proliferation leads to a 5-10 fold increase in erythroid iron demands leading to the paradoxical phenomenon of iron deficient erythropoiesis in the presence of enlarged iron stores.[4,5] Hepcidin is thought to be the common final mediator of both the *stores* and *erythroid* regulators.[6,7] Anemia, hypoxia and increased iron stores have opposing effects on hepcidin expression, the negative effect of the former being dominant over the positive effect of iron.[7] Hepcidin mRNA expression was found to be decreased in the liver of C57Bl/6 $Hbb^{th3/+}$ mice (a murine model of human thalassemia)[8] and urinary hepcidin was found to be suppressed in patients with thalassemia major and thalassemia intermedia.[9,10] In this report, we estimated hepatic RNA expression and urinary levels of hepcidin and their correlation to clinical and biochemical indices in patients with thalassemia major.

Design and Methods

Patients

Nineteen regularly-transfused patients (14 females) with β-thalassemia major were included in the study. Five patients were seropositive for hepatitis C virus (HCV). Annual consumption of packed red blood cells was estimated for the year prior to the study. The study was approved by the institutional review board. Informed consent was obtained from all participants.

Methods

Total RNA was isolated from liver samples and preserved in RNA later (Qiagen, Hilden, Germany) using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). cDNA was synthesized by reverse transcription of 1.5 μg of quality-checked RNA. Real-time quantification of hepcidin mRNA transcripts was performed using Light-Cycler (Roche, Mannheim, Germany) and SYBR Green I fluorescent dye (Molecular Probes, Oregon, USA). Every sample was processed in duplicate and β-actin was used as an internal control to normalize all transcript values. Standard curves were generated using serial dilutions of known copy number of purified polymerase chain reaction (PCR) products of the gene of interest according to the Light-Cycler Operator's manual instructions. Primers used for hepcidin and for β-actin were: hepcidin forward:5'-ATGGCACT-GAGCTCCCAGAT-3', hepcidin reverse:5'-ACTTTGATCGATGACAGCAGCCG-3', β-actin_forward: 5'-AGGATGCAGAAG-GAGATCACT-3', β-actin_reverse: 5'-GGGTGTAACGCAACT AAGTCATAG-3'. Hematologic and biochemical parameters were determined by standard methods just before the liver biopsy. Liver iron concentration (LIC) was estimated by atomic absorption spectrometry. Urine N-acetyl-β-glucosaminidase (NAG), which is a sensitive indicator of renal injury, was measured using a colorimetric assay (Roche Diagnostics, Mannheim, Germany). Serum non-transferrin bound iron (NTBI) was determined in 13

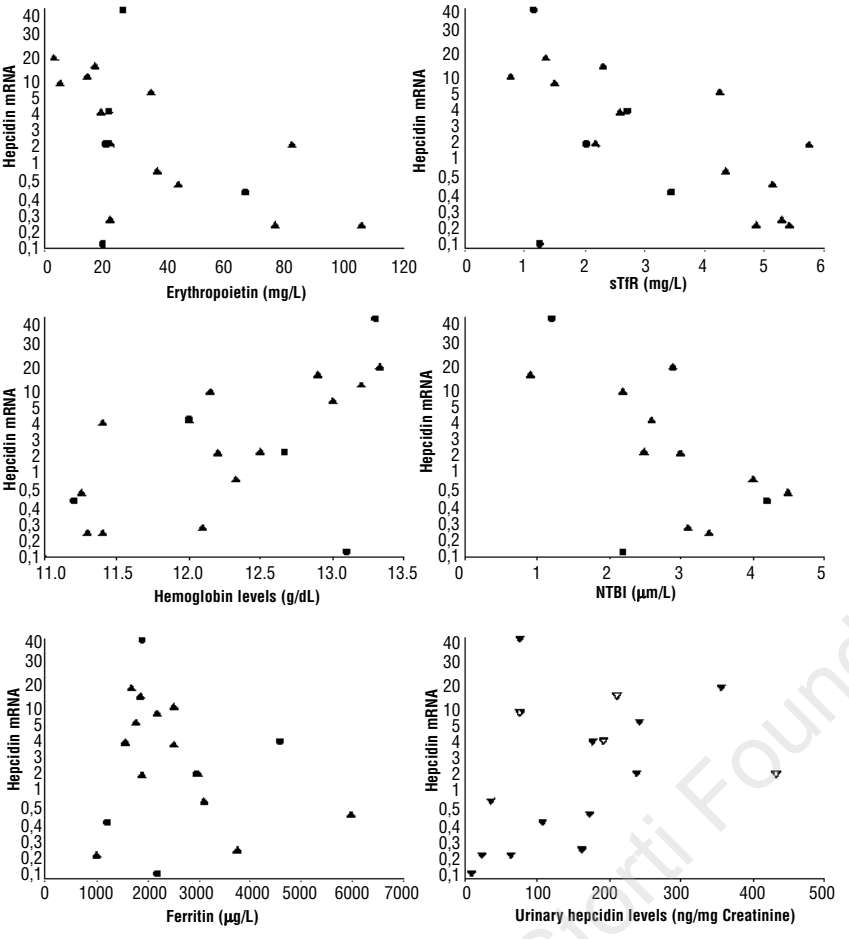

Figure 1. **Correlations between hepcidin mRNA and parameters of erythropoetic activity and iron overload. Hepcidin mRNA levels, expressed in arbitrary units (log_{10} scale), correlated inversely with EPO, sTfR, Hb, NTBI but they did not correlate with indices of iron overload such as LIC and ferritin levels. Triangles represent HCV seronegative patients, while closed squares represent HCV seropositive patients. Closed and open inverse triangles represent patients with either no measurable or measurable NAG in the urine, respectively.**

patients as previously described.[11] Briefly, NTBI was chelated using nitrilotriacetic acid (NTA) and then ultrafiltrated. NTBI from the Fe-NTA complex was measured by graphite furnace atomic absorption spectrometry (A-Analyst 800, Perkin Elmer AAS) at 2100°C element atomization. Urinary hepcidin levels were measured in spot urine samples collected just before the liver biopsy from 16 patients. The urinary hepcidin levels were measured at the University of California (Los Angeles, USA), as previously described.[10] Statistical analysis was performed using non-parametric tests. A p value of less than 0.05 was considered statistically significant.

Results and Discussion

Hepatic hepcidin mRNA levels were calculated as a ratio to the mRNA levels of the internal control and were expressed in arbitrary units (AU). They showed a wide distribution with a median value of 1.13 (range 0.08–38.48 AU). Urinary hepcidin levels varied accordingly (Table 1) and correlated well with hepatic hepcidin mRNA levels (Rho=0.57, p=0.022). When four patients with urine NAG>0.01 mg/dL, indicating proximal tubular dysfunction, were excluded from analysis, the correlation of urinary hepcidin with hepcidin RNA improved significantly (Rho=0.69, p=0.012) (Figure 1). The ratio of hepcidin over ferritin ranged from 0.004 to 0.21 (median 0.054). Erythropoetin (EPO) levels were elevated in 16 patients (normal range: 6.2-15.6 IU/L), while soluble transferrin receptor (sTfR) levels were elevated in 15 patients (normal range: 0.93-1.59 mg/L) (Table 1). LIC were in the expected range for thalassemic patients (mean: 8.6±3.8 μg Fe/d.w.tissue). NTBI levels were elevated in all patients. Correlations between clinical and laboratory variables are summarized in Table 1. Hepcidin mRNA levels were inversely correlated with NTBI and indices of erythropoietic activity, namely sTfR and EPO levels. They were positively correlated with hemoglobin concentration, but did not correlate with indices of iron stores, such as LIC, ferritin, iron and transferrin saturation (Figure 1). The statistical significance increased when HCV seronegative patients were analyzed separately, suggesting a more complex homeostatic regulation of hepcidin in patients with HCV infection. NTBI correlated well with both LIC (Rho=0.67, p<0.05) and indices of erythropoietic activity, namely EPO (Rho=0.63, p<0.05) and sTfR (Rho=0.71, p<0.01). LIC also correlated well with EPO (Rho=0.56, p<0.05) and sTfR (Rho=0.51, p<0.05). Correlations of NTBI and LIC with ferritin, transferrin saturation and transfusional iron load were not significant.

Conclusions

In accordance with previous reports, we showed a wide variation in urinary hepcidin levels, which were similar to those reported for normal adults.[9,10,12] The ratio of urinary hepcidin over ferritin, which in normal individuals is

Table 1. Correlation of liver hepcidin mRNA levels with clinical and laboratory variables in patients with thalassemia major.

Variable *Patients*	*Median (range)* *All*	*Hepcidin mRNA levels*[a] *All*		*HCV(-)*	
		rho	*p*	*rho*	*p*
Age (years)	17.5 (10.5–37)	–0.39	0.10	-0.55	0.043*
ALP (IU/L)	90 (43–448)	0.12	0.62	0.20	0.49
ALT (IU/L)	22 (12–43)	0.20	0.40	0.00	0.99
AST (IU/L)	15 (9–67)	–0.04	0.86	-0.08	0.77
γ-GT (IU/L)	11 (7–19)	0.12	0.64	0.01	0.97
CRP (mg/L)	0.1 (0.1–8.5)	–0.17	0.48	-0.36	0.20
Serum Iron (μmol/L)	45.5 (20.4–56.7)	0.20	0.41	0.39	0.17
Ferritin (μg/L)	2174 (990–5963)	0.01	0.97	-0.08	0.79
Transferrin saturation (%)	110 (61–162)	0.08	0.73	0.21	0.46
Transfusional iron (mg/kg/d)	0.44 (0.25–0.58)	–0.09	0.70	0.04	0.9
Hemoglobin (g/dL)	12 (11.2–13.4)	0.55	0.017*	0.76	0.002*
sTfR (mg/L)	2.64 (0.75–5.75)	–0.59	0.010*	-0.82	0.0006*
Erythropoietin (IU/L)	21.6 (2.9–106)	–0.61	0.007*	-0.85	0.0002*
NTBI (μmol/L)	3.1 (0.9–4.5)	0.56	0.047*	0.77	0.009*
LIC (μg Fe/d.w.tissue)	8.3 (3.1–18.9)	–0.27	0.26	-0.47	0.09
Urinary hepcidin (ng/mgCreatinine)	167 (9–433)	0.57	0.022*		

[a]*:Spearman's rho correlation coefficient; *:statistically significant; ALP: alkaline phosphatase; ALT: alanine aminostransferase; AST: aspartate aminostransferase; γ-GT: γglutamyltransferase; CRP: C-reactive protein; sTfR: serum transferrin receptor; NTBI: non-transferrin-bound iron; LIC: liver iron concentration.*

around 1, was significantly decreased. Urinary hepcidin concentrations have a good but not absolute correlation with mRNA levels.[12] Renal tubular dysfunction may affect the rate of hepcidin tubular reabsorption and thus, urinary hepcidin concentration. Many patients with thalassemia major have subclinical renal dysfunction due to chronic anemia, severe iron overload and chelation therapy.[13] When patients with altered renal function were excluded from analysis, the correlation of urinary hepcidin levels and mRNA improved considerably. We demonstrated a significant correlation between hemoglobin and hepcidin expression, similar to that in previous reports.[12] Current transfusion regimens improve but do not completely nullify anemia, which is more pronounced in the immediate pretransfusion period. It is of note that urinary hepcidin levels before and after transfusions have been shown to vary significantly, with a median magnitude of 164%.[9] Thalassemic patients have markedly enhanced gastrointestinal iron absorption, which is related to erythropoietic activity and which decreases when erythroid marrow hyperplasia is suppressed by transfusions.[4] In our patients, hepcidin mRNA levels were inversely correlated to EPO and sTfR levels, indicating that down-regulation of hepcidin is proportional to the increase of erythropoietic activity. The inverse relationship between hepcidin and EPO levels raises the question whether EPO directly regulates hepcidin expression, as has been previously suggested.[7,14] Administration of EPO to mice resulted in down-regulation of hepcidin and increased iron absorption,[7] while administration of EPO to irradiated animals with bone marrow suppression had no effect on iron absorption.[4] Furthermore, patients with aplastic anemia do not have increased iron absorption despite high levels of erythropoietin.[4] These observations suggest that EPO probably affects hepcidin expression indirectly through the bone marrow expansion it provokes. Hepcidin could be directly suppressed by NTBI, a hypothesis which is also supported by the paradoxical down-regulation of hepcidin in hepatic cells cultured in the presence of non-transferrin iron.[15] Alternatively, decreased hepcidin levels may enhance iron release in the circulation, resulting in over-saturated transferrin and NTBI formation. NTBI represents circulating forms of iron not tightly bound to transferrin, which, unlike the receptor-mediated uptake of transferrin-bound iron, translocate across membranes in a non feedback-regulated process, possibly via alternative iron delivery pathways.[16] NTBI seems to play a significant role in iron accumulation in specific tissues resulting in considerable tissue toxicity. Notably, cardiomyopathy and hypogonadism occur long before cirrhosis in patients with thalassemia major or juvenile hemochromatosis.[17] Hepcidin expression did not correlate with iron load indices, such as LIC, ferritin, iron and transferrin saturation. This was to be expected, as it is known that regulation of iron balance according to iron stores breaks down in disorders with massive erythroid proliferation and ineffective erythropoiesis. LIC reflects cumulative liver iron and is influenced by age, chelation therapy, transfusions, diet, infections and blood losses. Cazzola *et al*. demonstrated the predictive significance of sTfR in the development of iron overload in patients with ineffective erythropoiesis,[5] while Pootrakul *et al*. showed a strong relationship between plasma iron turnover, absorption and liver iron.[18] One hypothesis that may explain our findings is that tissue hypoxia triggers the production of EPO, which results in pronounced erythroid proliferation accompanied by increased sTfR levels. Hypoxia and yet-undefined signals from the robust erythroid activity down-regulate hepcidin production. Low hepcidin levels result in increased iron absorption and greater release of stored iron into the circulation, leading to high transferrin saturation and NTBI formation, which contributes to iron accumulation in the liver, endocrine glands, and the heart. Should this hypothesis be confirmed in larger studies, it will further delineate the pathogenesis of iron toxicity in patients with thalassemia and may change the therapeutic approach used for these patients. Novel therapies and adjustment of transfusion schedules aiming to increase hepcidin expression may reduce intestinal iron absorption and deter release of iron from reticuloendothelial cells into the circulation, thus reducing transferrin saturation and NTBI formation. This approach might prove more effective than iron chelation alone in preventing iron overload toxicity.

AK: designed the study, analyzed the data and wrote the manuscript; IP: performed the chemistry analysis, analyzed the data and wrote the manuscript; DP: performed the molecular studies; FA: performed the chemistry analysis; AG: contributed to the sample collection, data analysis and the patients' care; VL: contributed in the sample collection, data analysis and the patients' care; NS: co-ordinated the molecular studies and edited the manuscript; GP: designed the study, performed the molecular analysis and wrote the manuscript. The authors declare that they have no potential conflicts of interest. *This study was financially supported by University of Athens Grants No 70/4/4256 and 70/3/6900 (to AK) and the General Secretariat for Research and Technology of Greece (GSRT) grant SP YB 13 (to NS).*

We are grateful to Elizabeta Nemeth and Tomas Ganz (University of California, USA) for evaluating urinary hepcidin.

Manuscript received January 13, 2006. Accepted April 7, 2006.

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