Increased plasma transferrin, altered body iron distribution, and microcytic hypochromic anemia in ferrochelatase-deficient mice

Saïd Youmí,1 Marie Abitbol,2 Valérie Andrieu,3 Dominique Henin,4 Elodie Robert,5 Caroline Schmitt,1 Laurent Gouya,1 Hubert de Verneuil,5 Jean-Charles Deybach,1 Xavier Montagutelli,2 Carole Beaumont,1 and Hervé Puy1

1Institut National de la Santé et de la Recherche Médicale (INSERM) Unité (U) 773, Centre de Recherche Biomédicale Bichat Beaujon CRB3, Paris; Université Paris 7 Denis Diderot; and Centre Français des Porphyries, Hôpital Louis Mourier, Colombes, France; 2Institut Pasteur, Unité de Génétique des Mammifères, Paris, France; Assistance Publique–Hôpitaux de Paris (AP–HP), Hôpital Bichat, Laboratoire d’hématologie and Laboratoire d’anatomie-pathologie, Paris, France; 3INSERM E0217 and Université V Segalen Bordeaux 2, Bordeaux, France

Patients with deficiency in ferrochelatase (FECH), the last enzyme of the heme biosynthetic pathway, experience a painful type of skin photosensitivity called erythropoietic protoporphyria (EPP), which is caused by the excessive production of protoporphyrin IX (PPIX) by erythrocytes. Controversial results have been reported regarding hematologic status and iron status of patients with EPP. We thoroughly explored these parameters in Fechm1Pas mutant mouse of 3 different genetic backgrounds. FECH deficiency induced microcytic hypochromic anemia without ringed sideroblasts, little or no hemolysis, and no erythroid hyperplasia. Serum iron, ferritin, hepcidin mRNA, and Dcytb levels were normal. The homozygous Fechm1Pas mutant involved no tissue iron deficiency but showed a clear-cut redistribution of iron stores from peripheral tissues to the spleen, with a concomitant 2- to 3-fold increase in transferrin expression at the mRNA and the protein levels. Erythrocyte PPIX levels strongly correlated with serum transferrin levels. At all stages of differentiation in our study, transferrin receptor expression in bone marrow erythroid cells in Fechm1Pas was normal in mutant mice but not in patients with iron-deficiency anemia. Based on these observations, we suggest that oral iron therapy is not the therapy of choice for patients with EPP and that the PPIX–liver transferrin pathway plays a role in the orchestration of iron distribution between peripheral iron stores, the spleen, and the bone marrow. (Blood. 2007;109:811-818)

Introduction

Erythropoietic protoporphyria (EPP; Mendelian Inheritance in Man [MIM] 177000) is an inherited disorder caused by partial deficiency of ferrochelatase (FECH; EC 4.99.1.1.), the last enzyme of the heme biosynthetic pathway.1 FECH is an inner membrane mitochondrial enzyme catalyzing the insertion of ferrous iron into protoporphyrin IX (PPIX) to form heme. FECH deficiency in bone marrow erythroid cells is responsible for the primary overproduction of PPIX, leading to an accumulation of protoporphyrin in the bone marrow, plasma, erythrocytes, skin, bile, and feces.2 Because of its hydrophobic nature, PPIX can be removed from the body only through the liver, where it is secreted into bile and then is excreted by fecal elimination.3 More than 100 mutations in the FECH gene, including missense, nonsense, splicing, deletions, and insertions, have been identified in EPP families (Human Gene Mutation Database, http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). Usually, EPP is inherited as an autosomal pseudodominant disorder, and clinical penetrance is mainly modulated by the presence of a common intronic single-nucleotide polymorphism (SNP), IVS3–48C, in trans to a dominant mutation.4,5

The most common clinical manifestation of EPP is lifelong acute photosensitivity of sun-exposed skin appearing in early childhood.6 Although EPP is generally a benign disease, hepatic complications such as cholelithiasis or, in rare cases (approximately 2%), rapid fatal liver disease with cirrhosis may occur.7,9 Chronic liver disease is associated with marked PPIX accumulation in liver, which can begin insidiously.10 The source of the excessive amounts of PPIX in patients with EPP is, according to most authors, the bone marrow, and mild anemia is observed in 20% to 50% of EPP patients.11,12 Pathogenesis of the hematologic symptoms is not yet fully understood, and controversial hypotheses have been reported about its origin. Two EPP mouse models have been reported. A FECH exon 10 deletion was generated by gene targeting, resulting in a dominant-negative effect and embryonic lethality of homozygotes.13 The best animal model is an ENU-induced point mutation, ferrochelatase deficiency (Fechm1Pas; fch). The mutation shows a fully recessive transmission. A T→A substitution at position 293 replaces a methionine with a lysine at residue 98.14,15 In the BALB/cByJCrI genetic background, to which the mutation was originally backcrossed, homozygote mice show 5% residual FECH activity in the liver and spleen and develop skin lesions, jaundice, and severe hepatic dysfunction with massive PPIX deposits. This model, which mimics the severe forms of the disease, has been used to show that gene therapy and cellular therapy may greatly improve the condition,16 and it represents a useful model for studying the pathophysiological feature of the human disease.17 However, iron metabolism has never been extensively investigated in this model. Ferrochelatase has 2...
substrates, iron and PPIX, and only PPIX seems to accumulate in excess.

Therefore, we decided to characterize more details of the hematologic and iron phenotypes of the Fech<sup>min/</sup> mutation on 3 different genetic backgrounds, namely BALB/cByJCr, C57BL/6JCr, and SJL/JOrJCr. Our results showed that the impact of the mutation on erythropoiesis was primarily microcytic hypochromic anemia without ringed sideroblasts or significant reticulocytosis. This study provides evidence for normal total body iron associated with redistribution of iron from peripheral tissues to the spleen, which is unexplained by hemolysis or spleen erythropoiesis recovery. We investigated this peculiar pathophysiological process and found that PPIX might act as a signaling molecule, stimulating the production of transferrin by the liver to facilitate the mobilization of tissue iron stores.

Materials and methods

Production and maintenance of congenic strains

Wild-type BALB/cByJCr, C57BL/6JCr, and SJL/JOrJCr mice (hereafter BALB/c, C57BL/6, and SJL, respectively) were purchased from Charles River Laboratories (L’Arbresle, France). The original Fech<sup>min/</sup> mutation had been previously backcrossed to the BALB/c inbred background for more than 10 generations. Congenic strains were developed similarly on C57BL/6 and SJL mice, with 10 generations of backcrossing to the recipient strain. At each backcross generation, and in further crosses, mouse genotypes were identified by amplification of a genomic segment encompassing the point mutation, which removed a BspHI restriction site. PCR products were produced and digested as previously described.17 Mutant mice were maintained in the Animal Facility of Institut Pasteur. According to standard husbandry procedure, they were maintained in filter-top cages with artificial fluorescent light, under a 12-hour light/dark cycle. They received unlimited amounts of autoclaved water and irradiated food pellets (standard laboratory mouse chow; AO3; SAFE, Augy, France). Two series of mice were bred and analyzed. The first series included 6 groups (+/+ and fch/fch for each congenic strain) of 12 to 14-week-old female mice analyzed for hematologic parameters. In the second series, BALB/c+/+ and fch/fch mice were analyzed at 12 to 14 weeks of age (6 females per group) for biochemical and iron parameters. For phenthyradiamine, phlebotomy, or PPIX treatments, 12- to 14-week-old control female mice were purchased from Charles River Laboratories. All procedures on animals were performed in compliance with the French and European regulations on Animal Welfare and with Public Health Service recommendations.

Phenylhydrazine, phlebotomy, and PPIX treatments

Phenylhydrazine hydrochloride (Sigma Chemical, St Louis, MO), a potent hemolytic agent, was dissolved in phosphate-buffered saline (PBS) at 20 mg/mL and was pH adjusted to 7.4 with NaOH. Wild-type BALB/c female mice, 12 to 14 weeks of age (n = 10), received intraperitoneal injection of freshly prepared phenylhydrazine (80 μg each). Animals were killed 2 days after the final injection. Blood was collected through the vena cava and was centrifuged, and serum was frozen at −20°C until assayed for haptoglobin. A 20-mM stock solution of PPIX (Sigma) was prepared by dissolving PPIX in 0.2 M trisodium orthophosphate buffer and was adjusted to pH 7.6, filtered through a 2-μm bacteriologic filter, and stored in the dark. Twelve- to 14-week-old wild-type BALB/c female mice (n = 7) received intraperitoneal injection of 500 μL of this solution daily for 14 consecutive days and were killed 24 hours after the final injection.

Cell culture and PPIX treatment

Human HepG2 hepatoma cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and seeded at 10<sup>6</sup> cells/mL. After 24 hours in culture, the cells were either scraped directly in RNA Plus Extraction Solution Kit (Quantum Biotechnologies, Illkirch, France) (T0) or were grown for another 24 or 48 hours in the presence of 30 μM PPIX before RNA extraction.

Hematologic and iron status parameters

Mice were anesthetized by intraperitoneal injection of a xylazine/ketamine mixture and were weighed. Blood was collected by puncture of the orbital sinus. Red blood cell (RBC) count, hemoglobin (HB) level, hematocrit (Hct), mean cell volume (MCV), and mean cell content in Hb (MCHC) were measured with an SCIL Vet’ABC counter (SCIL, Viernheim, Germany). Protoporphyrin levels in RBCs and in stool were determined by a method adapted from Poulos and Lockwood.18 FECH activities were determined by synthesis of mesoporphyrin-Zn, adapted from Li et al.20 Final mesoporphyrin-Zn concentration was measured with the use of a spectrophotometer (RF540; Shimadzu, Kyoto, Japan) with 410-nm excitation and 580-nm detection. Serum iron, ferritin, and transferrin levels were measured (AU400 automate; Olympus, Tokyo, Japan). Human reagents calibrated with commercial mouse transferrin or recombinant mouse ferritin were used as previously described.20 Serum haptoglobin level was measured with rabbit antihaptoglobin antibodies. Direct sandwich ELISA for mouse serum haptoglobin was developed with the use of affinity-purified reagents, as previously described.21 The working range of the haptoglobin standard curve was 0.02 to 0.5 μg/mL. Tissues were isolated from fch/fch mice and their control wild-type littermates and were fixed in 3.5% formaldehyde for 3 to 5 hours. Fixed tissues were then subjected to routine histologic processing, and the sections were stained with Perls Prussian blue for the detection of tissue iron. Bone marrow smears were analyzed in the same conditions. Tissue iron content was determined by acid digestion of tissue samples, as described by Torrance and Bothwell,22 followed by iron quantification (IL test; Instrumentation Laboratory, Lexington, MA) on an AU400 automate (Olympus).

Electron microscopy

For electron microscopy, tissues were cut in 1-mm<sup>3</sup> blocks and were immediately fixed in 2.5% glutaraldehyde-buffered solution (PBS, pH 7.4) for 2 hours at 4°C. After washing in PBS, blocks were postfixed for 2 hours in 1% buffered osmium tetroxide solution, dehydrated in graded series of ethanol, and embedded in epoxy resin. Semithin sections stained with toluidine blue were made on each block for orientation. Ultrathin sections stained with uranyl acetate and lead citrate were examined under an electron microscope (JEOL 1010; JEOL, Tokyo, Japan) equipped with an SIS MegaView digital camera (Olympus, Münster, Germany). In some cases, counterstaining was omitted to identify electron-dense iron-containing granules.

Assay of ferrireductase activity in duodenal brush-border membranes

A 2-cm fragment of upper intestine was excised, thoroughly rinsed with sterile 9 g/L NaCl, and scraped with a glass blade according to the Kessler method, as adapted by Simpson and Peters.23 Briefly, the scraped mucosa was suspended in 50 mM mannitol and 2 mM HEPES-NaOH, pH 7.1 (30 mL/g tissue). The mucosal suspension was homogenized for 2 minutes in a chilled blender, solid MgCl<sub>2</sub> was added to a final concentration of 10 mM, and the homogenate was stirred on ice for 20 minutes before centrifugation at 3000g for 10 minutes. The resultant supernatant was centrifuged at 40 000g for 40 minutes, and the pellet was then subjected to resuspension buffer (100 mM mannitol, 100 mM NaCl, 100 mM MgSO<sub>4</sub>, 20 mM HEPES-NaOH, pH 7.4, 20 μL/g mucosa weight). The suspension was centrifuged at 6000g for 20 minutes, and the resultant supernatant was centrifuged at 40 000g for 40 minutes. The final vesicle pellet was suspended in resuspension buffer at an approximate concentration of 10 mg/mL and was stored at −80°C. Protein concentration was determined in triplicate with a protein assay (Bio-Rad, Hercules, CA) and BSA as standard. Ferricyanide-reducing activity was determined at room temperature by measuring the disappearance of the chromogenic substrate ferricyanide, as optimized by Pouniet et al.24 Briefly, 50 μL and 20 μL, respectively, freshly prepared NADH and FMN solutions were added to a 1.5-mL cuvette containing 1 mL of 0.16 mM ferricyanide/100 mM HEPES–NaOH (pH 7.1) and 1 mL 4% lauryl maltoside (0.03% wt/vol), and
the basal ferricyanide reduction rate was recorded. Fifty-microliter aliquots of brush-border membrane vesicles were then added, and, with the use of an extinction coefficient of 1020 M\(^{-1}\) cm\(^{-1}\) for ferricyanide, the specific activity was calculated in μmoles · e\(^{-}\) transferred × min\(^{-1}\) × μg protein\(^{-1}\). A basal rate of reduction attributable to the direct reduction of ferricyanide by NADH was subtracted from the assay rate before specific activity was calculated.

### RNA extraction and quantitative RT-PCR

Total RNA from liver and duodenum were isolated using RNA Plus Extraction Solution Kit (Quantum Biotechnologies, Illkirch, France). The purity and yield of total RNA were determined spectrophotometrically, and the integrity of RNA bands (18S and 28S) was checked on agarose gel electrophoresis. Single-stranded cDNA was synthesized using SuperScript RNase H\(^{-}\) Reverse Transcriptase (Invitrogen Life Technologies, Cergy-Pontoise, France). Real-time quantification of transcripts was performed in 25 μL in ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Courtaboeuf, France) using SYBR Green PCR master mix (PE Applied Biosystems), 5 pmol forward and reverse primers, and 2.5 μL reverse transcriptase reaction mixture, as previously described. Sequences of the primers were as follows: Hepc1 (171 bp), 5'-CTCATCCCTCAACAGGATG-3' (forward) and 5'-AAGAATCCCATACACTGAAA-3' (reverse); transferrin (Tf; 69 bp), 5'-TGTAGCCTTTGTGAAACACCAGA-3' (forward) and 5'-TCGGCAGGGTTCTTTCCTT-3' (reverse); Dcytb (98 bp), 5'-GAGCTTCCCGAGACCCCTG-3' (forward) and 5'-CATGCCCATCATTGAGCACCTTTG-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase (Gapdh; 177 bp), 5'-TGACACCAACTGTTAG-3' (forward) and 5'-GAATGCGGATGATGTTC-3' (reverse).

The result was normalized arbitrarily on the sample with the lowest C\(_T\) value for Gapdh (named C\(_T\)\(_{\text{Gapdh R}}\)). For all other samples, relative quantification was calculated using the comparative C\(_T\) method with the arithmetic formula \((1 + E_{\text{Gapdh}}/C_{\text{Gapdh}}) \times C_{\text{Gene}} - C_{\text{Gene}}\) \((1 + E_{\text{Gapdh}}/C_{\text{Gapdh}})\) \((1 + E_{\text{Gapdh}}/C_{\text{Gapdh}})\), where \(E_{\text{Gapdh}}\) is the efficiency of Gapdh target amplification and \(E_{\text{X}}\) the efficiency of amplification of the gene of interest. \(C_{\text{Gene S}}\) and \(C_{\text{Gene X}}\) are the respective threshold cycles for Gapdh and for the gene of interest of every sample (S) except the reference sample (R); \(C_{\text{Gene S}}\) and \(C_{\text{Gene X}}\) are the respective threshold cycles for Gapdh and for the gene of interest of every sample (S) except the reference sample (R). Amplification efficiency of each target was determined using serial 2-fold dilutions of cDNA.

### Cell preparation and flow cytometry analysis

Bone marrow cells were extracted from the femurs and tibias of BALB/c fch/fch and their wild-type littermates \((n = 6\) for each group), and a single-cell suspension was made by gentle passage of the bone marrow cells through an 18-gauge needle. Cells were pelleted by centrifugation, washed, and resuspended at 10\(^6\) cells/mL in 37°C DMEM containing 2% FBS and 1 mM HEPES and were incubated for 90 minutes at 37°C. Cells were pelleted and maintained at 4°C before fluorescence-activated cell sorter analysis (FACS). FACS analysis for transferrin receptor (CD71) of bone marrow erythroid cells was performed on a FACSCalibur (BD Biosciences, Le Pont de Claix, France). Bone marrow cells were labeled with PE-conjugated anti-Ter-119 (BD Biosciences) and FITC-conjugated anti-CD71 (Serotec, Oxford, UK) antibodies. RBCs (small cells), intermediate normoblasts (cells of intermediate size), and early normoblasts (large cells) were gated based on their FCS and SSC profiles. A ratio of the mean fluorescence intensity (MFI) of fch/fch cells and their wild-type littermates was used to normalize Ter-119 expression by FACS. Comparative analyses of fluorescence intensity were performed on the same FACS machine with regular calibration standards and constant voltage for each cell genotype, as previously described.

### Statistical analysis

Statistical significance was evaluated using the unpaired, 2-tailed Student t test for comparison between 2 means. Correlations were performed by linear regression. GraphPad Prism software (GraphPad Software, San Diego, CA) was used for statistical evaluation.
Table 1. Hematologic parameters in 14-week-old +/+ and fch/fch mice in the three congenic strains

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ht, proportion of 1</th>
<th>Hb level, g/L</th>
<th>RBC count, × 10^12/L</th>
<th>MCV, fl</th>
<th>RET, % RBC</th>
<th>MCHC, g/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>0.481 ± 0.001</td>
<td>164 ± 3</td>
<td>10.2 ± 0.2</td>
<td>51.1 ± 2.1</td>
<td>2.5 ± 0.6</td>
<td>33.6 ± 0.4</td>
</tr>
<tr>
<td>fch/fch</td>
<td>0.356 ± 0.017*</td>
<td>104 ± 6</td>
<td>7.7 ± 0.5*</td>
<td>43.9 ± 1.0*</td>
<td>8.8 ± 3.4†</td>
<td>28.7 ± 1.2†</td>
</tr>
<tr>
<td>SJL/J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>0.434 ± 0.016</td>
<td>140 ± 9</td>
<td>9.3 ± 0.4</td>
<td>46.8 ± 1.0</td>
<td>3.9 ± 1.8</td>
<td>31.9 ± 0.8</td>
</tr>
<tr>
<td>fch/fch</td>
<td>0.338 ± 0.017*</td>
<td>126 ± 4†</td>
<td>7.1 ± 0.3*</td>
<td>44.1 ± 1.0†</td>
<td>6.5 ± 1.1†</td>
<td>29.9 ± 0.5‡</td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>0.405 ± 0.035</td>
<td>127 ± 1</td>
<td>8.6 ± 0.5</td>
<td>47.8 ± 1.4</td>
<td>4.8 ± 2.6</td>
<td>31.4 ± 0.5</td>
</tr>
<tr>
<td>fch/fch</td>
<td>0.325 ± 0.025†</td>
<td>114 ± 2‡</td>
<td>7.3 ± 0.3†</td>
<td>42.6 ± 1.9†</td>
<td>6.5 ± 1.0‡</td>
<td>30.3 ± 1.3‡</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of 6 mice per group. RBC, Hb, Ht, MCV, RET, and MCHC in female FECH-deficient mice (fch/fch) and wild-type (+/+ ) mice.

*P < .001.
†P < .01.
‡Not significant.

There was a modest increase in reticulocyte count only in the BALB/c genetic background (2.5% ± 0.6% in +/+ mice compared to 8.8% ± 3.4% in fch/fch mice; P < .001), suggesting that this anemia is poorly regenerative. However, bone marrow smears did not reveal major abnormalities in the erythroid precursors, nor did they reveal an increased proportion of erythroblasts (Figure 2). Perls staining was negative in the 3 genetic backgrounds, with no iron-loaded macrophages and no ringed sideroblasts. Electron microscopy of the spleen did not show any iron deposits in the cytosol or in the mitochondria of erythrocytes. The FECH defect, which inhibits iron incorporation into PPIX, was not associated with abnormal accumulation of iron in erythroid cells.

Given that the accumulation of free protoporphyrin in RBCs could generate oxidative stress and membrane alterations, we measured serum haptoglobin levels as a marker of hemolysis. In BALB/c mice in which anemia was more severe, the haptoglobin levels were identical to those in fch/fch mice (0.26 g/L ± 0.1) compared with their control littermates (0.30 ± 0.05 g/L). On the contrary, phenylhydrazine-induced hemolysis led to a complete drop in haptoglobin level, to an almost undetectable level (less than 0.002 g/L).

These data indicate that ferrochelatase deficiency alters blood homeostasis and induces microcytic hypochromic anemia with an absence of sideroblasts, little hemolysis, and no bone marrow or spleen erythroid hyperplasia. This pattern is more pronounced in BALB/c fch/fch mice.

Iron metabolism and ferrochelatase deficiency

We explored iron metabolism in the BALB/c genetic background, which shows the more pronounced microcytic hypochromic anemia. The results of iron metabolism studies are shown in Table 2 and Figure 3. No difference was observed in serum iron levels between control (29.7 ± 3.5 μM) and fch/fch mice (31.8 ± 2.7 μM), and no difference was observed in serum ferritin levels (46 ± 13.2 μg/L in controls vs 66 ± 19.5 μg/L in mutant mice; Table 2). Surprisingly, there was a significant 2-fold reduction in tissue iron load in fch/fch mice compared with wild-type (~46.6% in the liver, ~45.8% in kidney, and ~57.9% in heart; P < .001 in all 3 tissues), whereas there was no significant difference in spleen iron between both groups (Figure 3A). Spleens of the mutant animals were grossly enlarged, with a 2- to 3-fold increase in the spleen index (spleen weight/body weight × 100), as previously reported. This prompted us to calculate the iron load of each organ and the total body iron by taking into account the weight of the organ and the tissue iron concentration. The total amount of iron in the liver, heart, and kidney remained lower in the fch/fch than in wild-type animals (Figure 3B), whereas in the spleen, the total amount of iron was increased 2.3-fold. However, when we added up the total amount of iron in the liver, spleen, heart, and kidney of these animals, and considering that these are the major sites of iron stores, it clearly appeared that there was no difference in overall body iron stores between wild-type and fch/fch mice (Figure 3B). We also performed histologic examination of the spleen, which did not show major modifications between the 2 groups, with no increased proportion of erythroid precursors in the fch/fch mice. However, Perls staining revealed changes in spleen iron distribution in the mutant mice (Figure 3C). In agreement with the results of iron quantification, staining intensity was not increased in mutant mice, but the blue iron deposits appeared concentrated in the perifollicular zone surrounding the white pulp follicle, whereas they were scattered throughout the red pulp in the control mice. The perifollicular zone is thought to be the preferred site of spleen erythropoiesis. However, by electron microscopy, electron-dense iron deposits were found in macrophages in wild-type (not shown) and fch/fch mice (Figure 3C). Altogether, these data demonstrate that the microcytic anemia associated with ferrochelatase deficiency was not caused by iron deficiency but was accompanied by a redistribution of iron from the liver to the spleen, which could be mediated by the onset of spleen erythropoiesis.

Figure 2. Bone marrow smears. Hematoxylin-eosin staining of bone marrow smear. Original magnification: × 25 (A, C) × 63 (B, D) from wild-type (A-B) and fch/fch (C-D) 14-week-old BALB/c mice. No structural abnormalities, no erythroid hyperplasia, and no ringed sideroblasts were observed in fch/fch mouse. EB indicates erythroblast; PMN, polymorphonuclear cell; L, lymphocyte. Images were taken with a DMRB microscope equipped with a Leica objective lens (40×/1.00; Leica Microsystems, Rueil Malmaison, France) and connected to a Sony CCD DXC 950P camera (Sony, Cliche, France). Images were processed using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA).
Hepcidin mRNA and ferrireductase activity in duodenal enterocytes are not modified in fch/fch mice

Hepcidin is a regulatory peptide secreted into the plasma by hepatocytes. Its expression is repressed in the course of anemia or iron deficiency. Because the mutant mice were anemic but not iron deficient, we measured liver hepcidin mRNA by quantitative RT-PCR. When the results were normalized to Gapdh expression, hepcidin mRNA levels were similar in fch/fch (1.2 ± 0.1) mice and in their control littersmates (0.8 ± 0.5) (Table 2), indicating that the signaling pathway that triggered hepcidin repression in conditions of anemia was altered when heme synthesis was impaired. Similar results were obtained when the results were normalized by S14 mRNA (not shown). Although we found no difference in the total amount of iron between wild-type and mutant mice, suggesting that iron absorption was normal, we wanted to rule out the possibility that in FECH-deficient mice, heme would become rate limiting for Dcytb, a di-cytochrome b5 reductase thought to be an important component of duodenal ferrireductase activity. We measured ferrireductase activity of duodenal brush-border membranes but found no difference between wild-type and mutant duodenum (Table 2). Dcytb mRNAs were also similar in both conditions. These data confirm the absence of iron deficiency and suggest that there is no up-regulation of intestinal iron absorption in FECH-deficient mice.

Relationship between erythrocyte protoporphyrin and transferrin levels

A new and striking observation is the elevation of serum transferrin levels in FECH-deficient mice. In the course of our investigations on iron homeostasis, we also measured serum transferrin levels by

### Table 2. Iron parameters in 14-week-old +/- and fch/fch BALB/c mice

<table>
<thead>
<tr>
<th>Biological parameter</th>
<th>BALB/c +/+</th>
<th>BALB/c fch/fch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin, g/L</td>
<td>1.4 ± 0.1</td>
<td>2.8 ± 0.1*</td>
</tr>
<tr>
<td>Transferrin saturation, %</td>
<td>87 ± 8</td>
<td>44 ± 3*</td>
</tr>
<tr>
<td>Ferritin, µg/L</td>
<td>46 ± 13.2</td>
<td>66 ± 19.5†</td>
</tr>
<tr>
<td>Iron, µM</td>
<td>29.7 ± 3.5</td>
<td>31.8 ± 2.7†</td>
</tr>
<tr>
<td>Haptoglobin, g/L</td>
<td>0.3 ± 0.05</td>
<td>0.26 ± 0.1††</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepcidin/Gapdh mRNA</td>
<td>0.8 ± 0.5</td>
<td>1.2 ± 0.1††</td>
</tr>
<tr>
<td>Duodenum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dcytb/Gapdh mRNA</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.4††</td>
</tr>
<tr>
<td>Ferrireductase activity, µmoles · e⁻ transferred · min⁻¹ · µg protein⁻¹</td>
<td>0.25 ± 0.03</td>
<td>0.21 ± 0.05††</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of 10 mice per group in FECH-deficient (fch/fch) and wild-type (+/+) BALB/c female mice.

*P < .01. †Not significant.

Figure 3. Tissue nonheme iron contents in BALB/c fch/fch mice and their control littersmates. (A) Tissue nonheme iron per gram weight tissue was measured in liver, spleen, kidney, and heart of wild-type (■) or fch/fch (□) 12- to 14-week-old females. (B) Total iron content in each organ calculated as tissue nonheme iron content per weight of tissue × organ weight. Total iron contents in fch/fch compared with wild-type were significantly decreased in liver, kidney, and heart and increased in spleen, leading to a redistribution of iron from the organs to the spleen in fch/fch mice. Mean ± SD is shown for 6 mice per genotype. Asterisks indicate the significance of the influence of the fch mutation on the modifications of each parameter (*P < .01; **P < .001; n.s., not significant). (C) Perls staining of the spleens of BALB/c wild-type and fch/fch mice. Comparable intensity of the iron stain is observed in fch/fch mice compared with their wild-type littermates, associated with regular iron coleration, more condensed in the perifollicular zone surrounding the white pulp follicle. Original magnification, × 25. Images were taken with a 10 × 0.1 PL Fluotar objective lens attached to a DMRB microscope (Leica Microsystems). The numerical aperture of the lens was 10 × 0.30. Image digitization was performed with a Tri CCD Sony camera (Sony) with TRIBVN ICS Software (TRIBVN, Châtillon, France). Images were processed using Adobe Photoshop 5.0. wp indicates white pulp; pz, perifollicular zone; gc, germinal center; Ta, T lymphocyte area. (D) Electron microscopy of the spleen from a fch/fch mouse. At this low magnification, several spleen cells are visible. M indicates macrophage; L, lymphocyte; N, neutrophil. Original magnification, × 3000. Electron-dense intracytoplasmic vesicles containing iron deposits are visible in macrophages (arrows), especially at higher magnification (inset; original magnification, × 10 000).
qRT-PCR in HepG2 cells grown in the absence (0) or in the presence (24 h, 48 h) of PPIX-injected mice (n studied, including controls, wild-type animals, FECH mutants, and erythrocyte protoporphyrin when considering all the animals highly significant correlation between serum transferrin levels and iron-deficient anemia, they had increased protoporphyrin levels. Therefore, we hypothesized that protoporphyrin might induce transferrin expression by the liver. We injected PPIX intraperitoneally into normal BALB/c mice and measured serum transferrin levels. Erythrocyte protoporphyrin levels increased from 3000 nM in control mice to 8000 nM in PPIX-injected mice, as opposed to 8% in control mice to 44% ± 3% in fch/fch mice (P < .001). Similarly, hepatic transferrin mRNA was increased 2-fold in mutant mice (Figure 4A). Iron-deficient anemia is known to increase serum transferrin levels and to reduce transferrin saturation in human patients and in mouse models. Our results showed that the FECH-deficient mice that had hypochromic microcytic anemia but were not deficient in iron also had increased serum transferrin levels. However, like mice with iron-deficient anemia, they had increased protoporphyrin levels. Therefore, we hypothesized that protoporphyrin might induce transferrin expression by the liver. We injected PPIX intraperitoneally into normal BALB/c mice and measured serum transferrin levels. Erythrocyte protoporphyrin levels increased from 3000 nM in control mice to 8000 nM in PPIX-injected mice, as opposed to 30 µM PPIX for 24 or 48 hours.

Iron deficiency is also known to increase transferrin receptor 1 (TfR1 or CD71) expression at the cell surface of erythroid cells. Therefore, we used FACS analysis to compare TfR1 expression on bone marrow erythroid cells between wild-type and fch/fch mice. Three populations containing cells with increasing stages of erythroid differentiation were detected by gating with side scatter and forward scatter (Figure 5), consisting of early normoblasts (III), intermediate normoblasts (II), and RBCs (I).

Erythroid cells were identified by their positive Ter-119 expression (Figure 5). The number of CD71+ Ter-119+ cells was not significantly different between wild-type and fch/fch mice at each stage of differentiation. Furthermore, the mean fluorescence intensity of double-positive cells was not significantly different between wild-type and fch/fch mice (not shown), suggesting that, despite the presence of microcytic anemia in fch/fch mice, TfR1 expression in erythroid cells was not increased.

Discussion

Mild anemia is known to occur in 20% to 50% of patients with EPP but controversial hypotheses have been put forward about its origin. In addition, little is known about iron, the other substrate of FECH enzyme, whereas PPIX overproduction has been extensively studied in human and animal models.

In this study, we characterized the anemia of the FECH mouse mutant and provided some evidence of a concomitant increase in serum transferrin levels and iron redistribution from storage sites to sites of erythropoiesis. Usually the anemia in EPP has been described as microcytic and normochromic to slightly hypochromic, without sideroblasts. In a few patients, anisocytosis has been observed among the RBCs. The erythrocytes do not seem to have increased fragility or osmotic resistance. Indeed, hemolysis has been reported in only a few patients with splenomegaly associated with cirrhosis of...
end-stage protoporphyria hepatopathy, in whom the destruction of fragile porphyrin-loaded RBCs may give rise to hemolytic anemia.\textsuperscript{12} Accordingly, we found no evidence of abnormal erythocyte shape on bone marrow smears and no major signs of hemolysis because haptoglobin levels were normal. A few reports have been published of sideroblastic anemia in patients with clinical EPP.\textsuperscript{36–40} However, in these reports, either the decreased ferrochelatase activity was not formally established, the nature of the defect was not reported,\textsuperscript{36} or the cases were not classical EPP.\textsuperscript{40} We found no evidence of ringed sideroblasts in the bone marrow smears of the \textit{fch/fch} mice or in spleen erythrocytes.

The anemia we observed in these animals was hypochromic and microcytic, and the severity of the anemia was more pronounced in BALB/c mice than in C57BL/6 or SJL/L mice. Heme deficiency is the likely cause of this microcytosis, but one critical issue is whether it results from iron mismanagement or from ferrochelatase deficiency.

Depletion of iron stores is reported in the literature as a relatively common event in EPP,\textsuperscript{41} although no extensive data are available in this disorder, neither on tissue iron nor on the regulation of iron. Although we did not measure intestinal iron absorption, several lines of evidence suggest that it was not modified in the \textit{fch/fch} mice. First, no tissue was iron deficient because the total body iron, estimated by adding the total amount of iron in the 4 main iron storage compartments (liver, spleen, heart, and kidney) was identical in wild-type and mice. Second, ferrireductase activity of the duodenal brush border membranes was not modified in the mutant mice, suggesting that in FECH-deficient mice, the rate of heme absorption, several lines of evidence suggest that it was not modified in the \textit{fch/fch} mice. First, no tissue was iron deficient because the total body iron, estimated by adding the total amount of iron in the 4 main iron storage compartments (liver, spleen, heart, and kidney) was identical in wild-type and \textit{fch/fch} animals. However, a redistribution of iron with a 2- to 3-fold reduction in tissue iron did occur in most organs, in concentration and total amount per organ, with a concomitant increase in spleen iron. Second, ferrireductase activity of the duodenal brush border membranes was not modified in the mutant mice, suggesting that in FECH-deficient mice, the rate of heme synthesis in nonerythroid tissue was sufficient to sustain the enzyme activity of the heme-containing b-type cytochrome reductase(s). Third, hepcidin mRNA levels were not modified in the ferrochelatase mutant mice, contrary to what was observed in thalassemia. Hepcidin is considered the negative regulator of intestinal iron absorption, and its synthesis is repressed by iron deficiency, stimulation of erythropoiesis, and hypoxia but is increased by liver iron overload and inflammation (for a review, see Ganz and Nemeth\textsuperscript{48}). In dyserythropoietic syndromes such as thalassemia, hepcidin expression is suppressed\textsuperscript{49} (hence, the characterization of these syndromes as iron-loading anemia). However, there were no signs of dyserythropoiesis in our ferrochelatase mutant mice, as shown by the increased reticulocytosis and the absence of erythroid maturation defects in the bone marrow. This is compatible with normal hepcidin levels. Furthermore, normal haptoglobin levels assessed the absence of significant extramedullary hemolysis. Normal hepcidin mRNA levels could also have resulted from conflicting signals that could neutralize each other. On the one hand, the discrete reduction in hepatic iron stores might trigger hepcidin synthesis, and protoporphyria accumulation in the liver might create a proinflammatory response and contribute to the stimulation of hepcidin expression. On the other hand, an apparent systemic iron deficiency, highlighted by a 2-fold reduction in transferrin saturation, might counterbalance these stimulating signals and achieve normal hepcidin gene expression. It has indeed been proposed that transferrin saturation—more specifically, di-ferric transferrin—plays a major role in directing changes in hepcidin expression.\textsuperscript{53}

The apparent systemic iron deficiency results in fact from a 2-fold increase in transferrin expression, at the mRNA and the protein levels, whereas serum iron levels are unchanged. This is reminiscent of what is observed in true iron-deficiency anemia, in which erythrocyte PPIX and serum transferrin levels are elevated.\textsuperscript{40} Therefore, we tested the hypothesis that PPIX could stimulate transferrin synthesis. Indeed, PPIX injection into normal mice increased serum transferrin levels. An almost perfect correlation existed between erythrocyte PPIX and serum transferrin levels in all animals, including wild-type, FECH mutant, and commercial BALB/c mice before and after intraperitoneal PPIX injection. These results raise the intriguing possibility that serum PPIX acts as a sensor of iron supplies to erythroid cells, signaling to the liver to stimulate transferrin synthesis when these supplies are insufficient. However, we were unable to reproduce these results in vitro in cultured hepatoma cells, suggesting that this signaling pathway might be indirect. Finally, the absence of abnormal iron deposits in erythroblast mitochondria is intriguing because a defective step in intramitochondrial iron use usually leads to abnormal iron deposits.

Molecular defects in the erythroid-specific 5-aminoluvulinate synthase gene in humans\textsuperscript{45} and in mice (e-Alas)\textsuperscript{45} and in mutations in \textit{ABC7},\textsuperscript{46} a mitochondrial exporter of iron-sulfur clusters, are characterized by the formation of ringed sideroblasts. In yeast, mutants defective in one enzyme of the iron-sulfur cluster assembly also contain abnormal mitochondrial iron deposits.\textsuperscript{47} Therefore, in ferrochelatase deficiency, some negative feedback mechanisms might down-regulate the iron uptake pathway in the developing erythroid precursors. Despite partial heme deficiency, we found a normal density of TfR1 at the cell surface of \textit{fch/fch} Ter119\textsuperscript{+} bone marrow cells. By contrast, partial e-Alas deficiency in mice induced high levels of TfR1 expression and the formation of abnormal iron deposits.\textsuperscript{45}

In rapidly proliferating cells, when intracellular concentrations of iron are low, TfR1 mRNA is stabilized by the high-affinity binding of trans-acting factors IRP1 and IRP2 to iron-regulatory elements (IREs) in the 3' UTR.\textsuperscript{48} However, it has been suggested recently that in erythroid cells, the stability of TfR1 mRNA is no longer modulated by the IRE/IRP system\textsuperscript{49} but rather that heme deficiency contributes to the regulation of \textit{TfR1} gene expression.\textsuperscript{45} Therefore, it is tempting to speculate that in the FECH mutant erythroid cells, because heme deficiency is not severe, TfR1 mRNA expression is not up-regulated as it is in e-ALAS–deficient cells.

In conclusion, the observation that there is no real iron deficiency in FECH-deficient mice suggests that the benefit of iron therapy to treat the microcytic anemia of EPP patients should be reevaluated, especially because it is likely to be deleterious by exacerbating the cutaneous symptoms.\textsuperscript{50}

**Acknowledgments**

We thank Laetitia Micheli, Olivier Thibaudeau, Françoise Muzeau, and Muriel Rocancourt for excellent technical assistance and Alain Grodet of the Electron Microscopy Service at CRB3.

This work was supported by Groupement d’Intérêt Scientifique (GIS)–Maladie Rares 2005 Research Network on Rare Disorders grant A04155HS.
Authorship

Contribution: All authors participated in designing and performing the research. S.L., C.B., and H.P. wrote the paper. All authors checked the final version of the manuscript.

References


Conflict-of-interest disclosure: The authors declare no competing financial interests.
S.L. and M.A. contributed equally to this work.
Correspondence: Carole Beaumont, INSERM U773, Faculté Xavier Bichat, 16 rue Henri Huchard, 75018, Paris, France; e-mail: beaumont@bichat.insERM.fr.
Increased plasma transferrin, altered body iron distribution, and microcytic hypochromic anemia in ferrochelatase-deficient mice

Saïd Lyoumi, Marie Abitbol, Valérie Andrieu, Dominique Henin, Elodie Robert, Caroline Schmitt, Laurent Gouya, Hubert de Verneuil, Jean-Charles Deybach, Xavier Montagutelli, Carole Beaumont and Hervé Puy