Recent Advances in Erythroid Iron Homeostasis: Implications for Pathophysiology of Microcytic Anemias

Prem Ponka

Department of Physiology
Lady Davis Institute, Jewish General Hospital
McGill University, Montréal, QC, Canada

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Iron

- Indispensable for life
  - O₂ transport (Hb)
  - Electron transfer (Cytochromes)
  - DNA synthesis (Ribonucleotide reductase)
  - Neurotransmitter production (tyrosine hydroxylase)

- Insoluble (10⁻¹⁷M)

- Toxic (Fenton chemistry):
  \[ \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^- \]
Forms of Iron Within Proteins

Two major groups:

- heme

- non-heme, mostly Fe-S clusters

2Fe-2S

4Fe-4S
## Proteins of Iron Metabolism

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAS2/eALAS</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; enzyme of heme synthesis; deficiency leads to x-linked sideroblastic anemia</td>
</tr>
<tr>
<td>Ceruloplasmin (Cp)</td>
<td>Plasma protein with ferroxidase activity; cellular export</td>
</tr>
<tr>
<td>DMT1/DCT1/Nramp2</td>
<td>Membrane Fe&lt;sup&gt;2+&lt;/sup&gt; transporter; cellular uptake</td>
</tr>
<tr>
<td>Duodenal cytochrome b</td>
<td>Membrane ferric reductase; cellular uptake</td>
</tr>
<tr>
<td>(Dcytb)</td>
<td></td>
</tr>
<tr>
<td>Ferritin (Ft; H and L)</td>
<td>Cytosolic Fe storage protein</td>
</tr>
<tr>
<td>Ferrochelatase</td>
<td>Mitochondrial protein; insert Fe into protoporphyrin IX ring to form heme</td>
</tr>
<tr>
<td>Ferroportin1/Ireg1/MTP1</td>
<td>Membrane Fe&lt;sup&gt;2+&lt;/sup&gt; transporter; cellular export</td>
</tr>
<tr>
<td>Frataxin</td>
<td>Involved in mitochondrial iron export</td>
</tr>
<tr>
<td>Heme oxygenase 1</td>
<td>Microsomal protein; recycle Hb iron</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>Plasma peptide; deficiency leads to iron hyperabsorption</td>
</tr>
<tr>
<td>Hephaestin</td>
<td>Membrane Cp homolog; enterocyte export</td>
</tr>
<tr>
<td>HFE</td>
<td>Unknown; binds TfR; mutated in &gt;85% of hereditary hemochromatosis</td>
</tr>
<tr>
<td>IRP (-1 and –2)</td>
<td>Cytosolic iron sensors; post-transcriptional regulation</td>
</tr>
<tr>
<td>Mitochondrial ferritin</td>
<td>Mitochondrial Fe storage; H-Ft homolog</td>
</tr>
<tr>
<td>Mitoferrin/Mrs3/4</td>
<td>Mitochondrial inner membrane Fe transporter</td>
</tr>
<tr>
<td>Sec15l1/Sec15</td>
<td>Mutated in “haemoglobin deficit mouse”; yeast homolog is part of exocyst pathway</td>
</tr>
<tr>
<td>Steap3</td>
<td>Possible (?) endosomal ferrireductase</td>
</tr>
<tr>
<td>Transferrin (Tf)</td>
<td>Plasma Fe&lt;sup&gt;3+&lt;/sup&gt; carrier</td>
</tr>
<tr>
<td>Tf Receptor</td>
<td>Cognate membrane receptor for Tf</td>
</tr>
<tr>
<td>Tf Receptor 2</td>
<td>Unknown; similar to “classical” Tf receptor</td>
</tr>
</tbody>
</table>
Overview of Heme Synthesis

The Mammalian Iron Cycle

- ~3 g of Fe is in Erythrocytes:
- 80% Total Body Iron (3.8 g Fe)
- Total Plasma Fe Turnover = 30 mg/d
<table>
<thead>
<tr>
<th>Type</th>
<th>Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (pH 7.0)</td>
<td>maximum [Fe$^{3+}$] 0.000 000 000 000 000 000 01</td>
</tr>
<tr>
<td>Plasma</td>
<td>Fe$_2$-Tf          0.000 002</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>Heme               0.020</td>
</tr>
<tr>
<td></td>
<td>Non-heme           0.000 001</td>
</tr>
</tbody>
</table>
The first step in the transfer of iron from the plasma iron-carrying protein, transferrin, to reticulocytes and probably to other erythopoietic cells, is the binding of transferrin to the cells\textsuperscript{1,2} ... We report here that transferrin actually penetrates into the interior of the cell.

References

Fe Transport across Biological Membranes

Fe Import

- **DMT1** (divalent metal transporter 1);
- Belongs to a larger family of Nramp prot. (yeast → man);
- 12 membrane-spanning regions;
- Acts as a $\text{H}^+$-coupled $\text{Fe}^{2+}$ transporter across the brush border of the intestine and endosomal membranes;
- Mutated in *mk* mice and Belgrade rats: hypochromic microcytic anemia.
How Iron (Fe$^{2+}$) Reaches Ferrochelatase?

The prevailing opinion is that, after its export from endosomes, the redox-active Fe$^{2+}$ spreads into the cytosol and mysteriously finds its way into mitochondria through passive diffusion.
Iron must reach the inner mitochondrial membrane (ferrochelatase)

Decreased protoporphyrin IX (the 2nd substrate for ferrochelatase) levels cause iron accumulation in mitochondria

Free Fe$^{2+}$ in the oxygen rich cytosol is toxic via Fenton chemistry

$$(\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} \text{OH}^- + \text{OH}^-)$$

Iron is delivered directly from endosomal protein to mitochondrial protein, by-passing the cytosol

*Ponka, Blood 89:1, 1997*
Most “Iron People” Hate “Kiss-and-Run” Hypothesis

- The intracellular path of iron from endosomes to mitochondrial ferrochelatase is still obscure or, at best, controversial.

- The prevailing opinion: After its export from endosomes, iron spreads into the cytosol and mysteriously finds its way into mitochondria.

- An alternative view: The highly efficient transport of iron toward ferrochelatase in erythroid cells requires direct interaction between transferrin-endosomes and mitochondria.

- Despite the longevity of the prevailing opinion, experimental evidence only supports the latter hypothesis (“Kiss-and-Run”), which sees favorable reception among cell biologists.

- However, the role of endosomes in distributing intracellular iron is accepted without enthusiasm, misinterpreted or simply ignored.
Association of Transferrin-Endosomes with Mitochondria

Endosomes (Alexa 488 Tf)
Mitochondria (MitoTracker Deep Red)
CD1 mice injected with PHZ (50 mg/kg/day, 3 days)

1. Incubation of reticulocytes with MTDR (37°C, 30 min)

2. Incubation with AGTf (4°C, 30 min)

3. Incubation of double-labeled reticulocytes (37°C, time intervals) with or without Test Agents

4. Blocking endocytosis with cold PBS

5. Treatment of cells with pronase

6. 4 cycles of rapid freeze and thaw

7. Centrifugation of reticulocyte lysates (800 g, 10 min)

8. Flow sub-cytometry of supernatant

The Flow-Subcytometry Workflow
Endosome-Mitochondria Interactions as Displayed by Subcellular Flow Cytometry of Lysates
The Double-labeled Population Was Sorted by FACS and Shown by 2D Confocal Microscopy to be Composed of Endosomes Associated with Mitochondria

FACS-sorting

AGTF: Alexa 488 Tf
MTDR: MitoTracker Red

2D Confocal Microscopy
Flow Sub-Cytometry Analysis of the Endosome-Mitochondria Interaction


**A**

**Minus transferrin**

Time (min)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage</td>
<td>17.04%</td>
<td>23.83%</td>
<td>21.98%</td>
<td>21.64%</td>
<td>18.71%</td>
<td>18.89%</td>
</tr>
</tbody>
</table>

**Fe₂-transferrin**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage</td>
<td>17.50%</td>
<td>16.80%</td>
<td>12.03%</td>
<td>10.38%</td>
<td>6.15%</td>
<td>4.40%</td>
</tr>
</tbody>
</table>

**B**

**Holotransferrin Causes the Detachment of Endosomes from Mitochondria**
Scheme of Iron Delivery to Mitochondria for Heme Biosynthesis in Erythroid Cells
PATHOGENESIS OF HYPOCHROMIC MICROCYTIC ANEMIAS

- Fe deficiency
- Chronic inflammation or malignancy

PROTOPORPHYRIN IX

HEME + GLOBIN

Thalassemia (α or β)

Sideroblastic anemia

Adapted from Hoffbrand et al. Essential Haematology, Blackwell, 2006
Prussian-Blue Staining of Ring Sideroblasts
(a perinuclear ring of iron granules)
TEM of the Ring Sideroblast
Pathogenesis of Ring Sideroblast Formation in Congenital Sideroblastic Anemias

- The efficient delivery of iron to ferrochelatase in erythroid cells requires the direct interaction of endosomes with mitochondria.

- Mitochondrial iron cannot be adequately utilized due to the lack of protoporphyrin IX (erythroid-specific ALAS defects; SLC25A38 defects).

- Erythroid cells, but not non-erythroid cells, are equipped with a negative feedback mechanism in which ‘uncommitted’ heme inhibits iron acquisition from transferrin. The lack of heme plays an important role in mitochondrial iron accumulation in erythroid cells.

- Iron can leave erythroid mitochondria only after being inserted into protoporphyrin IX.
Red cells are anucleated sacs involved in gas exchange and most importantly oxygen delivery. Over 90% of their protein content is hemoglobin. We inadvertently determined that the feline leukemia virus C receptor (FLVCR), a cytoplasmic membrane transporter that is required for proerythroblast survival, exports heme (1). Mice in which Flvcr is ablated die in utero from erythroid failure and adult mice in which Flvcr is deleted develop a severe macrocytic anemia (2)...Why should developing erythroid cells need to export heme when its adequate supply in mature red cells is so critical? Although FLVCR is ubiquitously expressed in different human tissues by northern analyses and RT-PCR, western blot data suggest it is most abundant in liver, duodenum, brain, placenta, spleen, marrow, and uterus, which are sites of high heme synthesis or flux, and thus are cells that might need protection from heme toxicities.
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WHAT ABOUT HEME OXYGENASE 1?
Heme Oxygenase

Heme, oxidative stress, hypoxia, cytokines, heat shock, ROS, NO, cAMP, and oxidized lipids

Cytochrome P450 Reductase

NADPH

NADP⁺

CO

Iron

Biliverdin

NADPH

Biliverdin reductase

NADP⁺

Bilirubin
Heme Oxygenase

Key Points

• Heme oxygenase-1 is expressed in erythroid progenitors

• Heme oxygenase-1 levels increase during erythroid differentiation

• Heme oxygenase-1 actively participates in maintaining appropriate hemoglobinization rates

Garcia-Santos et al.  
Blood 123: 2269, 2014
Inherited Human Hemoglobin Disorders

- Structural hemoglobin variants: Hemoglobin S, C and E (over 500)

- Defective synthesis of hemoglobin: α- and β-thalassemias:
  
  • β-thalassemia: diminished expression of β-globin genes → excess of α-globin chains; heme molecules attached to them

  • vice versa in α-thalassemia

Main symptoms: Ineffective erythropoiesis (erythroid expansion with apoptosis); peripheral hemolysis; anemia; iron overload
NORMAL

HEMOGLOBIN F
α₂ γ₂

HEMOGLOBIN A
α₂ β₂

β-THALASSEMIA

α₂ γ₂
PERSISTS BEYOND INFANCY

α₂ β₂
EXCESS
PRECIPITATES INCLUSION

α-THALASSEMIA

α₂ γ₂
EXCESS
γ₄
Hb Bart’s

α₂ β₂
EXCESS
β₄
Hb H
Hypothesis: HO-1 Expression in Thalassemia

Unbalanced production of α- or β-globin

↓

Accumulation of heme?

↓

Heme degradation and iron release

↓

Formation of ROS and cell death?

Is HO-1 induced in thalassemia? Does HO-1 and/or its reaction products contribute for the thalassemic phenotype?
HO-1 Expression Increases in Fetal Liver Cells Isolated from β-Thal Mice
Tin Protoporphyrin IX (SnPP):
Competitive inhibitor of HO

SnPP (I.P.)
40μmol/kg/d

3 injections per week
4 weeks

(1 year old mice)

1) wild type(wt) + saline
2) wild type(wt) + SnPP
3) Thalassemic(th3) + saline
4) Thalassemic (th3) + SnPP

n = 3

* Blood collected
* Organs harvested
* Bone marrow isolated
Tin-Protoporphyrin IX

Spleen Index

Reticulocytes

- Wt + saline
- Wt + SnPP
- TH3 + saline
- TH3 + SnPP

Spleen weigh/mice weigh

% of Reticulocytes

Tin-

Protoporphyrin IX

- Wt + saline
- Wt + SnPP
- TH3 + saline
- TH3 + SnPP

Spleen weigh/mice weigh

% of Reticulocytes
**In Vivo**

H&E (10x)  Pearls (20x)

wt + saline

wt + SnPP

th3 + saline

th3 + SnPP

Liver Fe

Fe (µg/mg)

wt saline  wt SnPP  th3 saline  th3 SnPP
SnPP Significantly Increases the Survival of RBC Derived from Th3/+ mice
Conclusions

Our strategy has multiple beneficial effects in the context of thalassemia; it decreases iron release and oxidative damage in the erythroid precursors and limits iron efflux from “iron-donor” cells. This double-punch strategy stands out as an alternative and advantageous approach for treatment of thalassemia compared to hepcidin.

In a recent review Makis et al. (Am J Hematol. 91:1135, 2016) refer to DGS & AH work:

“The expression of HO-1 has been found especially augmented in EPO-dependent fetal liver erythropoietic cells from β-thalassemic mice fetuses, demonstrating the potential role of HO-1 in the mechanism of cellular damage of thalassemic erythroblasts. In the same mouse model, the administration of tin-protoporphyrin IX (SnPP), an HO-1 inhibitor, significantly improved ineffective erythropoiesis, decreased spleen size and liver iron and increased hemoglobin levels. This approach of HO-1 induced iron damage of β-thalassemic erythroblasts is a very promising field for further research.”