Blood Cells, Molecules and Diseases 59 (2016) 63–70

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Recombinant human erythropoietin-induced erythropoiesis regulates hepcidin expression over iron status in the rat

Sandra Ribeiro [a], Patrícia Garrido [b], João Fernandes [b,c], Susana Rocha [a], Petronila Rocha-Pereira [a,d], Elísio Costa [a], Luís Belo [a], Flávio Reis [b,c], Alice Santos-Silva [a,*]

[a] Research Unit on Applied Molecular Biosciences (UCIBIO), REQUIMTE, Department of Biological Sciences, Laboratory of Biochemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal
[b] Laboratory of Pharmacology & Experimental Therapeutics, Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal
[c] Center for Neuroscience and Cell Biology - Institute for Biomedical Imaging and Life Sciences (CNC.IBILI) Research Unit, University of Coimbra, Coimbra, Portugal
[d] Health Sciences Research Centre, Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal

ARTICLE INFO

Article history:
Submitted 23 January 2016
Revised 13 April 2016
Accepted 13 April 2016
Available online 19 April 2016

Editor: Mohandas Narla

Keywords:
Erythropoiesis
Hepcidin
Iron
Matriptase-2
Transferrin saturation

ABSTRACT

The crosstalk between several factors controlling hepcidin synthesis is poorly clarified for different physiological and pathological conditions. Our aim was to study the impact of increasing recombinant human erythropoietin (rHuEPO) doses on erythropoiesis, iron metabolism and hepcidin, using a rat model.

Male Wistar rats were divided in 5 groups: control (vehicle) and rHuEPO-treated groups (100, 200, 400 and 600 IU/kg body weight/week), 3 times per week, during 3 weeks. Hematological and iron data were evaluated. The expression of several genes involved in iron metabolism was analyzed by qPCR. Liver hepcidin protein was evaluated by Western Blot.

The rHuEPO treatment induced erythropoiesis and increased transferrin saturation (TSAT) in a dose dependent manner. Tf receptor 2 (TfR2), hemojuvelin (HJV) and bone morphogenetic protein 6 (BMP6) were up-regulated in rHuEPO200 group. Matriptase-2 was down-regulated in rHuEPO200 group, and up-regulated in the other rHuEPO-treated groups. Hepcidin synthesis was increased in rHuEPO200 group, and repressed in the rHuEPO400 and rHuEPO600 groups.

Our study showed that when a high erythropoietic stimulus occurs, hepcidin synthesis is mainly regulated by TSAT; however, when the erythropoiesis rate reaches a specific threshold, extramedullary hematopoiesis is triggered, and the control of hepcidin synthesis is switched to matriptase-2, thus inhibiting hepcidin synthesis.

1. Introduction

Erythropoiesis and iron metabolism are deeply linked, as iron is needed for hemoglobin (Hb) synthesis. An erythropoietic stimuli increases iron demand into the bone marrow. Body iron is acquired through the diet and is mostly contained in Hb of circulating red blood cells (RBC) [1]. Heme-iron is absorbed through a receptor on the apical membrane of the duodenal enterocyte, releasing iron after digestion. The non-heme iron is absorbed in the enterocyte through the divalent metal iron transporter 1 (DMT1) and released into the circulation by the iron exporter ferroportin (FPN), present on the basolateral surface of the enterocytes, and also present in macrophages and hepatocytes [1]. Iron is transported by transferrin (Tf) to the locals where it is needed for heme synthesis or to be stored. Iron levels are physiologically controlled by the balance between iron influx and iron demands, being hepcidin the major regulator. Hepcidin is a liver peptide that binds to FPN, inducing its internalization and degradation [2]; thus, hepcidin regulates iron absorption and mobilization to avoid iron deficiency or overload. Several factors can regulate hepcidin synthesis, such as iron stores, inflammation, erythropoiesis and hypoxia [3]. High concentrations of diferric Tf and inflammation stimulate hepcidin synthesis, whereas increased erythropoiesis and hypoxia inhibit hepcidin synthesis; however, the crosstalk between these factors is not well established.

Erythropoiesis stimulating agents (ESA) are mainly used to treat anemia in chronic kidney disease patients (CKD) and in patients receiving chemotherapy [4]. The discovery of non-hematopoietic actions for ESA [5] opened the possibility of their use in the treatment of other pathologies [6–8]; however, to achieve those effects, higher doses of ESA are usually needed [9]. The stimulating effect on erythropoiesis is also illicitly used in sports doping to increase RBC mass, improving sports performance. A successful treatment with ESA increases erythropoiesis and iron demand into the bone marrow, leading to an increase in iron absorption and mobilization. The interaction between erythropoiesis, iron absorption and iron stores, namely which of them prevails in the regulation of hepcidin synthesis, is not fully elucidated. Actually,

* Corresponding author at: Laboratory of Biochemistry, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira n.° 228, 4050-313 Porto, Portugal.
E-mail address: assilva@ff.up.pt (A. Santos-Silva).

http://dx.doi.org/10.1016/j.bcmd.2016.04.008
1079-9796/

some studies showed that stimulated erythropoiesis, even in the presence of iron overload, inhibits hepcidin synthesis [10,11], whereas others reported a dominance of liver iron stores over erythropoiesis [12]. In this study we aimed to study the effect of recombinant human erythropoietin (rHuEPO) therapy on erythropoiesis and on iron metabolism, and the effect of each factor on hepcidin regulation, using a rat model.

2. Materials and methods

2.1. Animals and experimental protocol

Male Wistar rats (Charles River Lab. Inc., Chatillon-sur-Chalaronne, France), with 320–350 g of body weight (BW), were maintained in ventilated cages, in an air conditioned room, subjected to 12 h dark/light cycles and given free access to rat laboratory chow (SAFE-A03, Augy, France) and tap water. All animals received human care and animal experiments were conducted according to the European Communities Council Directives on Animal Care. The experiments were approved by the Portuguese Foundation for Science and Technology and the Local Ethics Committee (ORBEA: Organ Responsible for Animal Welfare) of the Faculty of Medicine from the University of Coimbra.

The rats were randomly divided in five groups (7–8 rats each group) receiving a subcutaneous (sc) injection of saline solution (control group) or of rHuEpo (NeoRecormon®, Roche, Basel, Switzerland) 100, 200, 400 or 600 IU/kg BW/week (rHuEPO100, rHuEPO200, rHuEPO400 and rHuEPO600 groups, respectively), 3 times per week, during 3 weeks.

2.2. Sample collection

Blood samples were collected at baseline, and 1 and 3 weeks (end of protocol) after starting rHuEPO treatment, with the rats under anesthesia (intraperitoneal) with 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal). Blood samples were collected by venipuncture, from the jugular vein, into Vacuette® tubes without anticoagulant (to obtain serum) or with K_3EDTA (to obtain plasma) for hematological and biochemical studies. Plasma and serum aliquots were immediately stored at −80 °C until assayed.

At the end of protocol, the rats under anesthesia were sacrificed by cervical dislocation; liver, spleen and duodenum were immediately removed and placed in ice-cold Krebs-Henseleit buffer, cleaned and weighted. In order to isolate total RNA, 0.2 g of liver, spleen and duodenum samples, from each rat, were immersed in RNAlater® solution (Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA) upon collection and stored at 4 °C for 24 h; afterwards, samples were frozen at −20 °C. For Western Blot analysis organs were immediately frozen with liquid nitrogen and stored at −80 °C. A bone marrow aspirate from the femur was also performed; bone marrow smears were stained with Wright's stain for cytological analysis.

2.3. Hematological and iron metabolism studies

RBC count, Hb, hematocrit (Ht), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red cell distribution width (RDW) and platelet count were assessed in whole blood K_3EDTA, using an automated counter (HORIBA ABX, Amadora, Portugal). Reticulocytes were evaluated by microscopic counting on blood smears, after vital staining with New methylene blue (reticulocyte stain; Sigma-Aldrich Co. LLC. St. Louis, Missouri, USA).

Reticulocyte production index (RPI) was calculated as previously described by Hillman and Finch [13] [(reticulocyte % / maturation index) ∗ (Ht / normal Ht)], where normal Ht was the mean value presented by the control group, and the maturation index (maturation time of circulating blood reticulocytes that increase with premature release of reticulocytes from the bone marrow) was 1 for all groups.

Serum iron and ferritin were analyzed through automatic methods and equipment (ROCHE Integra 400, Roche Diagnostics, Basel, Switzerland). Serum Tf levels were evaluated by rat specific ELISA kit (Transferrin Rat ELISA Kit, abcam, Cambridge, UK). Tf saturation (TSAT) was calculated using the formula (Iron μg/dL ∗ 100) / (Tf mg/dL ∗ 2).

2.4. Spleen histological analysis

Spleen samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 4 μm thick sections were stained with hematoxylin and eosin for extramedullary erythropoiesis analysis. All samples were examined by light microscopy (Microscope Nikon Eclipse Ci) and images were captured using a digital microscope camera (Nikon DS-Ri2).

2.5. Perls' Prussian blue staining

Liver samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 3 μm thick sections were stained with Perls' Prussian blue stain. In brief, liver tissue sections were deparaffinised, hydrated and immersed in a freshly prepared potassium ferrocyanide solution (10 g/L in 0.1 M hydrochloric acid) at room temperature. Safranin was used as counterstaining. The samples were examined by light microscopy (Microscope Nikon Eclipse Ci, Tokyo, Japan) and images were captured using a digital microscope camera (Nikon DS-Ri2, Tokyo, Japan).

2.6. Gene expression analysis

Liver, spleen and duodenum RNA isolation and integrity control were performed as previously described by us [14]. One microgram of total RNA was reversely transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA), according to manufacturer instructions. One nanogram of cDNA was used for gene expression analysis with qPCR, using a Mini-Opticon instrument (Bio-Rad Laboratories), the KAPA SYBR® FAST qPCR kit (Kapa

Table 1
List of primer sequences and annealing temperatures.

Gene	Primer sequences	Annealing temperature
actb	F: 5′- TAC AGC TTC ACC ACC ACA GC-3′ R: 5′- AAG GAA GGC TGG AAG AGA GC-3′	57 °C
bmp6	F: 5′- GGT GGA GTA CGA CAA GGA GTT-3′ R: 5′- GTC ACA ACC CAC AGA TTG CTA-3′	56 °C
epor	F: 5′- CCG GGA TGG GCT TCA ACT AC-3′ R: 5′- TCC AGT GGC ACA AAA CTC GAC-3′	59 °C
hamp	F: 5′- GGC AGA AAG CAA GAC TGA TGA C-3′ R: 5′- ACA GGA ATA AAT AAT GGG GCG-3′	58 °C
hfe	F: 5′- TGG GCA AGA TCA CCT TGA ATT-3′ R: 5′- GGA TCC TGT GCT CTT CCC ACT-3′	58 °C
hfe2	F: 5′- TTC CAA TCC TGC CTC TTT GAT-3′ R: 5′- GGA AAA GGT GCA AGT TCT CCA A-3′	58 °C
slc11a2	F: 5′- ATA GCA GAC GCC CCC ATG-3′ R: 5′- AGG CCC GAA GTA ACA TCC AA-3′	58 °C
tfrc	F: 5′- GGG AGC CAT TGT CAT ACA CC-3′ R: 5′- GTC GCA AAG CAG AGT CTT CC-3′	58 °C
tfr2	F: 5′- AGC TGG GAC GGA GGT GAC TT-3′ R: 5′- TCC AGG CTC ACG TAC ACA ACA G-3′	58 °C
tmprss6	F: 5′- AGA AGG TGG ATG TGC AAC TGA TC-3′ R: 5′- CTT GCC CTT GCG ATA ACC A-3′	59 °C
tuba	F: 5′- CAC CCG TCT TCA GGG CTT CTT GGT TT-3′ R: 5′- CAT TTC ACC ATC TGG TTG GCT GGC TC-3′	59 °C

F: forward; R: reverse; *actb* - beta – actin; *bmp6* - bone morphogenetic protein 6; *epor* – erythropoietin receptor; *hamp* – hepcidin; *hfe* – hemochromatosis protein; *hfe2* - hemojuvelin; *slc11a2* - divalent metal transporter 1; *tfrc* – transferrin receptor 1; *tfr2* – transferrin receptor 2; *tmprss6* – matriptase-2; *tuba* – alpha-tubulin.