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EPO-dependent induction of erythroferrone drives hepcidin suppression and systematic iron absorption under phenylhydrazine-induced hemolytic anemia

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ABSTRACT

Hemolytic anemia is a common form of anemia due to hemolysis, resulting in disordered iron homeostasis. In this study, a dose of 40 mg/kg phenylhydrazine (PHZ) was injected into mice to successfully establish a pronounced anemia animal model, which resulted in stress erythropoiesis and iron absorption. We found that serum erythropoietin (EPO) concentration was dramatically elevated by nearly 5000-fold for the first 2 days, and then drop to the basal level on day 6 after PHZ injection. Mirrored with serum EPO concentration, the mRNA expression of erythroferrone (ERFE) was rapidly increased in the bone marrow and spleen 3 days after injection of PHZ, and then gradually decreased but was still higher than baseline on day 6. In addition, we also found that the hepcidin mRNA levels were gradually reduced almost up to 8-fold on day 5, and then was ameliorated compared to the untreated control. Mechanistic investigation manifested that the increase of serum EPO essentially determined the induction of ERFE expression particular at the first 3 days after PHZ treatment. Lentiviral mediated ERFE knockdown significantly restrained hepcidin suppression under PHZ treatment. Thus, our data unearthed EPO-dependent ERFE expression acts as an erythropoiesis-driven regulator of iron metabolism under PHZ-induced hemolytic anemia.

1. Introduction

Hemolytic anemia is characterized by a shortened red blood cells (RBCs) due to an inherent abnormality, environmental factors, or exposure of foreign chemicals [1]. Hemolytic anemia could induce stress erythropoiesis in bone marrow and a remarkable alteration of iron homeostasis in order to meet the elevated demand of iron supply for hemoglobin synthesis and erythroid cell maturation [2,3]. Erythropoiesis and iron homeostasis have been proved to regulate each other to ensure an optimal amount of oxygen and iron supply to cells and tissues [4,5]. To define the mechanisms by which erythropoietic demand modulates the iron supply is crucial for the understanding of the pathogenesis of ineffective erythropoiesis.

Hepcidin, the hepatocyte-derived 25-amino-acid peptide hormone, is the main circulating regulator of iron absorption and tissue distribution [6–8]. The molecular basis of hepcidin function is to induce iron exporter ferroportin internalization and degradation within lysosomes through an ubiquitin-dependent manner, by which iron efflux out of macrophages and intestinal absorption would be inhibited [9,10]. Hepcidin expression is promoted by excess iron and inflammation, and is reversely suppressed by erythropoiesis and hypoxia [6,9–12]. These different ways in the regulation of hepcidin concertedly limit intestinal iron absorption during iron overload, and increase iron availability during erythropoiesis, e.g. under iron deficiency [4,5]. Although increased erythropoietic activity has long been known to suppress hepcidin expression, the specific mechanisms of regulation of iron metabolism by erythropoiesis activity are still poorly understood. Two members of the transforming growth factor-β superfamily, growth differentiation factor 15 (GDF15) and twisted gastrulation (TWSG1), have been previously recognized as pathological suppressors of hepcidin in ineffective erythropoiesis [13,14]. Additionally, a recently identified erythroid hormone erythroferrone (ERFE) was demonstrated to conduct a suppression of hepcidin at the early stage after endogenous or exotic erythropoietic stimulation [15]. ERFE ablation resulted in a more severe anemia with inappropriately elevated hepcidin expression in heat-killed *Brucella abortus*-induced inflammation anemia [16]. Moreover, ERFE also mediates pathological hepcidin suppression in conditions of ineffective erythropoiesis, such as β–thalassemia [17].

Previous studies indicated hepcidin expression was repressed in phenylhydrazine (PHZ)-induced hemolytic anemia [18–22]. Nonetheless,

Abbreviations: PHZ, phenylhydrazine; ERFE, erythroferrone; EPO, erythropoietin; FAC, Ferric Ammonium Citrate; RBC, red blood cells; HGB, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

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the mechanisms for the pathogenic role of erythroid regulator in hepcidin suppression upon PHZ-induced hemolysis are elusive, and specifically, no insight has been gained into physiological hepcidin suppression of hemolytic anemia in a long term course. We, therefore, utilized PHZ-induced hemolytic anemia as a model to address this question. Overall, we uncovered that ERFE incurred hepcidin suppression during stress erythropoiesis under PHZ-induced hemolysis, and elevated serum EPO content but not serum heme and non-heme iron accounted for the induction of ERFE expression under PHZ treatment. Our combined data therefore highlighted a crucial role of ERFE in regulating hepcidin expression and systematic iron homeostasis under PHZ-induced hemolytic anemia.

2. Materials and methods

2.1. Animal experiments

Eight-week-old male C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China) and housed under a sterile and pathogen-free condition. All animal care and experimental protocols were approved by the Animal Ethics Committee at the Tianjin Medical University and the Research Center for Eco-Environment Science, Chinese Academy of Science. PHZ (Sigma Aldrich, Canada) was peritoneally injected into mice at the dose of 40 mg/kg at 12 PM on day 1, and additional two injections were administrated at 9 AM. and 4 PM on day 2 (Fig. 1A), as previously described [18]. In addition, 200 units of human EPO (Epogen, Amgen), 50 uM Hemin (Sigma Aldrich, Canada), or 50 μM Ferric Ammonium Citrate (FAC) (Sigma Aldrich, Canada) was individually administrated into mice for 12 h to assess the expression level of ERFE.

2.2. Hematological profiles

Whole blood was collected by cardiac puncture of anesthetized mice using heparin as anticoagulant (BD Biosciences, USA). RBC, hemoglobin (HGB), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were determined using the SYSMEX XT-1800i analyzer (Kobe, Japan).

2.3. Determination of serum EPO, heme iron and non-heme iron

Serum EPO level was determined using a Quantikine ELISA mouse erythropoietin immunoassay kit following the instructions provided by the manufacturer (R&D Systems, USA). Serum heme concentration was assayed by the pyridine hemochrome method by a QuantiChromTM Heme Assay Kit (Bioassy Systems, USA). Serum non-heme iron was measured according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, China).

2.4. Flow cytometry (FACS) analysis of distinct sub-population over differentiation

Differentiation of erythroid progenitors was assessed by FACS analysis using PE-conjugated anti-Ter119 and FITC-conjugated anti-CD71 antibodies (Abs) (BD Biosciences, USA). Briefly, bone marrow cells were collected from mouse femurs and washed with cold PBS, and cells were then stained with these Abs for 30 min at 4 °C. Finally, 1×10^4 cells were subjected to FACS analysis (BD Biosciences, USA). Different sub-populations of nucleated cells in bone marrow could be gated by FACS analysis, proerythroblasts progressed from $CD71^{high}/Ter119^{low}$ (R3); a mixture of basophilic and polychromatic erythroblasts from $CD71^{high}/Ter119^{high}$ (R4); $CD71^{intermediate}/Ter119^{high}$, containing polychromatic erythroblasts (R5), and $CD71^{high}/Ter119^{low}$ corresponding to orthochromatic erythroblasts (R6), as described previously [23].

2.5. Quantitative real-time PCR

Total RNAs were extracted from mouse tissues using Trizol reagent following the manufacturer's instructions (Invitrogen, USA). Gene expression was quantified using SYBR Green master mix (Qiagen, German) on qRT-PCR machine (Bio-Rad, USA). All sequences for PCR primers were as follows. Human ERFE: forward, 5′-ATGGGGCTGGAG

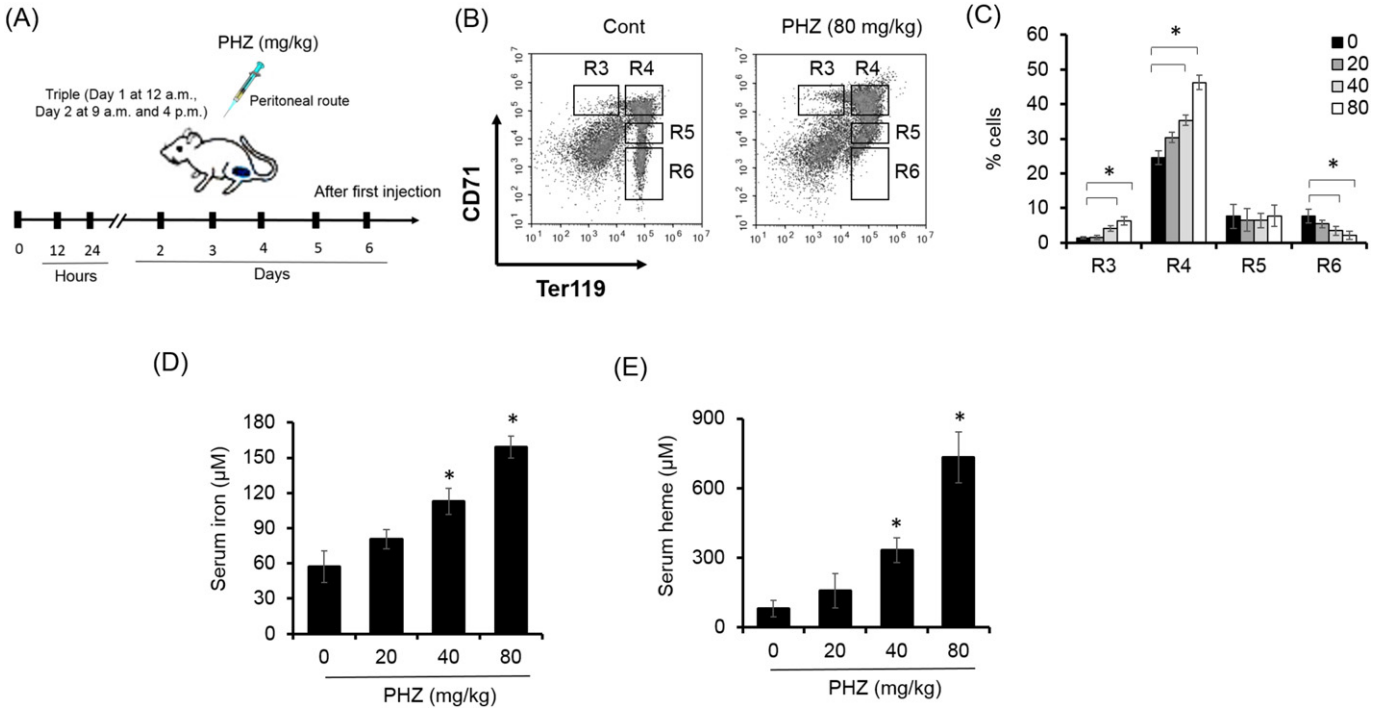

Fig. 1. Establishment of hemolytic anemia mouse model. (A) A schematic depicting the experimental design. Mice were intraperitoneally administered with PHZ (20, 40 and 80 mg/kg), and were then analyzed at different time points (n = 4). (B) FACS analysis of bone marrow erythroid cells from mice treated with PHZ at 80 mg/kg for 48 h and untreated mice using Abs againstTer119 and CD71. (C) Proportions of sub-populations at different stages. (D) Serum iron and (E) Serum heme levels were determined. Asterisk (*) indicates $P < 0.05$.