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.

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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 (TWG1), 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 administered 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 μ M Hemin (Sigma Aldrich, Canada), or 50 μ M Ferric Ammonium Citrate (FAC) (Sigma Aldrich, Canada) was individually administered 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 QuantiChrom™ Heme Assay Kit (Bioassay 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 against Ter119 and CD71. (C) Proportions of sub-populations at different stages. (D) Serum iron and (E) Serum heme levels were determined. Asterisk (*) indicates $P < 0.05$.

AACAGC-3', reverse, 5' -TGGCATTGTCCAAGAAGACA-3'; human Hepcidin: forward, 5' -AGAAAGCAGGGCAGACATTG-3', reverse, 5' -CACTGGGAATTGTTACAGCATT-3'; human Hprt: forward, 5' -CAGCAGTGAGCTCTTACCA-3', reverse, 5' -CAAGAA_CACGGAGGTCCACT-3'. HPRT was used as an internal control.

2.6. shRNA lentivirus transduction and injection

Lentivirus-based transduction particles were purchased from Sigma Aldrich, Canada. For lentiviral transductions, erythroid precursor cells were grown to 70% confluence and inoculated with lentivirus in the presence of 8 µg/mL polybrene. After 24 h, cells were treated with 10 U/mL EPO or saline. *In vivo*, lentiviral particles were injected via tail vein to C57BL/6 mice. Two weeks later, mice were treated with 40 mg/kg PHZ or saline.

2.7. Statistical analysis

Statistical analysis was performed to analyze the statistical significance of experimental data using SPSS 13.0 package. All results were presented as mean ± standard error. A p value less than 0.05 were considered to be statistically significant.

3. Results

3.1. Establishment of hemolytic anemia mouse model

To establish PHZ-induced hemolytic anemia model in mice, PHZ was peritoneally administrated into mice at different doses (0, 20, 40 and 80 mg/kg). The mice were then sacrificed 48 h after PHZ administration. Decreased body weight, enhanced spleen and liver weight were observed in PHZ-treated group at 40 mg/kg and 80 mg/kg, compared to untreated mice (Table 1, $P < 0.05$). Consistent with previous observations, a significant reduction of RBC count and HGB concentration were observed in PHZ-treated mice, relative to untreated mice (Table 1, $P < 0.05$). Moreover, elevated MCH and reduced HCT were also found in PHZ-treated mice. These results indicated acute hemolytic anemia in PHZ-treated groups, especially for those administrated at 40 mg/kg and 80 mg/kg (Table 1, $P < 0.05$). Afterwards, we evaluated bone marrow-mediated erythropoietic activity upon PHZ treatment (Fig. 1B). The proportion of cells at stage 3 and stage 4 (referring to CD71^{high} cells) was significantly enriched in PHZ-treated group at 40 mg/kg (4.12% and 35.34%) and 80 mg/kg (6.25% and 46.23%), compared with that in untreated group (1.41% and 24.61%), indicating that hematopoietic system of bone marrow was remarkably activated under hemolytic stimulation (Fig. 1C, $P < 0.05$). By contrast, consistent with reduced RBC content, the percentage of R6 cells was also significantly decreased in PHZ-treated group at 40 mg/kg and 80 mg/kg, compared with the control group (4.41% and 3.2% vs. 5.57%) (Fig. 1C, $P < 0.05$).

Table 1

The general and hematology parameters of mice under different dosage of PHZ administration.

	0 mg/kg	20 mg/kg	40 mg/kg	80 mg/kg
<i>General parameters</i>				
Body weight (mg)	21.14 ± 0.12	20.07 ± 0.09	19.18 ± 0.33*	17.18 ± 0.14*
Spleen weight (mg)	0.08 ± 0.01	0.08 ± 0.01	0.11 ± 0.01*	0.17 ± 0.01*
Liver weight (mg)	1.09 ± 0.11	0.98 ± 0.03	1.02 ± 0.04*	1.12 ± 0.10*
<i>Hematology</i>				
RBC (10 ¹² /L)	8.64 ± 0.27	8.27 ± 0.28	7.36 ± 0.34*	3.63 ± 0.27*
HGB (g/dL)	12.53 ± 0.16	11.77 ± 0.23	11.20 ± 0.11*	9.86 ± 0.21*
MCH (pg)	14.43 ± 0.15	14.36 ± 0.49	15.97 ± 0.17*	28.42 ± 3.21*
HCT (%)	36.90 ± 1.73	36.90 ± 1.21	36.63 ± 1.08*	14.33 ± 1.03*

Data are expressed as ± standard error.

* Indicated $p < 0.05$.

Previous studies have suggested that PHZ-induced hemolytic anemia could promote iron absorption in duodenum of experimental animals [19,20]. In the current study, we found that serum heme and non-heme iron levels were significantly increased in mice with PHZ administration at 40 mg/kg (2-fold and 3-fold) and 80 mg/kg (2.5-fold and 7-fold) compared to control (Fig. 1D–E, $P < 0.05$). These results demonstrated that PHZ successfully induced significant hemolytic anemia along with disordered iron homeostasis at the dosage of 40 and 80 mg/kg.

3.2. Enhanced ERFE expression controls iron mobilization under PHZ-induced hemolytic anemia

To better clarify the relationship between PHZ-stimulated compensatory erythropoiesis and iron re-distribution, a dose of 40 mg/kg PHZ was used in the long-term course administration. As shown in Table 2, reduced body weight and increased spleen weight and liver weight were observed over the time course post PHZ treatment. Moreover, decreased RBC content and HGB concentration as well as elevated MCH and reduced HCT were found after a long term course of PHZ administration, indicating severe hemolytic anemia (Table 2). However, after 5 days of PHZ treatment, the general and hematological parameters of mice were ameliorated, which demonstrated that hemolytic anemia began to recovery. Afterwards, we investigated iron homeostasis upon PHZ-induced hemolytic anemia. As illustrated in Fig. 2A–B, serum heme and non-heme iron concentrations were significantly elevated and peaked earlier on day 2 (1-fold and 3-fold, respectively, $P < 0.05$) after the first injection of PHZ, and also began to decrease afterwards. Interestingly, serum EPO content was dramatically elevated by nearly 5000-fold for the first 2 days, and then gradually drop to the basal level on day 6 after PHZ injection (Fig. 2C).

Hepcidin expression has been shown to be suppressed on day 3 after PHZ administration in order to promote iron absorption [19,20]. In present study, we found that the hepcidin mRNA levels were gradually reduced in over the time course, almost up to 8-fold on day 5, a then was ameliorated compared to the untreated control (Fig. 2D, $P < 0.05$). Moreover, in order to reveal the possible molecular link between erythropoiesis and iron metabolism, we detected the expression of three known hepcidin regulators in PHZ-administrated mice. Mirrored with serum EPO concentration, the mRNA expression of ERFE (Fam132b) was rapidly increased by 6 fold in the bone marrow and 5-fold in the spleen 3 days after injection of PHZ, and then gradually decreased but was still higher than baseline on day 6 (3-fold and 4-fold) (Fig. 2E–F, $P < 0.05$). Nevertheless, no significant change was demonstrated for the mRNA expression of *Gdf15* and *Twsig1* (Supplemental data). Collectively, these data indicated that enhanced ERFE expression contributes to elevated iron mobilization under PHZ-induced hemolytic anemia.

3.3. EPO-dependent induction of ERFE expression drives hepcidin suppression

Based on the abnormal changes of erythropoiesis and iron parameters within the first 2 days after PHZ injection, we looked into the relationship between erythropoietic activity and iron re-distribution at the earlier stage of PHZ-induced hemolysis. As we observed, no significant alterations were found in the contents of serum heme and non-heme iron until the first 24 h after PHZ administration (Fig. 3A–B, $P < 0.05$). However, serum EPO content was dramatically increased almost 4000 fold at the first 12 h, compared to untreated control (Fig. 3C, $P < 0.05$). Surprisingly, the ERFE (Fam132b) mRNA levels in bone marrow and spleen were both significantly increased by 4 fold and 5 fold at 12 h after the first injection of PHZ, relative to untreated mice (Fig. 3D–E, $P < 0.05$). Next, in order to investigate whether EPO governed the induction of ERFE expression under PHZ treatment, we detected the expression of ERFE upon EPO, hemin or FAC administration for 12 h. In analogy to PHZ-induced ERFE expression, exogenous EPO

Table 2

The general and hematology parameters of mice at different time point after PHZ administration.

	0 day	2 day	3 day	4 day	5 day	6 day
<i>General parameters</i>						
Body weight (mg)	22.10 ± 0.32	21.07 ± 0.29	20.01 ± 0.25*	19.28 ± 0.34*	18.33 ± 0.28*	18.22 ± 0.23*
Spleen weight (mg)	0.08 ± 0.02	0.10 ± 0.02	0.15 ± 0.03*	0.21 ± 0.04*	0.26 ± 0.32*	0.32 ± 0.32*
Liver weight (mg)	0.98 ± 0.05	1.05 ± 0.03	1.09 ± 0.04*	1.13 ± 0.09*	1.15 ± 0.04*	1.15 ± 0.09*
<i>Hematology</i>						
RBC (10 ³ /L)	7.49 ± 0.37	5.38 ± 0.15*	3.88 ± 0.11*	3.84 ± 0.23*	3.74 ± 0.21*	4.52 ± 0.27*
HGB (g/dl)	12.23 ± 0.19	11.06 ± 0.34*	9.23 ± 0.37*	9.03 ± 0.37*	7.67 ± 0.40*	7.97 ± 0.33*
MCH (pg)	14.31 ± 0.25	16.36 ± 0.37*	22.82 ± 1.57*	23.81 ± 2.13*	21.01 ± 1.13*	17.34 ± 0.82*
HCT (%)	34.51 ± 2.02	25.82 ± 1.73*	20.06 ± 2.32*	17.04 ± 1.43*	23.08 ± 1.64*	26.14 ± 1.79*

Data are expressed as ± standard error.

* Indicated $p < 0.05$.

administration significantly stimulated ERFE expression in bone marrow, compared to untreated control (Fig. 3F, $P < 0.05$). By contrast, either hemin or FAC failed to stimulate the induction of ERFE (Fig. 3F). Afterwards, we also examined ERFE expression across the stage of erythroid differentiation in bone marrow after sorting distinct sub-populations. Fig. 3G showed that *Fam132b* mRNA levels were higher after PHZ and EPO injection in all four sub-populations of erythroid precursors, with the highest expression level in R3 and R4 stage ($P < 0.05$).

To test whether enhanced ERFE expression drives hepcidin suppression under PHZ treatment, we administrated C57BL/6 mice a lentiviral-based vector encoding ERFE or control vector. Two weeks later, mice were then treated with PHZ or saline. Although no significant change in bone marrow at the baseline, the mRNA expression of ERFE was remarkably decreased almost 50% and 60% at 24 h and 48 h after the first injection of PHZ (Fig. 4A, $P < 0.05$). As expected, hepcidin suppression was largely ameliorated after PHZ treatment (Fig. 4B, $P < 0.05$). Moreover, we also knockdown ERFE expression in R4

stage of erythroid precursor cells. As shown in Fig. 4C, *Fam132b* expression was largely inhibited in *Fam132b* knockdown cells under EPO treatment ($P < 0.05$). After that, mouse primary hepatocytes were treated with supernatants in above growth medium for 24 h. As illustrated in Fig. 4D, hepcidin suppression was significantly restrained in ERFE-knockdown cells under EPO treatment. These findings together demonstrated that EPO essentially accounted for the induction of ERFE (*Fam132b*) mRNA expression to suppress hepcidin levels under PHZ-induced hemolytic anemia.

4. Discussion

Mammalian iron homeostasis is essentially a fine-tuned balance among the iron utilization for erythropoiesis, iron storage in the reticuloendothelial system and iron absorption through duodenum [4,5]. Under pathological conditions (e.g. hemorrhage or hemolysis), erythroid precursors request a delivery of sufficient iron in bone

Fig. 2. Elevated ERFE repressed hepcidin and enhanced iron levels under PHZ induced hemolytic anemia. Mice were intraperitoneally administered with PHZ at 40 mg/kg and sacrificed after different time. Time course analyses were performed for (A) serum iron, (B) serum heme iron and (C) serum EPO levels (pg/mL). Meanwhile, (D) hepatic hepcidin mRNA expression relative to that of *Hprt* is shown as $-\Delta\text{Ct}$ values (Ct *Hprt*-Ct *Hepcidin*), determined by qRT-PCR. ERFE (*Fam132b*) expression was also determined in (E) bone marrow and (F) spleen. Relative levels at each time point were compared to that in untreated control mice. Asterisk (*) indicates $P < 0.05$.

Fig. 3. Elevated EPO dictated the induction of ERFE expression under PHZ-induced hemolytic anemia. Mice were intraperitoneally administered with PHZ at 40 mg/kg and sacrificed at different time points. (A) Serum iron, (B) serum heme iron, and (C) serum EPO levels were assayed. ERFE (*Fam132b*) mRNA levels were measured by qRT-PCT in (D) bone marrow and (E) spleen. Levels at each time point were compared to that in untreated mice. (F) After treatment of mice with PHZ (40 mg/g), EPO (200 U), Heme (50 μM) and FAC (μM) for 12 h, *Fam132b* mRNA expression in bone marrow was assessed by qRT-PCR. (G) ERFE (*Fam132b*) mRNA levels were measured by qRT-PCR in different sub-populations sorted from erythroid cells in bone marrow. Asterisk (*) indicates $P < 0.05$.

marrow for the production of erythrocytes [2,3]. The liver-produced hormone hepcidin is identified as the main circulating regulator of systemic iron balance, and its suppression in anemia might result

from hypoxia or EPO [6–8]. However, the molecular mechanisms underlying hepcidin suppression remained elusive, and should be closely scrutinized.

Fig. 4. ERFE acted directly on liver to suppress hepcidin. Mice were transduced with a lentivirus encoding ERFE or controls and treated with PHZ or saline after 2 weeks. (A) *Fam132b* mRNA in bone marrow and (B) hepcidin mRNA in liver were detected by qRT-PCR. Lentivirus mediated knockdown of ERFE in R4 stage of erythroid precursor cells were treated with EPO or PBS. After that, mouse primary hepatocytes were cultured with supernatants from above growth medium for 24 h. (C) *Fam132b* expression in R4 cells and (D) hepcidin mRNA levels in mouse primary hepatocytes were measured by qRT-PCR. Asterisk (*) indicates $P < 0.05$.

Kautz L and colleagues first identified ERFE, a member of the C1q-tumor necrosis factor-related family of proteins, as the new erythroid regulator of hepcidin expression in 2014 [15]. The authors demonstrated that ERFE induction was responsible for early hepcidin suppression due to erythropoietic activity stimulated by endogenous or exogenous EPO stimulation. By contrast, phlebotomy or EPO injection failed to suppress hepcidin in ERFE knockout mice, indicating ERFE is necessary to initiate hepcidin suppression in response to increased erythroid activity. Lentiviral overexpression of ERFE in mice exerts an inhibitory effect on hepcidin transcription. Treatment of murine primary hepatocytes with recombinant ERFE showed that ERFE can act directly on hepatocytes to suppress hepcidin transcription, although direct interaction is still unknown thus far. Notably, ERFE deficient mice revealed similar alterations of hematologic and tissue iron parameters to those of wild-type mice, indicating that ERFE did not affect steady-state and long term course of erythropoiesis [15]. In addition, when ERFE deficient mice were subjected to heat-killed *Brucella abortus*, these mice developed more severe anemia of inflammation with inappropriately high levels of hepcidin and consequently lower serum iron concentrations [16]. Moreover, ERFE expression was also highly elevated in bone marrow and spleen of β -thalassemia intermedia mice, and deficiency of ERFE slightly ameliorated ineffective erythropoiesis with full restoration of hepcidin expression. However, correction of hepcidin levels in mice does not improve anemia in β -thalassemia [17]. In our study, we found ERFE (*Fam132b*) mRNA expression was highly induced in the bone marrow and spleen after PHZ administration, with the maximum expression for the first 3 days. Reduced hepatic hepcidin levels increased serum concentration under PHZ induced hemolytic anemia. However, without consistent stimulation of PHZ-induced hemolysis, serum EPO content started to decrease from day 2. In turn, ERFE expression was gradually reduced and the hepcidin level was ameliorated afterwards. These data demonstrated that ERFE plays a critical role in hepcidin suppression and iron re-distribution under hemolytic anemia.

Under conditions of stress erythropoiesis, elevated EPO levels are associated to suppression of hepcidin synthesis, a response that increases iron absorption and iron egress to provide sufficient iron for production and maturation of erythroid cells [24,25]. ERFE expression is regulated in an EPO-dependent manner gene through the JAK2/STAT5 signaling pathway [15]. However, both serum EPO concentration and iron tissue levels were significantly increased under stress erythropoiesis [15–17]. In the current study, serum EPO content, serum heme and non-heme iron were found to be significantly increased under PHZ-induced hemolytic anemia, coupled to the elevated ERFE expression. We, thus, speculated that serum heme or non-heme iron could also contribute to ERFE expression. However, among of them, both EPO content and ERFE expression were dramatically elevated at the first 12 h after PHZ administration. In addition, the expression of ERFE could not be regulated by increased serum heme or non-heme iron, referring to heme or FAC injection. These data together suggested EPO-dependent induction of ERFE expression under PHZ-induced hemolytic anemia.

It is well documented that PHZ-induced anemia is a classic model for the study of the pathogenesis of hemolytic anemia and the molecular mechanisms of anemia related to other physiological processes or diseases [19–21,26]. Here, PHZ-induced hemolysis model was used to analyze the relationship between stress erythropoiesis and iron metabolism. Our results showed that both 40 mg/kg and 80 mg/kg of PHZ could yield acute hemolytic anemia and significant iron metabolism disorder. However, the 80 mg/kg dose of PHZ caused more severe anemia, and as a result, 67% of these mice died on day 5 after the initial PHZ injection. Therefore, we selected the dose of 40 mg/kg of PHZ for closer investigation, at which PHZ incurred a reduction of hemoglobin to 10–12 g/dL. Decreased RBC content and HGB concentration, together with elevated MCH and reduced HCT indicated severe hemolytic anemia in a long time course after PHZ administration. Mechanistic investigation manifested that elevated serum EPO accounted for the induction of

Fig. 5. A schematic diagram deciphering the mechanism underlying ERFE-induced hepcidin suppression and adaptive iron homeostasis under PHZ-induced hemolytic anemia.

ERFE expression to suppress hepcidin expression and to enhance iron supply for erythropoiesis particularly for the first 3 days. Afterwards, with hemolytic anemia began to recovery, the serum EPO content and ERFE expression were gradually decreased. In turn, the suppression of hepcidin was attenuated to avoid excessive iron absorption.

Taken together, we here shed new light on the molecular mechanism responsible for hepcidin inhibition under PHZ induced hemolytic anemia. As illustrated in Fig. 5, elevated serum EPO concentration induced ERFE expression to suppress hepcidin expression and afterwards enhance iron absorption under PHZ-induced hemolysis. This study overall underscored an important role of ERFE in promoting RBC formation through enhancing iron supply for erythropoiesis by repressing hepcidin level.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bcmd.2016.02.005>.

Conflict of interest

No potential conflicts of interest were disclosed from the authors.

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