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Erythropoietin's inhibiting impact on hepcidin expression occurs indirectly

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Gammella E, Diaz V, Recalcati S, Buratti P, Samaja M, Dey S, Noguchi CT, Gassmann M, Cairo G. Erythropoietin's inhibiting impact on hepcidin expression occurs indirectly. *Am J Physiol Regul Integr Comp Physiol* 308: R330–R335, 2015. First published December 17, 2014; doi:10.1152/ajpregu.00410.2014.—Under conditions of accelerated erythropoiesis, elevated erythropoietin (Epo) levels are associated with inhibition of hepcidin synthesis, a response that ultimately increases iron availability to meet the enhanced iron needs of erythropoietic cells. In the search for erythroid regulators of hepcidin, many candidates have been proposed, including Epo itself. We aimed to test whether direct interaction between Epo and the liver is required to regulate hepcidin. We found that prolonged administration of high doses of Epo in mice leads to great inhibition of liver hepcidin mRNA levels, and concomitant induction of the hepcidin inhibitor erythroferrone (ERFE). Epo treatment also resulted in liver iron mobilization, mediated by increased ferroportin activity and accompanied by reduced ferritin levels and increased TfR1 expression. The same inhibitory effect was observed in mice that do not express the homodimeric Epo receptor (EpoR) in liver cells because EpoR expression is restricted to erythroid cells. Similarly, liver signaling pathways involved in hepcidin regulation were not influenced by the presence or absence of hepatic EpoR. Moreover, Epo analogs, possibly interacting with the postulated heterodimeric β common EpoR, did not affect hepcidin expression. These findings were supported by the lack of inhibition on hepcidin found in hepatoma cells exposed to various concentrations of Epo for different periods of times. Our results demonstrate that hepcidin suppression does not require the direct binding of Epo to its liver receptors and rather suggest that the role of Epo is to stimulate the synthesis of the erythroid regulator ERFE in erythroblasts, which ultimately downregulates hepcidin.

iron; ferroportin; erythropoietin receptor; liver; bone morphogenetic protein 6

INCREASED ERYTHROPOIESIS IS associated with an impressive alteration of iron homeostasis to satisfy the elevated need of iron for hemoglobin synthesis and erythroid cell proliferation. The coordination between erythropoietic activity and iron homeostasis is provided by hepcidin, a liver-derived peptide hormone that plays a key role in regulating iron balance in the body (reviewed in Refs. 2, 8, and 11). On target cells (primarily enterocytes, hepatocytes, and macrophages), hepcidin binds to the iron exporter ferroportin (Fpn) and induces its internalization and degradation, thus blocking iron efflux. Hepcidin levels are upregulated in response to iron overload through the bone morphogenetic protein (BMP)6/SMAD pathway in conjunction with hemojuvelin and decreased in response to iron deficiency (6, 11). Moreover, hepcidin expression is induced by inflammation (12), endoplasmic reticulum stress (21), and starvation (20). Conversely, under conditions of accelerated erythropoiesis, elevated erythropoietin (Epo) levels are associated with reduced synthesis of hepcidin. This physiological response increases iron availability to meet the enhanced iron demand of erythropoietic cells. The fact that suppression of erythropoiesis prevented the decrease of hepcidin synthesis triggered by bleeding or Epo administration suggested that hepcidin regulation strictly depends on erythropoietic activity (13, 15, 23). In the search for factor(s) communicating bone marrow iron requirements to the liver, the so-called "erythroid regulator," several candidates have been proposed, among them Epo itself (8, 11). Indeed, Epo administration to mice and humans resulted in strong repression of hepcidin synthesis, and under conditions of increased erythropoiesis, such as hypoxia or anemia, increased Epo levels were accompanied by hepcidin downregulation (8, 11). Moreover, the Epo receptor (EpoR) has been detected on the surface of a number of cell types, including liver cells (22). Accordingly, in vitro studies showed that the interaction of Epo with the EpoR on hepatoma cell lines resulted in inhibition of hepcidin transcription (4, 14). Furthermore, exposure to a high Epo dose led to a 50% inhibition of IL-6-dependent increase in hepcidin mRNA levels (16). However, our recent results showing that higher liver iron content is able to increase hepcidin expression in conditions of constantly high Epo serum levels (3) supported previous evidence obtained in mice with bone marrow suppression by cytotoxic agents or irradiation (13, 15, 23) to indicate that a direct effect of Epo on hepcidin expression is unlikely. This conclusion was supported by the recent identification of the erythroid factor erythroferrone (ERFE), which inhibits hepcidin expression under conditions of high erythropoietic activity (7).

The aim of this study was to further address this issue and test whether direct interaction between Epo and the liver is required to suppress hepcidin in erythropoiesis expansion.

MATERIALS AND METHODS

Animals. Mice were maintained under standard conditions with free access to water and food (170 mg iron/kg) in compliance with the "Principles of Laboratory Animal Care," as described previously (3). We used 6- to 8-wk-old male ICR CD1 and C57BL/6 wild-type mice, as well as TgEpoR mice (TgEPOR), with EpoR expression restricted to hematopoietic tissue (erythroid GATA-1 promoter/EpoR cDNA transgene) established on the EpoR$^{-/-}$ background (18). Genotypes

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were identified by PCR. Mice were injected with saline, or different amounts of Epo (epoetin alpha, Eprex; Janssen-Cilag) appropriately diluted in saline. Mice were also treated with the Epo-like peptide ARA290 that consists of 11 amino acids or with carbamylated Epo (CEpo) diluted about 1,000 fold in saline from 1.8 and 1.6 mg/ml stock solutions, respectively. Livers and spleens were harvested, frozen in liquid nitrogen, and stored at −80°C. All procedures were approved by the corresponding authorities and followed institutional guidelines of the University of Milano and the National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee.

Cell cultures. Human HepG2 and mouse c1c7 hepatoma cell lines obtained from the American Type Culture Collection were cultured in DMEM medium, 10% FBS, 0.5 units/ml penicillin/streptomycin, and 1 mM L-glutamine at 37°C and 5% CO_2 and exposed to various concentrations of Epo for different periods of time at a cell density of 1×10^4 cells/cm^2.

Blood analyses. Hematocrit of heparinized blood was measured in duplicate by using a microcentrifuge.

RNA analysis. Hepcidin, BMP6, and ERFE mRNA levels were measured by quantitative RT-PCR, as previously described (3). Total RNA isolated from liver and spleen using TRI reagent (Sigma) was reverse transcribed into cDNA with Proto Script M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs), and the obtained cDNA served as a template for RT-PCR, based on the TaqMan methodology (Life Technologies). Primers: mouse hepcidin: Mm00519025_m1; mouse BMP6 Mm01332882_m1; mouse 18S RNA Mm03928990_g1; mouse ERFE Mm00557748_m1; human hepcidin Hs00221783_m1; and human 18S RNA Hs03928985_g1 (Applied Biosystems). Thermal cycling parameters were 40 cycles at 95°C for 15 s and 60°C for 1 min. Samples were analyzed in triplicate and normalized to the housekeeping gene 18S RNA.

Protein analysis. Tissue lysates were prepared in RIPA buffer, incubated on ice for 30 min, and centrifuged at 13,600 *g* for 5 min. Proteins in the supernatant were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Bio-

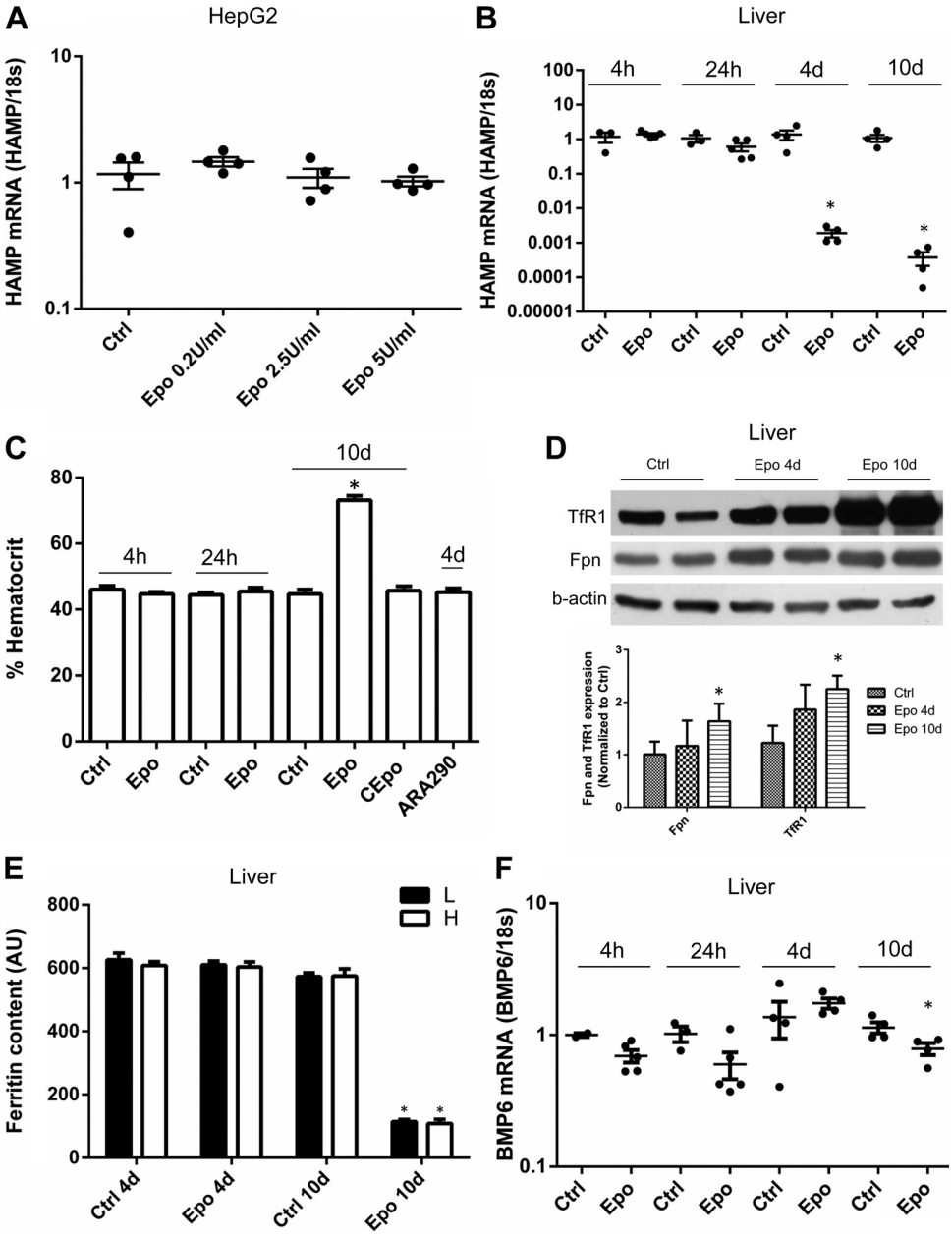

Fig. 1. *A*: HepG2 cells were left untreated (Ctrl) or treated with the indicated concentrations of erythropoietin (Epo) for 24 h. Hepcidin (HAMP) mRNA levels were measured by quantitative RT-PCR. Samples were analyzed in triplicate and normalized to the housekeeping gene 18S RNA. Dots and solid black line represent single animals and the mean, respectively. For better visualization, the *y*-axis scale has been set up using a logarithmic scale. *B–F*: data were obtained from ICR CD1 and C57BL/6 wild-type mice (without differences in response between strains) 4 h after injection of saline (Ctrl; $n = 3$) or 1,200 IU/kg of Epo (Epo; $n = 5$); 24 h after injection of saline (Ctrl; $n = 3$) or 1,200 IU/kg Epo (Epo; $n = 5$). Mice were also treated for four consecutive days with saline (Ctrl; $n = 4$) or 2,000 IU/kg daily Epo (Epo; $n = 4$), or 12 μg/kg daily ARA290 ($n = 3$); and 10 consecutive days with saline (Ctrl; $n = 4$), 1,200 IU/kg daily Epo (Epo; $n = 4$), or 10 μg/kg daily carbamylated Epo (CEpo; $n = 4$). *B*: hepcidin (HAMP) expression was measured as described above. *C*: hematocrit was measured using standard methods. *D*: transferrin receptor (TfR1) and ferroportin (Fpn) levels were detected by immunoblotting in liver extracts. Each panel shows a representative blot obtained with extracts of two animals for each experimental group, as well as the densitometric quantitation of the analysis of the extracts from all of the mice. The values were normalized to β-actin and to controls. *E*: heavy (H; gray bars) and light (L; black bars) ferritin subunits were measured using an ELISA, and the values were normalized to the protein content. *F*: BMP6 expression was measured by RT-PCR. Data are presented as means ± SE. $^*P < 0.05$.

sciences). Membranes were processed and incubated with primary antibodies (Ferroportin, Novus Biologicals NBP1-21502, 1:1,500; TfR1 Invitrogen 136800, 1:500; ERK1/2 Cell Signaling no. 9102, 1:1,000; phospho ERK1/2 Cell Signaling no. 9101, 1:1,000; Jak2 Cell Signaling no. 3230, 1:1,000; Phospho Jak2 Cell Signaling no. 3776, 1:1,000; β-actin Cell Signaling no. 5125, 1:5,000) and HRP-conjugated secondary antibodies. The antigens were detected using an immunodetection kit (ECL Basic, Amersham Biosciences). Each sample was loaded on 2–4 different gels, and the results were quantified using ImageJ software (v1.45q, National Institutes of Health). Background control was not applied, and the sum of all peak heights was used for quantification (5). Data were normalized to β-actin and to wild-type (Wt) controls.

Statistical analysis. Data are presented as means ± SE. Differences were analyzed using the Mann-Whitney *U*-test for unpaired samples. For those experiments involving three or more groups, the Mann-Whitney *U*-test was performed if prior Kruskal-Wallis analysis of variance revealed an effect of the treatment. All of the data were analyzed using SPSS 19.0 (SPSS Worldwide), and significance level was set to $P < 0.05$.

RESULTS AND DISCUSSION

As it has been shown that Epo administration to hepatic cells in vitro resulted in a 50% inhibition of hepcidin mRNA expression (4, 14), we decided to reinvestigate hepcidin expression in HepG2 cells exposed to varying Epo concentrations for two different time periods. The results reported in Fig. 1*A* show that in cells exposed to increasing concentrations of Epo for 24 h, hepcidin mRNA levels were not significantly changed; comparable results were obtained after 3 h of treat-

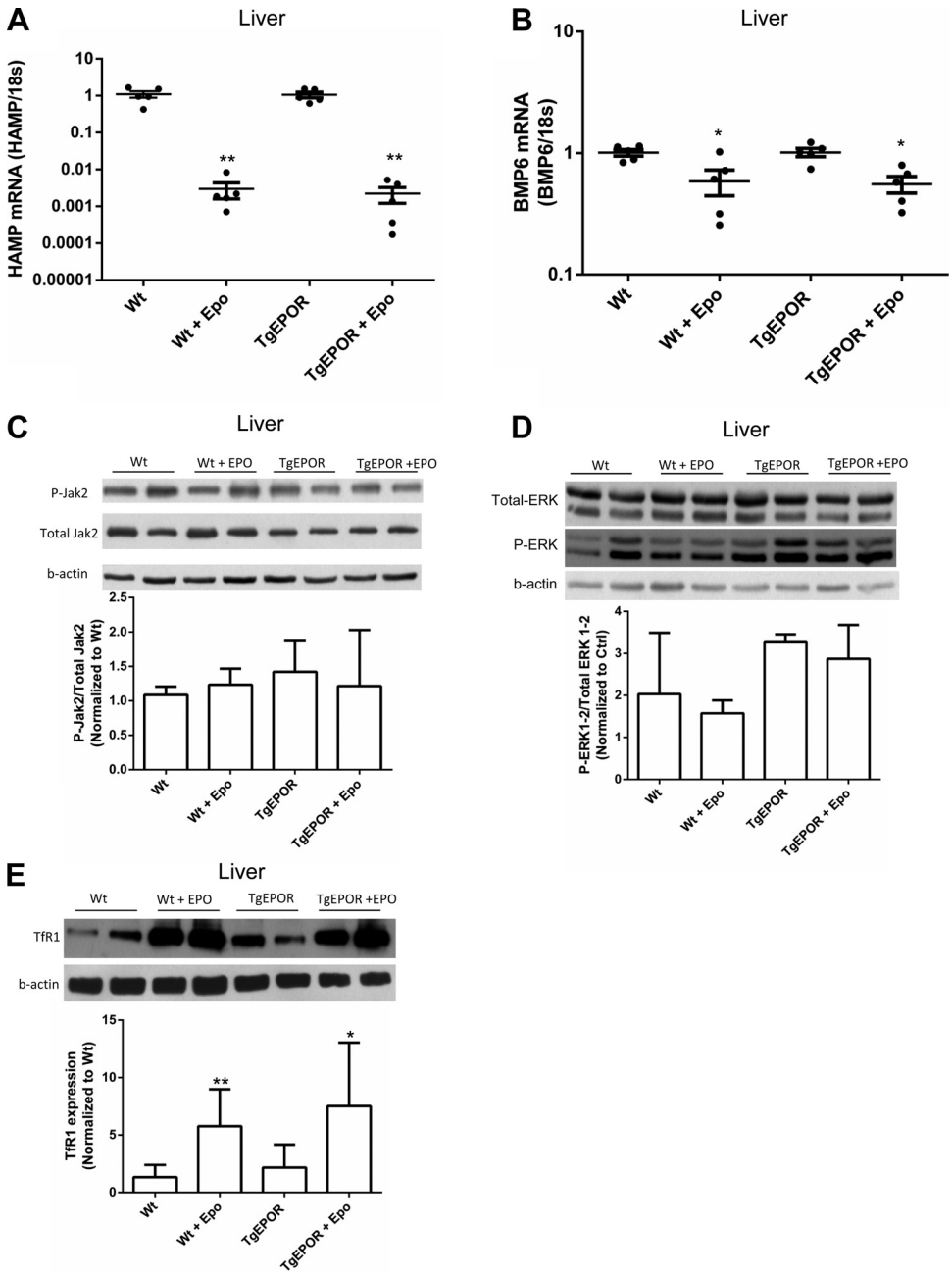

Fig. 2. Data were obtained from wild-type (Wt) controls, as well as from TgEpoR mice (TgEPOR) with EpoR expression restricted to hematopoietic tissue ($n = 5$) treated for 10 consecutive days with saline (Wt) or 1,200 IU/kg daily Epo. Hepcidin (HAMP) (*A*) and BMP6 (*B*) expression was measured by RT-PCR, as described in the legend to Fig 1. Dots and solid black line represent single animals and the mean, respectively. For better visualization, *y*-axis scale has been set up using a logarithmic scale. The levels of P-Jak2 and total Jak2 (*C*), P-Erk1/2 and total Erk1/2 (*D*), and TfR1 (*E*) were detected by Western blot analysis in liver extracts. Each panel shows a representative blot obtained with extracts of two animals for each experimental group and the densitometric quantitation of the analysis of the extracts from all of the mice. Data are presented as means ± SE. $*P < 0.05$; $**P < 0.01$.

ment (results not shown). Similarly, Epo treatment did not change hepcidin expression in the mouse liver cell line c1c7 (results not shown). Our results confirm recent studies reporting unaltered hepcidin mRNA levels in primary hepatocytes (10) and HepG2 cells exposed to Epo (17). The discrepancy between studies reporting no effect of Epo on hepcidin expression [present results, (10, 17)], and previous evidence of hepcidin repression (4, 14) could reflect differences inherent in the various experimental approaches.

We then evaluated the effects of Epo on hepcidin expression and liver iron metabolism in vivo. When Wt mice were injected with a single high dose (1,200 IU/kg) of Epo, liver hepcidin mRNA levels did not change at 4 h but showed a tendency to decrease at 24 h (Fig. 1*B*). As expected, no significant changes in hematocrit (Fig. 1*C*) were observed within this time period. Conversely, a strong decrease in hepcidin expression was found when the animals received repeated injections of high-dose Epo, either 2,000 IU/kg daily for 4 days or 1,200 IU/kg daily for 10 days (Fig. 1*B*). In animals receiving Epo for 4 days, hepcidin downregulation was accompanied by a small, nonsignificant increase of hepatic Fpn (Fig. 1*D*) and a visible splenomegaly (not shown), but the levels of transferrin receptor (TfR1), as well as of heavy (H) and light (L) ferritin subunits in the liver, were unaffected (Fig. 1, *D* and *E*, respectively). The strong erythropoietic stimulation caused by the 10-day treatment, which greatly increased hematocrit (Fig. 1*C*), similarly led to Fpn induction (Fig. 1*D*) and also resulted in increased TfR1 levels and decreased ferritin content (Fig. 1, *D* and *E*). Therefore, prolonged administration of high Epo doses leads to Fpn-mediated liver iron mobilization, obviously to support the erythroid demand, an observation in agreement with our previous results in Epo-overexpressing mice (3). In line with these findings, the hepatic mRNA levels of the iron stores regulator BMP6, which is key in the control of hepcidin expression (8, 11), were significantly decreased only after 10 days of treatment (Fig. 1*F*).

Next, we aimed to prove in vivo that direct interaction between Epo and the liver is not required to regulate hepcidin expression. To this purpose, we analyzed hepcidin mRNA levels in livers of TgEpoR transgenic mice expressing EpoR exclusively in hematopoietic tissue (18), which, therefore, represent an ideal model to assess the role of direct Epo signaling on hepatic hepcidin synthesis. Figure 2*A* shows that liver hepcidin mRNA levels were decreased after Epo administration (1,200 IU/kg daily for 10 days, leading to hematocrit increase, results not shown) to a similar extent both in TgEpoR mice and controls, thus indicating that the presence of EpoR on liver cells is not required for hepcidin suppression. BMP6 expression was also significantly decreased, but to a lower extent (Fig. 2*B*). Because it is well known that Epo binding to EpoR activates the Jak2 signaling pathway, we examined Jak2 phosphorylation, and we did not find any variations (Fig. 2*C*). Moreover, we investigated the ERK pathway, which is involved in hepcidin regulation (8, 11), but, once again, we did not observe changes in ERK phosphorylation upon Epo treatment (Fig. 2*D*). In line with the results shown in Fig. 1*C*, TfR1 was induced by Epo (Fig. 2*E*). We also determined the expression of ERFE, a recently identified protein that inhibits hepcidin expression during stress erythropoiesis (7). Epo administration led to a 10-fold increase in ERFE-encoding Fam132b mRNA levels in the spleen, which represents a site of extramedullary stress erythropoiesis upon Epo administration (8), in animals treated for 4 days, but not at earlier times (Fig. 3*A*). The discrepancy with the results reported by Kautz et al. (7), who found significant ERFE upregulation as soon as 4 h after phlebotomy or treatment with Epo, is possibly due to the more than five-fold higher dose of Epo given in their study. In addition, in our studies, Epo treatment for 10 days resulted in

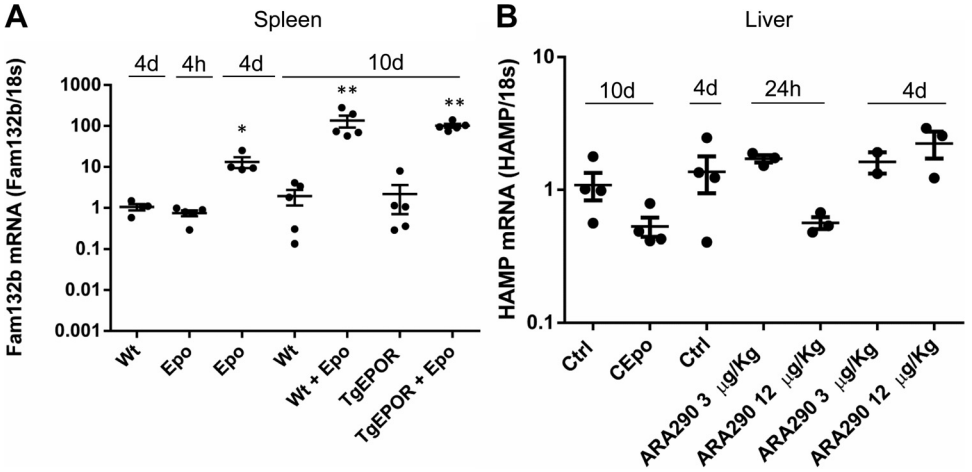

Fig. 3. *A*: data were obtained from ICR CD1 and C57BL/6 wild-type mice (without differences in response between strains). Mice were treated for four consecutive days with saline (Wt; $n = 4$) or 2,000 IU/kg daily Epo (Epo; $n = 4$), 4 h with 1,200 IU/kg of Epo (Epo; $n = 5$). Moreover, wild-type (Wt) controls and TgEpoR mice (TgEPOR) with EpoR expression restricted to hematopoietic tissue ($n = 5$) were treated for 10 consecutive days with saline or 1,200 IU/kg daily Epo. Erythroferrone (ERFE) expression was measured by RT-PCR. Samples were analyzed in triplicate and normalized to the housekeeping gene 18S RNA. Dots and solid black line represent single animals and the mean, respectively. For better visualization, the *y*-axis scale has been set up using a logarithmic scale. *B*: data were obtained from Wt mice treated for 10 consecutive days with saline (Ctrl; $n = 4$) or 10 μg/kg CEpo daily (CEpo; $n = 4$); mice treated for four consecutive days with a control peptide (Ctrl; $n = 4$); mice 24 h or 4 days after injection of 3 or 12 μg/kg daily of ARA290 ($n = 3$ for each group). Hepcidin (HAMP) expression was measured by RT-PCR. Samples were analyzed in triplicate and normalized to the housekeeping gene 18S RNA. Dots and solid black line represent single animals and the mean, respectively. For better visualization, *y*-axis scale has been set up using a logarithmic scale. Data are presented as means ± SE. $^*P < 0.05$; $^{**}P < 0.01$.

a nearly 100-fold stimulation of ERFE expression in both Wt and TgEpoR mice (Fig. 3*A*). These results further suggest that the role of Epo is to stimulate the synthesis of the erythroid regulator ERFE, which, in turn, inhibits hepcidin.

These findings clearly indicated that liver EpoR is not involved in hepcidin suppression; however, it cannot be formally excluded that in both Wt and TgEpoR mice, the inhibitory effect on hepcidin exerted by high doses of exogenous Epo was not mediated by its interaction on hepatocytes with its classical homodimeric receptor, but with the heterodimeric β common receptor (βcR), which has been proposed to mediate Epo's tissue-protective activity (9). This possibility is further supported by the suggestion that the affinity of βcR is much lower (1–20 nmol/l) than that of the classical receptor (100–200 pmol/l), and thus, the interaction with the heterodimeric receptor becomes active only upon application of high-Epo dosage (1). To address this issue, we treated the animals with CEpo, a compound thought to interact solely with the βcR, as shown by its inability to stimulate erythropoiesis (9) (Fig. 3*B*). In line with a study that used different treatment schedules (15), we did not observe significant down-modulation of hepcidin expression in animals treated for 10 days with doses of CEpo that were similar to those used for Epo and were shown to be tissue-protective (9). As expected, hematocrit was unchanged (Fig. 1*C*). Similarly ineffective in downregulating hepcidin (Fig. 3*B*) and increasing hematocrit (Fig. 1*C*) were our treatments for 1 or 4 days with two different doses of ARA290, a peptide that binds the β common receptor and shows the same protective properties of CEpo (19).

In summary, the present results showed that Epo does not directly down-modulate hepcidin expression. While this manuscript was in preparation, ERFE, a new hormone that inhibits hepcidin expression during stress erythropoiesis in mice, was characterized and published (7). In line with that study, our results (see Figs. 1*B* and 3*A*) support the idea that under conditions of markedly increased erythroid activity, Epo stimulates the synthesis of the regulator ERFE, which ultimately downregulates hepcidin. In fact, using mice with EpoR expression restricted to hematopoietic cells only, we demonstrated that hepcidin suppression does not require the binding of Epo to its receptor on the surface of hepatocytes. Moreover, administration of Epo analogs, possibly interacting with the postulated βcR, which is thought to mediate the tissue-protecting activity of Epo (1, 9), did not affect hepcidin expression either. In conclusion, our findings demonstrating that hepcidin suppression does not require the direct binding of Epo to its homodimeric or heterodimeric receptor on the surface of hepatocytes were supported by the unaffected hepcidin expression found in human and mouse hepatoma cells exposed to Epo.

Perspectives and Significance

Our data indicate that the effect of Epo on the liver's expression of hepcidin is not directly mediated by its interaction with high or low affinity EpoR on hepatocytes, but rather suggest that the role of Epo is to stimulate the production of the erythroid regulator ERFE in erythropoietically active tissue, which ultimately inhibits hepcidin expression.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: E.G., V.D., S.R., M.S., and G.C. conception and design of research; E.G., V.D., P.B., and S.D. performed experiments; E.G., V.D., S.R., M.S., C.T.N., and G.C. analyzed data; E.G., V.D., S.R., M.S., M.G., and G.C. interpreted results of experiments; E.G. prepared figures; E.G., C.T.N., M.G., and G.C. edited and revised manuscript; V.D., S.R., P.B., M.S., S.D., C.T.N., and M.G. approved final version of manuscript.

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