Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPα

Short Title: Erythropoietin and hepcidin expression in hepatocytes

Jorge P Pinto¹, Sara Ribeiro¹*, Helena Pontes²*, Shifaan Thowfeequ³*, David Tosh³, Félix Carvalho⁵ and Graça Porto¹,4,5

¹ Iron Genes and Immune System, IBMC - Instituto de Biologia Molecular e Celular, University of Porto, Porto, Portugal.
² REQUIMTE, Toxicology Department, Faculty of Pharmacy, University of Porto, Porto, Portugal.
³ Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY UK.
⁴ Clinical Hematology, Santo António General Hospital, Porto, Portugal.
⁵ Molecular Immunology and Pathology, ICBAS - Abel Salazar Institute for the Biomedical Science, University of Porto, Portugal.

* The authors contributed equally to the work.

Scientific heading: Red cells

Correspondence: Graça Porto, Iron Genes and Immune System, IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal; phone: 351-226074956; Fax: 351-226098480; e-mail: gporto@ibmc.up.pt.
Abstract

Hepcidin is the principal iron regulatory hormone, controlling the systemic absorption and re-mobilization of iron from intracellular stores. Recent in vivo studies have shown that hepcidin is down-regulated by erythropoiesis, anemia and hypoxia, which meets the need of iron input for erythrocyte production. Erythropoietin (EPO) is the primary signal that triggers erythropoiesis in anemic and hypoxic conditions. Therefore, a direct involvement of EPO in hepcidin regulation can be hypothesized. We report here the regulation of hepcidin expression by EPO, in a dose-dependent manner, in freshly isolated mouse hepatocytes and in the HepG2 human hepatocyte cell model. The effect is mediated through EPOR signaling, since hepcidin mRNA levels are restored by pre-treatment with an EPOR-blocking antibody. The transcription factor C/EBPα showed a pattern of expression similar to hepcidin, at the mRNA and protein levels, following EPO and anti-EPOR treatments. Chromatin Immunoprecipitation experiments showed a significant decrease of C/EBPα binding to the hepcidin promoter after EPO supplementation, suggesting the involvement of this transcription factor in the transcriptional response of hepcidin to EPO.
**Introduction**

Hepcidin has been described as the central regulator of iron homeostasis and deficiencies in hepcidin are associated with several iron-related disorders. Hepcidin modulates iron homeostasis by inducing the internalization and degradation of ferroportin, the single known cellular iron exporter, expressed by duodenal enterocytes as well as by macrophages and hepatocytes.

Hypoxia and anemia are the two main signals that trigger the erythroid-regulator of intestinal iron absorption, independently of iron stores. These signals also regulate the production of erythrocytes through synthesis of the hormone erythropoietin (EPO). The hypothesis that hypoxia could act both on erythropoiesis induction and on hepcidin down-regulation via EPO signaling was first advanced in 2002, based on the evidence that liver hepcidin gene expression is strongly decreased by EPO injection *in vivo*. The first evidence concerning a possible direct role of EPO on the regulation of hepcidin synthesis by hepatocytes, the main hepcidin-producing cells, was provided by Fein et al, who demonstrated a down-regulation of this protein in a hepatoma and in a pancreatic cell line after stimulation with EPO.

With the objective of clarifying the possible direct role of EPO on hepcidin regulation, we analyzed the dose-dependent effect of EPO on hepcidin levels on freshly isolated mouse hepatocytes and on the human hepatocyte cell line HepG2, which express endogenous hepcidin, EPO and EPOR. The involvement of EPOR signaling and of the transcription factor C/EBPα was also investigated.
Materials and Methods

Animals

C57BL/6 mice with 10-14 weeks of age were used as the source of hepatocytes. Animals were acclimatized in polyethylene cages lined with wood shavings, under a 12/12h light/dark cycle. Mice had free access to standard rat chow and drinking water. An acclimatizing period of at least one week was performed, before starting the experiments. Animals were anesthetized with diethyl ether before the start of the surgical procedures.

Isolation and incubation of hepatocytes

Hepatocyte isolation was performed by collagenase perfusion, as described by Moldéus et al., with the modifications described in Carvalho et al..

Immediately after isolation, cell viability was determined with the trypan blue exclusion test. Viability was always above 83%.

Since previous reports have shown that recombinant human EPO (rEPO) mimics the effect of murine EPO on mouse cells, mouse hepatocytes were incubated, immediately following isolation, with 0.01-2U/ml of rEPO (Sigma), and/or 1 or 5 µg/ml of goat anti-EPO receptor (EPOR) polyclonal antibody (Sigma) for 3 hours, which corresponds to the incubation period where hepcidin response to rEPO was maximum (data not shown). To test for responsiveness of hepcidin transcription to an exogenous stimulus, incubation with 20 ng/ml of human IL-6 (Sigma), for 3 hours, was performed.

Cell viability was determined after each experiment by the lactate dehydrogenase (LDH) leakage method, which was randomly confirmed by the trypan blue exclusion test. No statistical differences in cell viability were observed between
any of the treatments and the non-treated control (data not shown). Viability values of 74% ± 7% were obtained.

**HepG2 culture and treatments**

HepG2 cells were maintained in complete DMEM, (DMEM supplemented with 10% FCS and 1% penicillin/streptomycin/amphotericin). One day before treatments, 3x10^5 cells were seeded in 6-well plates and incubated overnight (O/N). Cells were then treated with 0.01-2.5 U/ml of rEPO for 3 hours, in complete DMEM. For anti-EPOR treatments, cells were incubated with 0.1-10 µg/ml of goat anti-EPOR for 30 min and then treated with 1 or 2 U/ml of rEPO, when appropriate, for 3 hours. Negative control was performed by replacing the primary antibody with an irrelevant antibody (polyclonal anti-CD3; A0452, DAKO). Specificity control was performed for EPOR by absorption of the primary antibody with a specific blocking peptide (sc-697-P, Santa Cruz Biotechnology, CA, USA). The blocking peptide was combined with the primary antibody with a 5-fold excess of the peptide in BSA/TBS buffer overnight at 4 °C. After treatment, cells were harvested by trypsinization, pelleted and frozen at -80°C until required. All experiments were performed at least three times.

**Determination of gene expression**

Total RNA was extracted from cells using RNeasy Midi Kit (Qiagen) and 0.5 µg used for reverse-transcription with SuperScript First-Strand kit (Invitrogen), following the manufacturer’s instructions. Assessment of mRNA levels was performed in an iCycler iQ5 PCR detection system (Bio-Rad), using specific oligonucleotides (Table 1, supplemental data). As an internal control, GAPDH mRNA levels were determined simultaneously. Relative expression levels were calculated as 2^(Ct human GAPDH)
endogenous control gene - Ct gene*10000). For every qRT-PCR, a dilution series consisting of four serial dilutions was used during optimization of the procedure. All qRT-PCR experiments were done at least in triplicate, with 2-3 replicates each.

**Western blot analysis**

Cells were trypsinized and lysed in gel loading buffer (500 mM Tris–HCl, pH 6.8, 5% SDS, 20% glycerol, 50 µl bromophenol blue). Forty micrograms of total cell lysate were boiled, separated by SDS-PAGE (12%) and transferred to a nitrocellulose Hybond-C membrane (Amersham Biosciences, UK). The blots were blocked at room temperature (R/T) with 5% dry milk/0.05% Tween 20 in PBS (TBS-T), incubated with rabbit polyclonal C/EBPa antibody (Santa Cruz Biotechnology), washed 3× with TBS-T and the signal was detected with goat anti-rabbit IgG–HRP conjugate (Molecular Probes, USA), followed by incubation with the enhanced chemiluminescence substrate Super Signal West Dura (Pierce, USA). For loading control, the membrane was stripped and hybridized with anti-β-actin (Abcam, UK) antibody. Quantification of band intensity was performed with ImageJ. In order to be able to observe C/EBPa expression even after treatment with high EPO doses, the maximum amount of total protein (40 µg) was loaded in each well, coupled with long autoradiography exposure times and use of a highly sensitivity detection substrate (Pierce’s SuperSignal West Dura Extended Duration Substrate) (see Figure 3A).

**Chromatin immunoprecipitation (ChIP)**

Two 90mm dishes of 70-80% confluent HepG2 cells, cultured and treated as previously described, were used per condition for the ChIP analysis. Cells were cross-linked with 0.38% formaldehyde at 37°C for 10 minutes. The fixation was stopped by
adding glycine to a final concentration of 0.125M and incubating at R/T for 5 minutes. Total cell lysate was extracted using 600 µl of ChIP lysis buffer (50 mM Tris-HCl, pH 8.1, containing 1% Triton X-100, 0.1% deoxycholate, 150 mM NaCl, and 5 mM EDTA), supplemented with 1:100 protease inhibitor cocktail (Sigma). The DNA was sheared to an average fragment size of 500 base pairs by sonication (10 pulses of 5 seconds with 55 seconds rest period between each). Cell lysates were pre-cleared with protein A-agarose beads (Santa Cruz) and 50 µg of chromatin were immunoprecipitated overnight, at 4°C, using the polyclonal rabbit anti-C/EBPα (14AA) antibody (sc-61, Santa Cruz) or incubated with normal rabbit serum for the serum control (5 µg of chromatin were retained for use as 10% input). Antibody-chromatin complexes were collected using protein A-agarose beads. The agarose was pelleted and washed sequentially in lysis buffer, lysis buffer with 500 mM NaCl, lysis buffer with 0.25 M LiCl and finally in Tris/EDTA buffer (10mM/1mM, pH 8.0). DNA and protein were eluted from the beads by washing in the elution buffer (0.1 M NaHCO₃ with 1% SDS and 20 µg/ml herring sperm DNA). The cross-links were then reversed by incubating at 65°C for 4 hours and 10 µg of proteinase K added to the samples and incubated for a further 1h, at 45°C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation, and dissolved in Tris/EDTA. DNA was amplified by Real-Time PCR using the oligonucleotides 5’-TCATCAAACTGCTTAAC-3’ and 5’-TCTGTCTGGCTGCTCCTGCT-3’, as forward and reverse primers respectively, at an annealing temperature of 64°C. The results are expressed as a ratio of the input (into the reaction) to the output (after immunoprecipitation) normalized to the no-EPO control.
Statistical analysis

Results are presented as the ratio of mRNA and protein levels to the average of controls. Control mRNA and protein levels were normalized to 1. In general, one-way ANOVA was used to test for significant differences among sample means. When comparisons included the simultaneous effect of more than one variable two-way ANOVA was applied. When significant differences were found, Fisher’s Least Significant Difference (LSD) was used to identify significantly different means. All statistical analysis was performed with STATGRAPHICS Centurion XV.

Results and Discussion

The effect of EPO on hepcidin expression was analyzed in the human liver cell line HepG2 and in freshly isolated mouse hepatocytes, which are useful cell systems for studies of hepcidin regulation in vitro.8,10 We found that hepcidin mRNA expression is modulated by EPO in HepG2 cells and in mouse hepatocytes in a dose-dependent manner (Figures 1A,C). In HepG2 cells, supplementation with 0.01 U induced a moderate but significant increase in hepcidin, while the highest EPO doses (0.5-2.5 U) caused a significant down-regulation (up to 60% decrease) of hepcidin expression (Figure 1A). Hepcidin 1 expression was down-regulated in mouse hepatocytes at all EPO concentrations examined, with the exception of the lowest 0.01 U (Figure 1C). Down-regulation was, however, less pronounced than in the HepG2 cell line, with a lowest value of ~ 40%, for 0.5 U EPO. Treatment with IL-6, a strong inducer of hepcidin transcription in both in vitro and in vivo settings,16 was performed to assess for a normal responsiveness of hepcidin transcription to exogenous stimulus in the conditions of our assays. A significant increase in hepcidin expression was observed both in HepG2 and in mouse hepatocytes (Figures 1A,C, right).
To test for the specificity of the EPO effect, we blocked the EPOR with an anti-EPOR antibody that recognizes the extracellular domain of this receptor and prevents EPO-EPOR interaction. On both models EPOR blocking abrogated the repressive effect of EPO on hepcidin expression (Figures 1B,C). The specificity of the blocking effect of anti-EPOR antibody was confirmed by the fact that pre-absorption of the primary antibody with an excess of a specific blocking peptide restored hepcidin mRNA to levels not different from those induced by the two doses of EPO alone (Figure 1B). Similarly, co-incubation of HepG2 cells with 1 and 2 U of EPO and with an irrelevant antibody did not induce significant differences in hepcidin mRNA levels in comparison with cells treated with EPO alone (data not shown). Interestingly, cells treated with anti-EPOR alone showed a dose-dependent up-regulation of hepcidin mRNA, relatively to the non-treated control cells (Figures 1B,C).

No significant effect of EPO supplementation or EPOR blocking on murine hepcidin 2 mRNA levels was observed (data not shown).

Analysis of the levels of CEBPA and Cebpa mRNAs showed that both CEBPA (Figure 2A) and Cebpa (Figure 2B) mRNA levels are significantly decreased after treatment with 0.5-2.5 U or 0.1-2 U of EPO, respectively for the HepG2 cell line and fresh mouse hepatocytes. Blocking of EPOR and EpoR restored mRNA expression to levels similar to the control cells and significantly higher than EPO-treated (1 U) cells. The specificity of the anti-EPOR antibody was confirmed by the observation that pre-absorption with a specific peptide restored CEBPA mRNA expression to levels not different from those of cells treated with 1U EPO alone. Similarly to hepcidin mRNA expression, no rescue of CEBPA mRNA levels was observed after co-treatment with 1U EPO and an irrelevant antibody. IL-6 treatment did not induce significant changes in CEBPA or Cebpa mRNA levels (Figures 2A,B; right).
Since hepcidin and *CEBPA* response to EPO and anti-EPOR is essentially the same in HepG2 and in fresh primary mouse hepatocytes, we decided to continue the experimental work using the HepG2 cell model. The expression of C/EBPα in response to EPO and anti-EPOR treatments was analyzed. The high sensitivity detection method adopted in this study allowed for the detection of C/EBPα expression even after down-modulation by EPO treatments. Although some discrepancy exists in the literature concerning the levels of C/EBPα in HepG2 cells,\textsuperscript{17-22} the levels detected in the present study were similar to those reported by Chew et al.\textsuperscript{20} and Yoon and Smart,\textsuperscript{21} using equally or less sensitive conditions.

The expression of C/EBPα protein closely mimics what was observed for *CEBPA* mRNA, with a mild increase in C/EBPα induced by the lowest dose of EPO and a decrease which is more pronounced from 0.5 U EPO onwards, reaching ~50% for 2.5 U EPO (Figures 3A,B). A down-modulation of C/EBPα protein levels by EPO after only 3 hours of treatment can be explained by the short half-lives of the mRNA and protein: two hours for mRNA (in HepG2 cells)\textsuperscript{23} and one hour for the C/EBPα protein.\textsuperscript{24}

To test if the EPO-induced down-modulation of C/EBPα would impact on the binding of this transcription factor to the *HAMP1* promoter, ChIP analysis was performed, using lysates from HepG2 cells treated according to the protocol followed for the previous experiments (Figure 3C). Results show that treatment with the lowest (0.01 U) EPO dose induces a mild, but significant, increase in the binding of C/EBPα to *HAMP1* promoter. In contrast, treatment with 0.01-2.5 U EPO causes a significant decrease of C/EBPα binding, with a maximum of 75% reduction for 2.5 U EPO.

Previous studies have invoked EPO in the regulation of hepcidin expression.\textsuperscript{6,25,26} However, they do not clarify the mechanism underlying this interaction, namely whether EPO is capable of directly signaling the hepatocyte to
modulate hepcidin levels. The study from Pak and colleagues referred above suggested that during erythropoiesis, “hepcidin is not directly regulated by EPO but rather by a signal downstream from EPO”, based on inhibition of erythropoiesis with carboplatin. Our results show that, at least in vitro, EPO, via signaling through EPOR, is able to modulate hepcidin expression in freshly isolated mouse hepatocytes and in human hepatoma cells. It is possible that the results obtained in vivo after blocking of erythropoiesis are reflecting carboplatin-induced changes in other parameters secondary to erythropoiesis inhibition. One hypothesis is that the increased hepatic non-heme iron levels observed in the study of Pak and colleagues may be counter-acting the down-regulation of hepcidin by EPO. To control for EPO-induced changes in iron levels in our in vitro models, we measured TFR1 mRNA expression in the same samples used for hepcidin and CEBPA/Cebpa assessment. No significant changes were observed after EPO or anti-EPOR treatments (data not shown), supporting the hypothesis that the mechanism underlying the modulation of hepcidin by EPO is independent of iron sensing by the hepatocyte.

We show that EPO-induced hepcidin regulation is achieved by the modulation of C/EBPα mRNA and protein levels, with significant changes on the transcriptional activation of hepcidin. Several studies have demonstrated a role for C/EBPα in the regulation of hepcidin transcription, but its involvement on the EPO-mediated regulation of hepcidin expression has not been suggested prior to this study. The mechanism underlying the C/EBPα regulation by EPO remains unknown. A previous study showed that the transcription factor CHOP (GADD153), which is known to impair C/EBPα binding to target promoters, is up-regulated in Rauscher murine erythroleukemia cells by treatment with high doses (50-100 U/ml) of rEPO. Such a mechanism could further amplify the role of C/EBPα in the EPO-mediated hepcidin
expression. However, we failed to observe an up-regulation of CHOP mRNA at least with the EPO doses used in our experimental protocol, some of which are already supra-physiological (results not shown). This suggests that CHOP may not be involved in the down-modulation of hepcidin by EPO in physiological conditions.

The relevance of the present results is further reinforced by previous reports showing that hepatocytes, which are the main hepcidin producing-cells, express EPO and EPOR. Also, a variety of malignant cell lines, including HepG2, was found to synthesize both this hormone and its receptor, which suggest the importance of the EPO signaling beyond erythropoiesis.

The EPO concentrations that in the present in vitro study were shown to inhibit hepcidin expression do not correspond to normal in vivo physiological concentrations but rather to concentrations that are found only in situations of severe anemia, hypoxia and ineffective erythropoiesis or when EPO is administered therapeutically. Schmidt et al. found that physiological (0.01 and 0.03) and supra-physiological (0.1 – 3 U) doses of EPO activate different signaling pathways, with effective MEK and Erk signaling only at physiological doses. Similarly, low and high EPO dosages have opposite effects on NO production and vasodilatation. We propose that a similar mechanism may underlie the distinct effects in hepcidin expression of the different EPO doses used, whereby the basal EPO signal would be modulated by other signaling pathways activated by higher EPO concentrations, resulting in a repression of hepcidin expression.

In conclusion, the present results support the hypothesis of a direct role for EPO in the regulation of hepcidin, via modulation of C/EBPα expression. Although this does not argue against the existence of additional mediators of hepcidin expression in the context of erythropoiesis, as has recently been demonstrated for the TGF-β
superfamily-member GDF15, we suggest that a direct signaling of EPO to the hepatocyte could be one of the mechanisms underlying the reported in vivo hepcidin modulation by erythropoiesis.

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Conflict-of-interest disclosure: The authors declare no competing financial interests.

Authorship:
Contribution: JPP conceptualized the idea, designed and performed the research, analyzed data and wrote the paper; SR, HP and ST performed the research; DT and FC contributed vital analytical tools and contributed to paper writing; GP conceptualized the idea, analyzed data and contributed to paper writing.
References


Figure Legends:

Figure 1. **EPO modulates hepcidin expression in a dose-dependent manner in HepG2 cells and in freshly isolated mouse hepatocytes.**

A. qRT-PCR analysis of HAMP mRNA expression in HepG2 cells treated with rEPO or IL-6. Figure represents the average + SD of three independent experiments; *denotes a statistically significant difference between treated samples and mock (P<0.05; one-way ANOVA).

B. qRT-PCR analysis of HAMP mRNA expression in HepG2 cells treated with rEPO (1 or 2 U/ml) and/or with 0.1-10 µg/ml of anti-EPOR. Bars on the right of each group correspond to HAMP mRNA levels after pre-absorption of the anti-EPOR antibody with a specific peptide (+ Block). *denotes a significant difference (P<0.05) between EPO(+)anti-EPOR(+) treated samples and the EPO(+)anti-EPOR(-) controls (one-way ANOVA) **stands for a significant difference P<0.05 level, between EPO(-)anti-EPOR(+).or EPO(-)/anti-EPOR(+)/Block(+) and the EPO(-)/anti-EPOR(-) control (two-way ANOVA). Figure represents the average + SD of three independent experiments.

C. qRT-PCR analysis of Hamp1 mRNA expression in freshly isolated mouse hepatocytes treated with 0.01-2.0 U/ml of rEPO (grey bars), and/or 0.1-5 µg anti-EPOR (white and black bars, respectively). Untreated HepG2 cells were used as control (streaky bar, left). Treatment with IL-6 was used to assess Hamp1 response to stimulation (grey bar, right). A total of seven animals were used for each treatment; *denotes a significant difference (P<0.05) between EPO(+)anti-EPOR(-) or EPO(-)anti-EPOR(+) or IL-6(+) and the non-treated control (one-way ANOVA). **stands for a significant difference at the 95% confidence level between (EPO(+)/anti-EPOR(+) and the EPO(1U)/anti-EPOR(-) control (one-way ANOVA).

Figure 2. **EPO modulates CEBPA expression in a dose-dependent manner in liver derived-cells.** qRT-PCR assessment of CEBPA (A) and Cebpa (B) mRNA expression
in HepG2 cells and in mouse hepatocytes treated with rEPO (grey bars, left) and/or anti-EPOR (white and black bars, respectively) and IL-6 (grey bar, right). Untreated cells were used as control (streaky bars, left). Figure represents the average ± SD of three independent experiments for HepG2 cells. A total of seven animals were used for each mouse hepatocyte treatment; *denotes a significant difference \( (P<0.05) \) between EPO(+)/anti-EPOR(-) or EPO(-)/anti-EPOR(+)) or EPO(-)/anti-EPOR(+)/Block(+) or IL6(+) and the EPO(-)/anti-EPOR(-) control (one-way ANOVA). **stands for a significant difference \( (P<0.05) \) between EPO(+)/anti-EPOR(+) or EPO(1U)/anti-EPOR(+)/Block(+) and the EPO(1U)/anti-EPOR(-) control (two-way ANOVA).

Figure 3. **C/EBPα protein levels and binding to HAMP promoter are modulated by EPO in a dose-dependent manner.** **A.** Western blot analysis of C/EBPα levels in HepG2 cells after treatment with physiological and supra-physiological dosis of rEPO, anti-EPOR antibody or IL-6. β-actin was used as a loading control (bottom panel). Numbers on the left of each panel correspond to the sizes of the MW marker bands. Figure shows a representative result out of three experiments. Vertical lines have been inserted to indicate repositioned gel lanes. **B.** Scanning densitometry of C/EBPα western blot. Data are normalized for β-actin levels and are expressed as arbitrary units (A.U.). **C.** qRT-PCR-ChIP analysis of the effect of rEPO and anti-EPOR on the binding of C/EBPα to the HAMP promoter. Figure shows the averages and SD of two experiments, each with three replicates. *signals a significant \( (P<0.05) \) difference between EPO-treated samples and the non-treated control (one-way ANOVA); **signals a significant \( (P<0.05) \) difference between anti-EPOR-treated samples and the 2.5U EPO-treated cells (one-way ANOVA).
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**Note:** For personal use only.
Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signalling and regulation of C/EBP α

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