ORIGINAL ARTICLE

Hypoxia induced downregulation of hepcidin is mediated by platelet derived growth factor BB

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ABSTRACT

Objective  Hypoxia affects body iron homeostasis; however, the underlying mechanisms are incompletely understood.

Design  Using a standardised hypoxia chamber, 23 healthy volunteers were subjected to hypoxic conditions, equivalent to an altitude of 5600 m, for 6 h. Subsequent experiments were performed in C57BL/6 mice, CREB-H knockout mice, primary hepatocytes and HepG2 cells.

Results  Exposure of subjects to hypoxia resulted in a significant decrease of serum levels of the master regulator of iron homeostasis, hepcidin and elevated concentrations of platelet derived growth factor (PDGF)-BB. Using correlation analysis, we identified PDGF-BB to be associated with hypoxia mediated hepcidin repression in humans. We then exposed mice to hypoxia using a standardised chamber and observed downregulation of hepatic hepcidin mRNA expression that was paralleled by elevated serum PDGF-BB protein concentrations and higher serum iron levels as compared with mice housed under normoxic conditions. PDGF-BB treatment in vitro and in vivo resulted in suppression of both steady state and BMP6 inducible hepcidin expression. Mechanistically, PDGF-BB inhibits hepcidin transcription by downregulating the protein expression of the transcription factors CREB and CREB-H, and pharmacological blockade or genetic ablation of these pathways abrogated the effects of PDGF-BB toward hepcidin expression.

Conclusions  Hypoxia decreases hepatic hepcidin expression by a novel regulatory pathway exerted via PDGF-BB, leading to increased availability of circulating iron that can be used for erythropoiesis.

INTRODUCTION

Hypoxic stress commonly occurs as a consequence of environmental or pathological disturbances resulting in reduced oxygen tension in the blood or in tissues.1 As compensation, the body tries to expand the oxygen transport capacities as reflected by a rapid increase in circulating erythropoietin (EPO) levels and stimulation of erythropoiesis during hypoxic stress.2 3 A prerequisite for efficient erythropoiesis is a sufficient supply of iron that is needed for the synthesis of haemoglobin.1 3 4 The higher iron needs for erythropoiesis during hypoxic challenge are met by mobilisation of the metal from body iron stores and increased duodenal iron absorption.5 6 It has been suggested that such adaptations of body iron homeostasis require regulation of the master regulator of iron homeostasis, hepcidin, a mainly liver derived 25-amino acid peptide.7−9 Hepcidin controls iron metabolism by binding to the only known cellular iron exporter ferroportin (also known as SCL40A1 or Ireg1), thereby inducing its internalisation and degradation. Accordingly, high circulating hepcidin levels are seen in transfusional iron overload or inflammation—block duodenal iron absorption and iron egress from macrophages/monocytes.9 10
 whereas low circulating hepcidin levels—as they are found in association with systemic iron deficiency—stimulate dietary iron uptake and iron efflux from monocytes/macrophages making the metal available for erythropoiesis.9–10 The regulation of hepcidin synthesis is thought to take place mainly at the transcriptional level. Two main upstream signalling pathways regulate hepcidin expression in the liver: the BMP6/SMAD1/5/8 and the interleukin 6 (IL-6)/JAK2/STAT3 pathway, respectively.11–16 Interestingly, endoplasmic reticulum stress affects hepcidin transcription in hepatocytes via activation of cyclic AMP response element binding protein H (CREB-H) or GATA binding protein 4.17–18 The multitudinousness of factors impacting hepcidin expression may be explained by the pivotal role of iron for cell proliferation, cell metabolism and survival,6,20 and by the key role of the metal in immunosurveillance and infection.21,22

Hypoxia has been shown to impact on iron homeostasis mainly via hypoxia-inducible factor (HIF) regulation either via direct transcriptional activation of iron metabolism genes or indirectly via induction of EPO.23,24 Although HIFs have been noticed to modulate iron homeostasis, the specific role of HIF signalling on hepcidin expression has not been fully disentangled so far.23 Moreover, in vitro studies using HepG2 and HuH7 cells suggest that hypoxia results in generation of reactive oxygen species and subsequent inhibition of C/EBPα and STAT3 mediated activation of the hepcidin promoter.25,26 Another study performed with HuH7/THP1 cocultures indicates that the interaction between monocytes and hepatocytes is necessary to achieve hypoxia mediated hepcidin suppression.27 Moreover, the BMP/SMAD4 responsive element of the hepcidin promoter appears to be of importance for the transmission of hypoxia mediated hepcidin regulation.13

So far, a few human studies have been published investigating changes in circulating hepcidin levels during hypobaric hypoxia.28–30 These studies provide interesting insights into hypoxia mediated changes of iron metabolism, although mechanistic data toward the underlying pathways are lacking. Thus, we performed a study with human volunteers who were challenged with exercise under normobaric hypoxic conditions and combined this study with investigations in mouse models and cell culture to identify the pathways being potentially responsible for hypoxic regulation of iron homeostasis and hepcidin expression, which led to the identification of platelet derived growth factor (PDGF)-BB as a novel regulator of hepatic hepcidin expression and systemic iron homeostasis.

**Results**

**Design**

Studies with human subjects

In all, 23 healthy volunteers participated in the study. Written informed consent was obtained from all participants. The studies were carried out in accordance with Helsinki guidelines and approved by the ethical committee at the Medical University of Innsbruck (approval number: AM2544, 239/4.12 and 273/5.7). Details of hypoxia studies and analysis of serum parameters are described in online supplementary methods.

Animal care and in vivo mouse studies

C57BL/6 mice were purchased from Charles River Laboratories (Germany); CREB-H knockout mice and corresponding wild-type littermates were generated as previously described and shipped to Innsbruck.17 We used male C57BL/6 mice at the age of 6–8 weeks that were housed at the Animal Care Unit of the Medical University Innsbruck under pathogen-free conditions with fixed day and night cycles and free access to water and food. All animal experiments were performed in accordance with the Austrian Animal Testing Act of 1988 and all experiments were approved by the Austrian ethics committee (BMWF-66.011/0073-II/3b/2011). Details of hypoxia studies are shown in online supplementary methods.

Cell culture experiments

Primary murine hepatocytes were isolated from male 6–8-week-old C57BL/6 mice as previously reported.31 Primary murine hepatocytes or human hepatocellular HepG2 cells were used in all cell culture experiments (for details see online supplementary methods).

**RNA preparation, reverse transcription, RT-PCR and western blotting**

RNA was extracted from adherent cells or tissue samples using a guanidinium-isothiocyanate-phenol-chloroform-based protocol as previously reported.32 Protein extraction and western blotting were performed as detailed elsewhere.33 Further details are given in online supplementary methods.

**Statistical analysis**

Statistical analyses were carried out using statistical analyses software package (SPSS V.19.0). Following descriptive data evaluation including tests for homoscedasticity and normal distribution, we used analysis of variance (ANOVA) and post hoc test or non-parametric Kruskal–Wallis and Mann–Whitney U test, as appropriate. Bonferroni–Holmes correction was used to correct for multiple testing. Correlations of two parameters were tested with Pearson (parametric data) or Spearman-r (non-parametric data) test.

**Results**

**Changes of circulating iron metabolism and inflammation markers under normobaric, hypoxic conditions in healthy human volunteers**

The baseline characteristics and baseline laboratory variables of healthy human volunteers are shown in table 1. As anticipated, the exposure of subjects to hypoxia for 6 h resulted in a significant decrease of arterial oxygenation (pO2) and oxygen saturation of the blood (SO2) (p<0.001, table 1 and figure 1A). We measured several soluble factors and proteins which have been described to be affected by hypoxia including EPO, soluble vascular endothelial growth factor receptor (sVEGF-R), PDGF-BB, IL-6, C reactive protein, tumour necrosis factor, transforming growth factor β and serum hepcidin (figure 1 and table 1).1 Accordingly, the hypoxic challenge was accompanied by a significant increase in serum concentrations of sVEGF-R, EPO and PDGF-BB, whereas hepcidin levels decreased significantly (p<0.001, table 1 and figure 1B,C). When evaluating the impact of gender, we found no significant differences with regard to changes of hepcidin or PDGF-BB concentrations in response to hypoxia (see online supplementary table S2). Interestingly, exposure of participants to hypoxic conditions resulted in a significant increase of serum IL-6 levels (p<0.001) within 6 h but not of other markers of inflammation investigated herein.

**Identification of factors that mediate the hepcidin response to hypoxia**

As we observed a significant reduction of serum hepcidin following hypoxic exposure, we performed correlation analysis to identify factors being associated with alterations of hepcidin levels (see online supplementary table S3). Hepcidin concentrations significantly correlated with serum iron and transferrin but
not with ferritin levels. Most interestingly, when we studied associations between specific hypoxia regulated factors and changes in hepcidin levels, we found a highly significant correlation with PDGF-BB (R=0.589, p=0.003, see online supplementary table S3) but not with other parameters such as serum EPO levels. In line with this, neither at baseline nor after 6 h of hypoxia EPO levels correlated with PDGF-BB (R=0.057; p=0.795; R=0.176, p=0.414, respectively). To further evaluate the role of EPO and erythropoietic activity for PDGF-BB induction, we performed additional experiments in C57BL/6 mice and stimulated them with EPO or ARA-290, a synthetic EPO analogue without erythropoietic activity which only targets extra-erythropoietic EPO receptors.34 We found a non-significant trend towards higher PDGF-BB serum concentrations following daily injections of either recombinant EPO or ARA290 for 1 week (see online supplementary figure S1). As we observed this slight increase of PDGF-BB concentrations with both substances, it is unlikely to originate from stimulation of erythropoietic activity. Impact of normobaric hypoxic challenge on PDGF-BB and hepcidin expression in an in vivo mouse model

Based on the in vivo results in humans obtained so far, we then explored the hypothesis that PDGF-BB is responsible for hypoxia mediated inhibition of hepcidin expression. Therefore, we first determined serum PDGF-BB concentrations in C57BL/6 mice that were housed in a hypoxic normobaric chamber for 48 h. We found significantly higher serum PDGF-BB levels in hypoxic mice as compared with control mice (p=0.004, figure 2A). In line with our human data, hypoxic mice presented with a significant reduction in hepatic hepcidin mRNA as compared with normoxic control mice (p=0.001 and p=0.005, figure 2B,D). Based on these results, we then injected mice with the specific PDGF receptor (PDGF-R) kinase antagonist AG1296 (tyrphostin) and exposed them to hypoxia for 48 h. Already, AG1296 administration to normoxic mice resulted in an increase of hepatic hepcidin mRNA expression (p=0.011, figure 2A) as compared with normoxic control mice. Notably, hypoxic mice receiving AG1296 showed hepcidin mRNA expression that was comparable with that of normoxic control

Table 1 Haematological, iron and inflammation parameters of patients at baseline and 6 h after hypoxia

| Parameter                  | Baseline | After 6 h of hypoxia | p Value |
|----------------------------|----------|----------------------|---------|
| Female/male (N=23)         | 10/13    |                      |         |
| Age (years)                | 27.7±6.4 |                      |         |
| pO2 (mm Hg)                | 96.3±13.0 (N=14) | 45.8±7.1 (N=17) | <0.001  |
| SO2 (%)                    | 96.8±0.9 | 67.1±12.1            | <0.001  |
| Haemoglobin (g/dL)         | 13.8±1.8 | 14.2±1.3             | 0.221   |
| Serum iron (μmol/L)        | 19.2±7.6 | 17.7±6.2             | 0.132   |
| Transferin (ng/dL)         | 318±51   | 317±61               | 0.953   |
| Transferin saturation (%)  | 24.2±9.4 | 23.0±9.4             | 0.350   |
| Serum ferritin (ng/mL)     | 100±73   | 114±86               | 0.197   |
| sTfR (mg/L)                | 3.30±0.84| 3.30±0.76            | 0.966   |
| C reactive protein (nmol/L)| 1540±3803| 2105±6363            | 0.633   |
| Serum IL-6 (pg/mL)         | 2.43±3.99| 12.09±19.32          | <0.001  |
| Serum TNF (pg/mL)          | 2.09±1.03| 2.09±0.95            | 0.924   |
| Serum TGF-β (ng/mL)        | 34.95±8.23| 35.97±9.13         | 0.438   |
| sVEGF-R (pg/mL)            | 83.5±16.7| 129.2±40.4           | <0.001  |
| Serum EPO (mU/mL)          | 10.1±3.7 | 21.5±6.0             | <0.001  |

Significant p values are indicated in bold. Data are shown as means±SD. p Values for time courses are shown as determined by paired t test or Wilcoxon test. p Value<0.05 was considered to be significant. Conversion factors for units: serum iron in μg/dL to μmol/L ×0.179; C reactive protein in mg/dL to nmol/L ×95.2.

EPO, erythropoietin; IL-6, interleukin 6; pO2, oxygen pressure; SO2, oxygen saturation; sTfR, soluble transferrin receptor; sVEGF-R, soluble vascular endothelial growth factor receptor; TGF-β, transforming growth factor β; TNF, tumour necrosis factor.

Figure 1 Normobaric hypoxia affects hepcidin and platelet derived growth factor (PDGF)-BB levels in humans. (A–C) Blood was collected prior to and after 6 h of hypoxic challenge of 23 healthy volunteers as detailed in Methods. Single values and means for (A) SO2, (B) serum hepcidin and (C) serum PDGF-BB levels are shown. p Values were calculated by Wilcoxon rank test.
mice but significantly higher than in hypoxic mice treated with solvent (p=0.003, figure 2B). In parallel, we found significantly higher serum iron levels in hypoxic mice as compared with normoxic controls or AG1296 treated hypoxic mice (p=0.014 and p=0.035 respectively, figure 2C). To rule out an unspecific effect of AG1296, we used a specific anti-PDGF-BB antibody. Hypoxic mice receiving the anti-PDGF-BB antibody showed a hepatic hepcidin mRNA expression which was comparable with normoxic control mice (p=0.206), whereas hypoxic mice receiving an IgG isotype antibody or phosphate-buffered saline (PBS) solvent presented with significantly lower hepcidin mRNA levels (p=0.002 and p=0.003, respectively, figure 2D).

**PDGF-BB but not PDGF-AA reduces hepcidin expression in HepG2 cells and primary hepatocytes**

HepG2 cells express both PDGF-Rα and PDGF-Rβ mRNA (see online supplementary figure S2). Their expression was not altered by treatment of cells with BMP6, PDGB-BB and/or AG1296 (see online supplementary figure S2). When testing the effects of two PDGF isoforms, we found that PDGF-BB but not PDGF-AA significantly reduced hepcidin mRNA levels (p=0.001, figure 3A; p=0.361, figure 3B). In contrast, treatment of cells with the PDGF-R kinase inhibitor AG1296 resulted in an approximately threefold increase of hepcidin mRNA levels (p<0.001, figure 3C) as compared with controls. Similar results were obtained in analogous experiments with primary hepatocytes isolated from C57BL/6 mice (figure 3D). To exclude unspecific effects of AG1296, we performed concentration dilution tests (1–50 μM, see online supplementary figure S3) resulting in the selection of 10 μM as an effective dosage for all performed experiments. In addition, we performed time course experiments in primary hepatocytes, demonstrating a significant reduction of hepcidin mRNA expression as early as 3 h after treatment with PDGF-BB (p=0.028), which was even more

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**Figure 2** Normobaric hypoxia affects hepcidin and platelet derived growth factor (PDGF)-BB levels in mice. (A–D) C57BL/6 mice were housed under normoxic conditions (Ctl) or were subjected to 48 h of hypoxic challenge (Hpx). (B and C) C57BL/6 mice were intraperitoneally injected with phosphate-buffered saline (PBS) solvent (Hpx) or AG1296 (AG1296) immediately before exposure to hypoxia, or (D) mice were injected anti-PDGF-BB antibody (Hpx+α-PDGF) or IgG isotype antibody (Hpx+IgG) immediately prior to and after 24 h of hypoxic challenge. (A) Serum protein levels of PDGF-BB in control and hypoxic mice, (B and D) hepatic hepcidin mRNA expression relative to untreated normoxic controls, and (C) serum iron levels are shown. (A–D) Boxes denote lower quartile, mean and upper quartile, and whiskers show maximum and minimum ranges. p Values were determined by post hoc exact two-sided Mann–Whitney U test.

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prominent after 24 h following PDGF-BB treatment (p<0.001, figure 3D). A significant induction of hepcidin expression was seen 6 h after treatment with AG1296 (p<0.001), whereas AG1296 treatment reversed PDGF-BB mediated hepcidin suppression when added 6 h after PDGF-BB stimulation (figure 3D).

Next, we treated HepG2 cells with huBMP6, a potent stimulator of hepcidin transcription, and studied the effects of PDGF-BB under these conditions. huBMP6 treatment resulted in an 18-fold induction of hepcidin mRNA expression in HepG2 cells. In contrast, co-incubation of cells with PDGF-BB and huBMP6 treatment significantly reduced BMP6 inducible hepcidin mRNA expression (p<0.001). This inhibitory effect of PDGF-BB could be reversed upon the addition of AG1296, suggesting that the effect of PDGF-BB is specific and transmitted after binding to its receptor (p=0.001, figure 4A).

Impact of PDGF-BB on hepcidin expression in a murine in vivo model

Next, we tested the effectiveness of PDGF-BB to suppress hepcidin expression in vivo. Injection of PDGF-BB in C57BL/6 mice resulted in a significant decrease of hepatic hepcidin mRNA levels as compared with PBS injected controls (p=0.009, figure 4B). In accordance with the in vitro results, intraperitoneal injection of huBMP6 resulted in an induction of hepcidin mRNA in the liver (p=0.029), an effect that could be completely reversed by coadministration with muPDGF-BB (p=0.008, figure 4C).

PDGF-BB treatment reduces hepcidin promoter activity in transiently transfected primary hepatocytes and HepG2 cells

Next, we transiently transfected primary murine hepatocytes and HepG2 cells with a PGL4 plasmid containing a 3 kb wild-type human hepcidin promoter construct. Stimulation of transfected primary murine hepatocytes with muPDGF-BB for 6 h resulted in a significant decrease of hepcidin promoter activity as compared with BMP6 treatment alone (p=0.005, figure 5A) as quantified by a dual-luciferase reporter system. In addition, co-stimulation of primary hepatocytes with BMP6 and PDGF-BB resulted in a significant lower hepcidin promoter activity as compared with BMP6 treatment alone (p<0.001, figure 5B). Remarkably, the inhibitory effect of PDGF could be completely reversed with AG1296 or α-PDGF-BB antibody treatment, whereas it was preserved when an IgG isotype antibody was added. In parallel, we performed the same experiments with HepG2 cells, obtaining comparable results (data not shown).

PDGF-BB mediates its effects via modulation of CREB and CREB-H expression

As PDGF-BB has not been shown to be involved in the regulation of hepcidin expression and iron homeostasis so far, we aimed to elucidate the signalling pathway underlying PDGF-BB

Figure 3  Platelet derived growth factor (PDGF)-BB treatment inhibits hepcidin expression in vitro. (A–C) HepG2 cells were left untreated (Ctl) or treated with 25 ng/mL huPDGF-BB, 25 ng/mL PDGF-AA or 10 μM AG1296 for 24 h. mRNA expression levels of hepcidin relative to untreated controls are shown. (D) Primary murine hepatocytes were left untreated (Ctl) or treated with 25 ng/mL muPDGF-BB, 10 μM AG1296 or combinations thereof at indicated time points. N=6 for each group and time point. Hepcidin mRNA expression relative to untreated controls are shown. (A–C) Data are plotted and statistics are calculated as detailed in figure 2 legend. (D) Symbols indicate means, whiskers depict±SEM and p values (*p<0.05, **p<0.001) were calculated with t tests using Bonferroni correction for multiple testing.
mediated hepcidin regulation. Using the in vivo murine hypoxia model, we first investigated protein expression of pSMAD1/5/8 and pSTAT3, main inducers of hepcidin transcription, in the liver by means of western blotting.

Intriguingly, pSMAD1/5/8 levels were not altered during hypoxia, and AG1296 treatment had no effect on SMAD phosphorylation (figure 6A,B). In contrast, pSTAT3 levels were significantly elevated upon hypoxic challenge but were not affected by AG1296 treatment (figure 6A,C). Thus, we searched for alternative signalling pathways that may translate the PDGF signal.

While the hepatic expression of phospho-Erk1/2 and mitogen-activated protein kinase (MAPK) was not altered upon hypoxic challenge of mice (data not shown), C/EBPα, CREB and CREB-H protein expression were significantly decreased in hypoxic mice as compared with mice kept under normoxic conditions (figure 6A,D–F). Remarkably, AG1296 treatment prior to hypoxic challenge completely reversed hypoxia inducible CREB and CREB-H suppression, whereas C/EBP-α levels were suppressed by hypoxia independently from AG1296 treatment (figure 6A,D–F).

To further elucidate the role of CREB-H signalling, we performed gene knockdown studies in primary hepatocytes using CREB-H siRNA. With this siRNA, we achieved a knockdown of CREB-H mRNA expression of approximately 60% (p=0.002, figure 7A). As a consequence, hepcidin mRNA baseline expression was reduced to 50% (p<0.002, figure 7B). Intriguingly, PDGF-BB treatment resulted in a significant reduction of hepcidin expression in primary hepatocytes with unaltered CREB-H expression (p=0.013, figure 7B), whereas in cells subjected to specific siRNA driven CREB-H knockdown PDGF-BB treatment had no significant effect on hepcidin mRNA expression (figure 7B). In line with this, PDGF-BB injection in vivo had no effect on liver hepcidin mRNA expression in CREB-H knockout mice (p=0.343, figure 7C), whereas a significant reduction of hepcidin mRNA levels was seen in PDGF-BB treated wild-type littermates as compared with solvent treated wild-type controls (p=0.029, figure 7C).

**DISCUSSION**

Our data obtained upon investigations of humans, mice and cells provide clear evidence that the tissue growth factor PDGF-BB is largely responsible for hypoxia mediated inhibition of hepcidin expression. Our observation of hypoxia driven hepcidin repression is in line with previous studies including...
observations made in healthy human volunteers after subjection to hypobaric hypoxia.\textsuperscript{28–30}

We introduce PDGF-BB as a novel regulator of hepcidin expression and thus of iron homeostasis, and further show that this regulatory effect is dependent on PDGF-BB binding to its specific receptor and transmitted via C/EBP\(\alpha\), CREB and CREB-H dependent signalling cascades. This is in line with the observation of increased PDGF-BB levels following hypoxic challenge that are associated with a decrease of CREB expression.\textsuperscript{36} PDGF-BB has been described to be a target of HIF-1 gene transcription\textsuperscript{37} and members of the CREB family have several binding sites within the hepcidin promoter and CREB-H is known to be a pivotal inducer of hepcidin expression.\textsuperscript{17} Intriguingly, we also found a reduction of C/EBP\(\alpha\) protein levels following exposure of mice to hypoxia. This effect was less pronounced than the impact of hypoxia on CREB signalling but might be an additional contributor to the reduced hepcidin expression following hypoxic challenge likewise independent of the PDGF-BB mediated regulation.\textsuperscript{14,38}

While we and others observed that PDGF-BB did not affect SMAD signalling\textsuperscript{39} it reduced STAT-3 activity. A study investigating hypoxic BALBc mice showing that the generation of reactive oxygen species resulted in inhibition of C/EBP\(\alpha\) and STAT3 mediated activation of the hepcidin promoter\textsuperscript{25} is in line with our results of reduced C/EBP\(\alpha\) expression following hypoxia. Thus, reduced C/EBP\(\alpha\), CREB and CREB-H expression following hypoxic challenge may also impact on the functionality of the STAT binding sites within the hepcidin promoter, and provides a possible explanation for the subordinate role of inflammation as far as hepcidin modulation under hypoxic conditions is concerned.

Although EPO expression is increased during hypoxia, we could neither find an association between EPO and hepcidin levels in sera of healthy volunteers exposed to hypoxia nor a direct effect of EPO on hepcidin expression in cell culture

**Figure 6** Hypoxia and platelet derived growth factor (PDGF)-BB cause suppression of CREB and cAMP responsive element-binding protein 3-like (CREBH; also known as CREB3L3), expression. Male C57BL/6 mice were left untreated (Ctl), subjected to normobaric hypoxia for 48 h (Hpx) or subjected to hypoxic challenge for 48 h following intraperitoneal injection of AG1296. (A) Representative western blots from cytoplasmic or nuclear extracts of the liver, as appropriate, are shown. \(\beta\)-Actin and TATA box binding protein (TBP) were used as loading control. (B–F) Densitometric quantification of protein expression in the liver of (B) pSMAD1/5/8, (C) pSTAT3, (D) C/EBP\(\alpha\), (E) CREBH and (F) CREB as reported by western blot analysis. Expression levels were normalised to an appropriate loading control, as indicated, and are shown relative to the expression levels in untreated mice kept under normoxic conditions. Exact two-sided \(p\) values are shown as determined by post hoc Mann–Whitney U test. Bars indicate mean\(\pm\)SEM, \(N=4\) for each group.

**Figure 7** Platelet derived growth factor (PDGF)-BB blocks hepcidin expression via suppression of cAMP responsive element-binding protein 3-like (CREBH; also known as CREB3L3) expression. (A and B) Primary hepatocytes were treated with phosphate-buffered saline (PBS) (Ctl), scrambled siRNA or CREBH specific siRNA duplex (\(\alpha\)CREBH), with or without addition of 25 ng/mL muPDGF-BB (PDGF). Data from three independent experiments are shown. (C) CREBH knockout mice (CREBH KO) and corresponding wild-type (WT) littermates were intraperitoneally injected with PBS solvent (Ctl) or 500 ng muPDGF-BB. Livers were extracted and hepcidin mRNA relative to expression levels of solvent treated controls is shown. (A–C) \(p\) Values are depicted as determined by exact two-sided post hoc Mann–Whitney U test.
experiments (not shown). Moreover, neither treatment of mice with EPO nor the EPO analogon ARA-290 for 1 week had a significant impact on serum PDGF-BB concentrations. This suggests that the alteration of PDGF-BB expression via EPO is only moderate and erythropoietic activity does not appear to play a role as both EPO and ARA-290 had indistinguishable effects on PDGF-BB serum concentrations and the latter targets only the extra-erythropoietic, heterodimeric EPOR and does not stimulate erythropoiesis.34 Though the underlying molecular mechanisms await further elucidation, one may speculate that the observation that prolonged administration of EPO results in a decrease of hepcidin levels in humans40 41 may be rather due to an indirect effect, as EPO induces erythropoiesis and stimulates iron consumption by erythroid progenitor cells.42 43 In addition, stimulation of erythropoiesis leads to the expression of soluble factors, such as GDF-15, which then can repress hepcidin expression.44 45 As we observed inhibition of hepcidin expression in humans as early as 6 h after hypoxic challenge, this effect is rather related to a protein or hormone, such as PDGF-BB, which is immediately released from preformed stores in platelets, macrophages and endothelial cells.46 47

Our data are in accordance with a recently described observation of inhibitory effects of the tissue growth factors hepatocyte growth factor (HGF) and EGF on BMP-6 induced hepcidin formation in hepatocytes, although in this specific system, PDGF-BB, used at much lower dosages as described herein, had only a paltry inhibitory effect on hepcidin formation.38 Further, these authors demonstrate that HGF and EGF regulate BMP-6 induced hepcidin expression via the P38 kinase MEK/ERK kinase pathways, whereas PDGF-BB affects hepcidin transcription by interaction with CREB and CREB-H signalling cascades.

In contrast to data from the recently performed HIGHCARE project which investigates hepcidin regulation during hypoxia induced erythropoiesis,28 we did not find a significant alteration of serum ferritin levels in our study. This is likely due to the fact that we investigated an acute model of hypoxic challenge lasting for 6 h, whereas Piperno et al focused on alteration following hypoxic challenge for several days which resulted in the observed changes of ferritin levels over time.

Another interesting observation is that hypoxia rather than iron deficiency downregulates hepcidin in response to anemia.20 This observation strengthens the idea that hypoxia impacts on hepcidin expression independent of the classical BMP/SMAD and IL-6/STAT3 pathway, which is mainly triggered by iron alterations and inflammation.11–16 48–50

To conclude, our findings describe a novel pathway for the regulation of hepcidin expression by a tissue growth factor (PDGF-BB) which has not been linked to iron homeostasis thus far, and shed new light on the hypoxic regulation of iron metabolism.

**Contributors**

TS, IT, AS, MN, DN, AM, DH, MB, MS, ED, AK, ST, CV and KM-S performed experiments. TS, IT, GW, DN, MB, MM, AP and AK designed the research. ATM, VW and DRW performed hepcidin protein measurement in humans. TS, IT, GW, DN and MM analysed the results. TS, GW and IT prepared the figures and wrote the paper; all authors checked the final version.

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**Competing interests**

None.

**Ethics approval**

Ethical Committee at the Medical University of Innsbruck.

**Provenance and peer review**

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