Hypoxia inhibits hepcidin expression in HuH7 hepatoma cells via decreased SMAD4 signaling

Timothy B. Chaston,1,2* Pavle Matak,1* Katayoun Pourvali,1 Surjit K. Srai,2 Andrew T. McKie,1 and Paul A. Sharp1

1Nutritional Sciences Division, King’s College London, London, United Kingdom; and 2Institute of Structural and Molecular Biology, University College London, London, United Kingdom

Submitted 6 April 2010; accepted in final form 31 January 2011

The liver-expressed peptide hepcidin (23) has emerged as a major regulator of systemic iron homeostasis. During inflammation, hepcidin is secreted into the serum, where it blocks iron release from duodenal enterocytes and reticuloendothelial macrophages, causing hypoferremia (8, 10, 12, 20, 36). Hepcidin transcription is also sensitive to systemic iron levels, body iron stores, erythropoietic factors, and hypoxia in vivo (14, 29, 33), and the regulated production and release of the peptide plays a central role in the maintenance of iron homeostasis. This is supported by the inappropriate regulation of hepcidin expression seen in transgenic animal models of iron overload that lack functional genes for HFE, hemojuvelin (HJV), or transferrin receptor 2 (1, 6, 28, 31).

The systemic and intracellular mechanisms that underlie negative regulation of hepcidin by hypoxia and oxidative stress are incompletely understood. Studies in rodent models have demonstrated dramatic effects of hypoxia on iron homeostasis: increased intestinal iron absorption (24, 25, 30, 37) and decreased expression of hepcidin (24, 29, 32). The molecular intermediates that signal the effects of hypoxia on hepcidin expression are unclear, but possible factors include erythropoietin (16, 34), hypoxia-inducible factors (HIFs) (32), and prolyl hydroxylases (5). While the hepcidin promoter contains putative HIF-binding sites (4, 42), recent evidence has demonstrated that the effects of hypoxia on hepcidin expression are not mediated directly by HIF-promoter binding (42). Reactive oxygen species produced as a consequence of hypoxia have also been implicated in the transcriptional repression of hepcidin and appear to operate via inhibitory effects on signal transducer and activator of transcription 3 (STAT3) and CCAAT/enhancer-binding protein (C/EBP) signaling (9). Furthermore, there is evidence that hypoxia inhibits the HJV/bone morphogenetic protein (BMP) signaling cascade and thereby decreases hepcidin expression (39). In the present study we have further investigated the roles of these pathways in the hypoxic regulation of hepcidin expression.

Experiments were performed using HuH7 hepatoma cells as a hepatocyte model. Cells either were used alone as monocultures or were cocultured with THP-1 macrophages. We have recently characterized this coculture model (26) and have shown along with others (17) that it ensures an appropriate hepatocyte hepcidin response to a number of stimuli.

METHODS

Cell culture. HuH7 human hepatoma cells were grown in DMEM containing 10% fetal bovine serum and were used for experiments at 80% confluence. THP-1 cells, grown in RPMI 1640 medium containing 10% fetal bovine serum, were seeded at 10⁶ cells per well on Transwell filters and treated overnight with phorbol myristate acetate (100 nmol/l) to induce differentiation and filter attachment. After differentiation, cells were washed and incubated in fresh media for 24 h before experimentation.

Coculture and treatments. HuH7 hepatoma cells were seeded at a density of 0.5 × 10⁶ cells per well in six-well plates and grown for 48 h. On the day of experiment, HuH7 cells were washed and given fresh medium and Transwell inserts containing differentiated THP-1 macrophages were overlaid onto six-well plates containing HuH7 cells. In some experiments, HuH7 cells were exposed either to conditioned medium from THP-1 macrophages cultured under normoxic condition or to IL-1β [10 ng/ml; this concentration was chosen to be consistent with our previous studies using this coculture model; (26)]. For hypoxia treatments, cell cultures were placed in a sealed Perspex chamber. The chamber was then flushed with air containing 1% O₂, 5% CO₂ and 94% N₂ and then sealed and incubated at 37°C.
for 24 h. In some experiments, cells were cultured under normoxic conditions in the presence of the oxidative stress agent H2O2 (100 μM). On the basis of Trypan blue exclusion studies, there was no effect of either treatment on cell viability (data not shown).

Real-time quantitative PCR. Total RNA was isolated from HuH7 or THP-1 cells using TRIzol reagent (Invitrogen). Following first-strand synthesis, expression levels of hepcidin, BMP2, BMP6, noggin, SMAD4, SMAD7, and 18S (used as a housekeeper gene) mRNA were analyzed by real-time PCR using an ABI Prism 7000HT PCR cycler with gene-specific primers (15, 21, 26) and a Quanti-Tect SYBR Green PCR kit (Qiagen), according to the manufacturer’s protocol. Quantitative measurements of each gene were derived from a standard curve constructed from known concentrations of PCR product.

Cell transfection and luciferase reporter assays. Full details concerning the generation and utilization of hepcidin promoter plasmid constructs have been described previously (26). Briefly, HuH7 cells were transfected with the wild-type, STAT3 mutant, C/EBP mutant, E-box mutant, BMP/SMAD4 mutant hepcidin-luciferase constructs or the empty pGL3-basic vector, using Fugene6 (Roche) according to the manufacturer’s instructions. As a normalization control, the pRL-SV40 Renilla luciferase plasmid (Promega) was cotransfected alongside the hepcidin constructs in a 1:50 ratio. Following immobilization on nitrocellulose, the proteins were exposed to commercially available anti-phospho-SMAD 1/5/8 antibody, SMAD4 antibody (1:1,000 dilution, Cell Signaling Technology), or HIV antibody (1:1,000 dilution, anti-RMGc, Santa Cruz; Supplemental Fig. S2; Supplemental Material for this article is provided in an online supplement at the Journal website). Cross-reactivity was observed using a horseradish peroxidase-linked secondary antibody (Dako) and ECL Plus (GE Healthcare). Band densities were semiquantified using Scion Image software (Scion, Frederick, MD).

Western blot analysis. HuH7 cells were harvested into ice-cold lysis buffer (PBS containing 1% sodium dodecyl sulfate and 10 μg/ml protease inhibitor cocktail) and homogenized by repeated passing through a 25-gauge needle. Protein samples (40 μg) were solubilized in sample loading buffer and subjected to polyacrylamide gel electrophoresis. The nuclear pellet was washed in sucrose buffer without NP-40 treatment. Approximately 0.6 volumes of high salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 800 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 25% glycerol, and 0.5% NP-40) were then added in 0.2 volume aliquots until nuclei were lysed.

**Fig. 1.** Hypoxia represses hepcidin expression in HuH7/THP-1 cell cocultures. HuH7 cells either cultured alone (A) or cocultured with THP-1 macrophages (B) were exposed to hypoxia (1% O2) or 100 μM H2O2. In HuH7 monocultures, hypoxia significantly increased hepcidin expression, whereas in the coculture model, HuH7 hepcidin levels were significantly decreased by both hypoxia and H2O2 treatment. Data are presented as hepcidin/18S ratios and are means ± SE of 6–12 observations in each group. Different letters above data bars indicate that these groups are significantly different (P < 0.05).

**Fig. 2.** Hypoxic repression of hepcidin mRNA is reconstituted in the presence of THP-1-conditioned medium. HuH7 monocultures were exposed to conditioned medium from activated THP-1 macrophages cultured under normoxic conditions (A) or were treated with IL-1β (10 ng/ml) (B) before exposure to hypoxia (1% O2) or 100 μM H2O2 for 24 h. Hepcidin mRNA (expressed as hepcidin/18S ratio) was significantly decreased in all groups compared with controls. Data are means ± SE of 6 observations in each group. Different letters above data bars indicate that these groups are significantly different (P < 0.05).
At the end of the experiment, the nitrocellulose membranes were stripped (Western Stripping Buffer, Perbio Science) and reprobed with antibodies to actin (1:2,000 dilution, Sigma-Aldrich), which acted as a loading control. In some experiments, β-tubulin (1:1,000 dilution, Cell Signaling Technology) was used as a cytosolic protein marker.

Statistical analysis. Data are presented as means ± SE. Statistical differences (P < 0.05) among groups were determined by one-way ANOVA, followed by Tukey’s post hoc test when F-test was significant at P < 0.05.

RESULTS

Hypoxia causes repression of hepcidin in HuH7 cells cultured in the presence of THP-1 macrophages. Hepcidin mRNA expression in monocultured HuH7 hepatoma cells was unchanged following exposure to 100 μM H2O2 but was increased in cells cultured under hypoxic conditions (Fig. 1A). In contrast, when HuH7 cells were cocultured with THP-1 macrophages, treatment with both H2O2 and hypoxia decreased hepcidin mRNA to 17 ± 6% and 29 ± 6% (P < 0.001) of the control, respectively (Fig. 1B).

Similar observations were also made in a second human hepatoma cell line (HepG2 cells); hypoxia increased hepcidin expression in monocultured HepG2 cells but significantly repressed hepcidin expression in THP-1-stimulated HepG2 cells (Supplemental Fig. S1).

Oxidative repression of HuH7 hepcidin mRNA is reconstituted in the presence of conditioned medium from THP-1 macrophages. The decrease in hepcidin mRNA observed in the coculture model (Fig. 1B) could arise from direct effects of hypoxia or H2O2 on either the THP-1 cells or the HuH7 cells or both. We have previously demonstrated that the generation and release of cytokines, particularly IL-1β, by activated THP-1 cells are required for induction of hepcidin mRNA in our coculture system (26). In the present study the expression of IL-1β mRNA in THP-1 cells was significantly decreased by treatment with H2O2 (−43.6 ± 8.5%, P < 0.05) and hypoxia (−53.8 ± 9.0%, P < 0.05), suggesting that suppression of cytokine production by THP-1 cells may be involved in the attenuated hepcidin response.

To determine whether there was also a direct influence of hypoxia or oxidative stress on HuH7 cells, we prestimulated HuH7 monocultures with conditioned medium derived from activated THP-1 macrophages grown under normoxic conditions. HuH7 cells were subsequently treated with H2O2 or...
hypoxia for 24 h. Under both of these conditions, hepcidin mRNA was significantly repressed (Fig. 2A). Furthermore, monocultured HuH7 cells prestimulated with IL-1β and subsequently exposed to 100 μM H2O2 or 1% O2 for 24 h also displayed a significant decrease in hepcidin mRNA (Fig. 2B).

**Mutation of proximal SMAD4-binding site on the hepcidin promoter prevents repression by hypoxia.** To identify the transcriptional mechanisms by which hypoxia and H2O2 regulate hepcidin expression, HuH7 hepatoma cells were transfected with a luciferase construct containing 0.9 kb of the wild-type hepcidin promoter. Consistent with the quantitative PCR (Q-PCR) experiments (Fig. 1), luciferase reporter activity in HuH7 monocultures was significantly increased by hypoxia (Fig. 3A), whereas in the coculture system it was significantly decreased (Fig. 3B) compared with the untreated control groups. Hepcidin expression was also decreased by H2O2 treatment in the coculture experiments (Fig. 3B).

To investigate further the mechanism for the transcriptional repression of hepcidin expression by hypoxia and H2O2 in the HuH7/THP-1 coculture system, we transfected HuH7 cells with mutant [ΔSTAT3, ΔC/EBP, ΔE-box, ΔSMAD4; (26)] luciferase constructs. Suppression of hepcidin promoter activity was sustained in the presence of mutations in the STAT3 (Fig. 3D) and C/EBP (Fig. 3E) response elements, and with the reporter construct containing mutations in the E-boxes (Fig. 3C). However, mutation of the SMAD4 response element prevented H2O2- and hypoxia-mediated repression of hepcidin promoter activity in cocultured HuH7 cells (Fig. 3F).

**Hypoxia diminishes BMP signaling in HuH7 cells.** Given the necessity of the SMAD4 response element for regulation of...
hepcidin by oxidative stress and hypoxia, we tested whether regulation of hepatic expression of BMPs may be responsible for hepcidin repression. However, mRNA expression of BMP2, BMP6, and the endogenous BMP inhibitor noggin was not regulated following treatment with H$_2$O$_2$ or hypoxia (Fig. 4, A–C).

Next, we determined whether hypoxia might directly influence the BMP/hepcidin signaling pathway. HJV is a coreceptor that is required for the initiation of the BMP receptor signaling cascade. However, Western blotting revealed that HJV protein levels were unaltered by H$_2$O$_2$ or hypoxia (Fig. 4D). Similarly, there was no significant change in the phosphorylation of HJV/BMP receptor-activated SMAD 1/5/8 in either treatment group (Fig. 4E).

The transcription factor SMAD4 is a co-SMAD that dimerizes with receptor-activated SMADs and enhances BMP and transforming growth factor-$eta$ signaling. We detected SMAD4 protein in nuclear and cytosolic extracts from HuH7 cells (cytosolic extracts were positive for the marker protein $\beta$-tubulin; nuclear extracts were $\beta$-tubulin negative; Fig. 5). In HuH7 monocultures there were no significant changes in either nuclear or cytosolic SMAD4 protein following treatment with H$_2$O$_2$ or hypoxia (Fig. 5A). However, both nuclear ($P < 0.001$) and cytosolic ($P < 0.05$) SMAD4 protein was significantly decreased by H$_2$O$_2$ and hypoxia in THP-1-stimulated HuH7 cells compared with control (Fig. 5B). SMAD4 mRNA in HuH7 monoculture was not significantly affected by either

---

**Fig. 5.** Hypoxia suppresses the expression of SMAD4 and SMAD7 in THP-1-stimulated HuH7 cells. Protein (A) and mRNA (C) expression levels of SMAD4, the coactivator of BMP signaling, were not altered by hypoxia or H$_2$O$_2$ in HuH7 monocultures. However, in THP-1-stimulated HuH7 cells, SMAD4 protein levels in both nuclear and cytosolic fractions were significantly decreased by hypoxia and oxidative stress (B). SMAD4 mRNA was also significantly decreased by hypoxia in THP-1-stimulated HuH7 cells (D). In addition, expression of the SMAD4-regulated inhibitory factor SMAD7 was decreased by both hypoxia and H$_2$O$_2$ in THP-1-stimulated HuH7 cells (F) but not in HuH7 monocultures (E). Data are means ± SE of 4–6 observations in each group. Different letters above data bars indicate that these groups are significantly different ($P < 0.05$). In B, lowercase letters denote differences in nuclear fractions and uppercase letters denote differences in cytosolic fractions.
treatment (Fig. 5C); however, in THP-1-stimulated HuH7 cells, SMAD4 mRNA was significantly decreased by hypoxia (Fig. 5D).

SMAD7 is a SMAD4-regulated gene (18) and has recently been identified as a negative regulator of hepcidin expression in hepatoma cells (27). We investigated whether SMAD7 expression might be upregulated by hypoxia in THP-1-stimulated HuH7 cells and thereby mediate the inhibition of hepcidin. However, while there was no effect of hypoxia or H2O2 on SMAD7 mRNA levels in monocultured cells (Fig. 5E), SMAD7 mRNA was significantly decreased by both hypoxia and H2O2 in HuH7 cocultures (Fig. 5F).

DISCUSSION

Hepcidin is a major regulator of systemic iron homeostasis and as such is responsive to changes in iron status (14, 33), erythropoietic requirements (13, 29), and hypoxia (24, 29, 32). In hepatic cell lines, hypoxia is linked to suppression of hepcidin expression (5, 9); however, this finding is not consistent and recent studies suggest that hypoxia increases hepcidin expression in HuH7 hepatoma cells (42). We have recently characterized a HuH7 cell/THP-1 macrophage coculture system in which cross talk between macrophages and hepatoma cell lines leads to the induction of hepcidin mRNA (26). In the present study we further explored the influence of macrophages on the regulation of hepcidin in the context of known negative stimuli, namely, hypoxia and oxidative stress (induced by H2O2). Interestingly, we found differential effects of hypoxia on hepcidin expression in our studies: an increase in expression in monocultures of HuH7 cells—consistent with the recent findings of Volke et al. (42)—but a significant decrease in hepcidin mRNA in the HuH7/THP-1 coculture model.

As shown in our previous study (26) and by others (22), activated THP-1 macrophages secrete a number of inflammatory cytokines including IL-1β and IL-6, which can subsequently stimulate hepcidin expression. We hypothesized that decreased macrophage production of these cytokines may contribute to hepatic suppression of hepcidin by hypoxia and oxidative stress. In preliminary experiments we observed a significant decrease in macrophage IL-1β mRNA following hypoxia and oxidative stress; however, it was clear from subsequent studies that the decrease in THP-1 IL-1β production alone could not account for the inhibitory effects on HuH7 cell hepcidin mRNA in the coculture model. When HuH7 cell monocultures were prestimulated with conditioned media derived from THP-1 cells grown under normoxic conditions, we also observed a dramatic hypoxic and oxidative suppression of hepcidin mRNA. Interestingly, we observed the same significant suppression of hepcidin mRNA in HuH7 monolayers prestimulated with IL-1β and subsequently exposed to hypoxia or H2O2. Hence it appears, in our in vitro model at least, that macrophage-derived factors are required to prime basal hepatic hepcidin expression, but that hypoxia and oxidative stress act directly on the stimulated HuH7 cells to suppress hepcidin mRNA. It remains unclear whether macrophage/hepatocyte cross talk is equally important in the regulation of hepcidin expression in vivo.

Next we focused on the transcriptional regulation of hepcidin/hepcidin expression. A number of putative transcription factor-binding sites have been identified in the proximal 0.9 kb of the human hepcidin promoter, including characterized response elements for STAT3 (41), SMAD4 (40), and members of the CCAAT enhancer-binding protein family (C/EBP) (11), as well as two E-boxes (4) which bind a number of basic helix-loop-helix factors and potentially HIFs (32). Using hepcidin promoter-luciferase reporter constructs containing mutations in these transcription factor-binding sites, we showed that the proximal SMAD4-binding site is required for the repression of hepcidin in this model. Within the hepatocyte the SMAD pathway is essential for the transmission of BMP signals and the regulation of hepcidin gene transcription (3, 7, 43). Importantly, an intact BMP/SMAD signaling pathway is essential for maintaining basal hepcidin expression (26, 40). We therefore hypothesized that a decrease in hepatic BMP/SMAD signaling may offer an explanation for oxidative and hypoxic hepcidin repression. There were no changes in hepatic BMP2, BMP6, or noggin levels, suggesting that regulation of endogenous hepatic ligands and inhibitors of this signaling pathway is not involved in this mechanism. Additionally, membrane levels of HJV, the coreceptor required for the

Hepatocyte

Macrophage

Fig. 6. Cross talk between macrophages and hepatocytes and the regulation of hepcidin expression. Data from this study and from previous work by our group (26) suggest that cross talk between macrophages and hepatocytes is important in controlling the production and release of hepcidin, the central regulator of iron homeostasis. These effects appear to be mediated by macrophage production of the proinflammatory cytokine IL-1β, which activates the C/EBP pathway in HuH7 cells. In the context of hypoxia, we observed a decrease in macrophage production of IL-1β. In addition, there are direct effects of hypoxia on SMAD4 protein levels in the cytosol and nucleus of HuH7 cells. Together, these mechanisms serve to abrogate the BMP/SMAD signaling pathway that is crucial for the regulation of hepcidin expression. HAMP, hepcidin antimicrobial peptide; B, BMP/SMAD-responsive element; C, CCAAT enhancer-binding protein responsive element; E, E-box elements; S, STAT3-responsive element.
initiation of the BMP receptor signaling cascade, were unaltered by exposure to H$_2$O$_2$ and hypoxia, and, furthermore, no significant changes in phosphorylation of the receptor SMAD 1/5/8 complex were observed.

We therefore investigated the effect of hypoxia and oxidative stress on expression of SMAD4, commonly referred to as the co-SMAD, which forms complexes with receptor SMADs to elicit their transcriptional response (44). SMAD4 mRNA was increased by hypoxia in monocultured HuH7 cells, but this did not reach statistical significance ($P=0.07$). However, in THP-1-stimulated HuH7 cells, SMAD4 mRNA was significantly decreased by hypoxia. SMAD4 protein (both nuclear and cytosolic) was also decreased in the THP-1-stimulated HuH7 cells following exposure to hypoxia and H$_2$O$_2$, suggesting that the decreased expression of SMAD4 might mediate the hypoxic repression of hepcidin expression in the coculture model.

SMAD4 is involved in chromatin remodeling, stabilizing transcriptional complexes, and recruiting coactivators (44). The complete blunting of hepcidin to all inflammatory cytokines and BMPs in SMAD4-knockout mice (43) suggests that decreased SMAD4 may be a central mechanism of hepcidin repression. Hypoxia and reactive oxygen species have been implicated in both the induction and the inhibition of receptor SMADs (2, 35, 38, 46) and SMAD4 nuclear localization (19). Furthermore, inhibitory SMADs and a number of E3 ubiquitin ligases are implicated in this process because they target SMAD4 for proteolytic degradation (44, 45). Further studies are needed to determine the precise mechanisms involved in the regulation of SMAD4 in our model.

In conclusion, hepcidin expression is decreased by hypoxia and oxidative stress in HuH7 hepatocytes (Fig. 6), but only when they are cocultured with conditioned medium from activated THP-1 macrophages. IL-18 appears to be the main mediator of this effect. Because hepatocytes are normally exposed to a stream of cytokines from Kupffer cells and circulating macrophages, our model may be physiologically relevant for the study of hepatic hepcidin expression. Under these conditions we have identified a transcriptional mechanism that, following stimulation, represents an intracellular hepatic regulatory circuit for direct SMAD4-dependent repression of hepcidin transcription.

**GRANTS**

This work was funded by the Biotechnology and Biological Sciences Research Council. P. Matak was funded by a studentship from the Medical Research Council. K. Pourvali is funded by a studentship from the Shaheed Beheshti Medical University, Tehran, Iran.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


