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

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Chaston TB, Matak P, Pourvali K, Srai SK, McKie AT, Sharp PA. Hypoxia inhibits hepcidin expression in HuH7 hepatoma cells via decreased SMAD4 signaling. Am J Physiol Cell Physiol 300:C888–C895, 2011. First published February 2, 2011; doi:10.1152/ajpcell.00121.2010.—Hepcidin negatively regulates systemic iron homeostasis in response to inflammation and elevated serum iron. Conversely, hepcidin expression is diminished in response to hypoxia, oxidative stress, and increased erythropoietic demand, though the molecular intermediates involved are incompletely understood. To address this, we have investigated hypoxic hepcidin regulation in HuH7 hepatoma cells either cultured alone or cocultured with activated THP-1 macrophages. HuH7 hepcidin mRNA expression was determined using quantitative polymerase chain reaction (Q-PCR). Hepcidin promoter activity was measured using luciferase reporter constructs containing a 0.9 kb fragment of the wild-type human hepcidin promoter, and constructs containing mutations in bone morphogenetic protein (BMP)/SMAD4, signal transducer and activator of transcription 3 (STAT3), CCAAT/enhancer-binding protein (C/EBP), and E-box-responsive elements. Hepatic expression of bone morphogenetic proteins BMP2 and BMP6 and the BMP inhibitor noggin was determined using Q-PCR, and the protein expression of 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 10^6 cells per well on Transwell inserts containing differentiated THP-1 macrophages. HuH7 cells were seeded at a density of 0.5 × 10^6 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, well 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...

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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. After 24 h, cells were exposed to H₂O₂ or hypoxia for a further 24 h and luciferase activity was determined in triplicate using the Promega Dual Luciferase Reporter Assay, according to the manufacturer’s instructions.

**Preparation of nuclear protein extracts.** HuH7 cells were scraped off plates, washed in ice-cold PBS, and lysed in sucrose buffer (in mM: 320 sucrose, 3 CaCl₂, 2 Mg acetate, 0.1 EDTA, 1 DTT, and 0.5 PMSF) containing 0.5% NP-40. After centrifugation at 1,500 g for 5 min, the cytosolic fraction (supernatant) was removed and stored at −80°C. The nuclear pellet was washed in sucrose buffer without NP-40 and centrifuged at 1,500 g for a further 5 min. After the pellet was dried, nuclei were resuspended in sucrose buffer. 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.

**Western blot analysis.** HuH7 cells were harvested into ice-cold lysis buffer (PBS containing 1% sodium dodecyl sulfate and 10 mg/ml protease inhibitor cocktail) and homogenized by repeated passing through at 25-gauge needle. Protein samples (40 µg) were solubilized in sample loading buffer and subjected to polyacrylamide gel electrophoresis. 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 HJV 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).

![Fig. 1. Hypoxia represses hepcidin expression in HuH7/THP-1 cell cocultures.](http://ajpcell.physiology.org/)

![Fig. 2. Hypoxic repression of hepcidin mRNA is reconstituted in the presence of THP-1-conditioned medium.](http://ajpcell.physiology.org/)
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 (1:10,000 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

Fig. 3. Human hepcidin promoter activity is repressed by hypoxia and oxidative stress. HuH7 cells were transfected with luciferase reporter constructs and 24 h later were exposed to hypoxia (1% O2) or 100 μM H2O2 for 24 h in the absence (A) or presence (B–F) of activated THP-1 cells. Luciferase activity was measured after 24 h. Hepcidin promoter activity was repressed by hypoxia and H2O2 treatments in HuH7 cocultures transfected with the wild-type (B) and E-box (C), signal transducer and activator of transcription 3 (STAT3) (D), and CCAAT/enhancer-binding protein (C/EBP)-mutant (E) constructs. No effect of hypoxia and H2O2 treatments was observed in HuH7 monocultures transfected with the wild-type promoter (A) or in cocultures transfected with bone morphogenetic protein (BMP)/SMAD mutant promoter (F). Data are means ± SE of 4–6 observations in each group and are expressed as a percentage of the control group for each experiment. Different letters above data bars indicate that these groups are significantly different (P < 0.05).
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

![Fig. 4. Endogenous expression of BMPs and receptor-activated SMAD 1/5/8 (R-SMADs) in HuH7 cells is not altered by hypoxia.](http://ajpcell.physiology.org/)

mRNA expression levels of the endogenous BMP/hemojuvelin (HJV) receptor ligands BMP2 (A) and BMP6 (B) together with the BMP receptor inhibitor noggin (C) were not altered by treatment with H2O2 or hypoxia in HuH7 cocultures. In addition, exposure to hypoxia (1% O2) or 100 μM H2O2 did not alter the protein expression of HJV (D), the coreceptor required for BMP-mediated regulation of hepcidin expression, or phospho-SMAD 1/5/8 (E) activated by BMP binding to the BMP receptor/HJV complex. AU, arbitrary units. Data are presented as means ± SE of 4–6 observations.
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-β signaling. We detected SMAD4 protein in nuclear and cytosolic extracts from HuH7 cells (cytosolic extracts were positive for the marker protein β-tubulin; nuclear extracts were β-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

![Image](http://ajpcell.physiology.org/)
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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 H2O2 and hypoxia, and, furthermore, no significant changes in phosphorylation of the receptor SMAD1/5/8 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 H2O2, 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-1β 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

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DISCLOSURES

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

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