Transferrin receptor 2 is crucial for iron sensing in human hepatocytes

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1Nutritional Sciences Division, King’s College London; 2Institute of Liver Studies, King’s College London School of Medicine, London; 3School of Biosciences, University of Westminster, London; 4Dr Hadwen Trust for Humane Research, Hitchin; and 5Centre for Infection, Immunity and Disease Mechanisms, Division of Biosciences, School of Health Sciences and Social Care, Brunel University, Uxbridge, United Kingdom

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Rapisarda C, Puppi J, Hughes RD, Dhawan A, Farnaud S, Evans RW, Sharp PA. Transferrin receptor 2 is crucial for iron sensing in human hepatocytes. Am J Physiol Gastrointest Liver Physiol 299: G778–G783, 2010. First published June 24, 2010; doi:10.1152/ajpgi.00157.2010.—Hepcidin expression in vivo is regulated in proportion to iron status (i.e., increased by iron loading and decreased in iron deficiency). However, in vitro studies with hepatoma cell lines often show an inverse relationship between iron status and hepcidin expression. Here, we investigated possible molecular mechanisms responsible for the differences in iron sensing between hepatoma cell lines and primary human hepatocytes. RNA was collected from primary human hepatocytes, and HepG2 and HuH7 hepatoma cells were treated with either transferrin-bound and nontransferrin-bound iron. Expression of hepcidin, transferrin receptor 2, HFE, and hemoljuvelin were quantified by real-time PCR. Hepcidin expression was increased in primary human hepatocytes following 24-h exposure to holoferric transferrin. In contrast, hepcidin mRNA levels in hepatoma cells were decreased by transferrin. Hepcidin expression was positively correlated with transferrin receptor 2 mRNA levels in primary human hepatocytes. Compared with primary hepatocytes, transferrin receptor 2 expression was significantly lower in hepatoma cell lines; furthermore, there was no correlation between transferrin receptor 2 and hepcidin mRNA levels in either HepG2 or HuH7 cells. Taken together, we suggest that transferrin receptor 2 is a likely candidate to explain the differences in iron sensing between hepatoma cell lines and primary human hepatocytes.

HEPCIDIN, a 25-amino-acid peptide produced predominantly by the hepatocytes, is a key regulator of iron homeostasis. The expression of hepcidin is controlled by several physiological and pathological factors including serum iron levels (6, 24), erythropoietic factors (5, 20), and inflammation (17, 31). Despite these convincing in vivo data, comparable in vitro studies using primary hepatocytes and hepatoma cell lines, with the aim of understanding the molecular mechanisms controlling hepcidin expression, have yielded conflicting results. Transferrin-bound iron treatment of mouse primary hepatocytes resulted in an increase in hepcidin expression (15, 26), whereas hepcidin mRNA levels were not altered in the presence of nontransferrin-bound iron (24). In contrast, one study using human primary hepatocytes found an unexpected decrease in hepcidin following exposure to both transferrin-bound and nontransferrin-bound iron (19). In hepatoma cell lines, iron loading either does not alter (11) or results in a decrease in hepcidin mRNA expression (9). Taken together, these data suggest that there are fundamental differences in the ability of different hepatocyte models to sense changes in transferrin-bound iron levels.

The mechanisms by which the hepatocytes sense changes in iron levels and respond by modulating hepcidin expression remain unclear. Three main candidate genes for iron sensing in hepatocytes have been identified, namely hemoljuvelin (HJV), transferrin receptor 2 (TTR2), and the hereditary hemochromatosis gene HFE. A mutation in any one of these genes is associated with inappropriately low expression of hepcidin and as a consequence the iron-loading disorder hemochromatosis (1, 2, 18, 21, 22). In the present study, to address the potential role of these genes as iron sensors, we have measured the expression of HJV, TTR2, and HFE in cultures of primary human hepatocytes and human hepatoma cells and compared this with the ability of the cells to regulate hepcidin following incubation with transferrin-bound iron.

MATERIALS AND METHODS

Cell isolation and culture. Primary human hepatocytes were isolated from liver resection tissues by a modified two-step collagenase perfusion technique (16). Patients gave informed consent for the use of tissue, and all protocols were approved by the Research Ethics Committee of King’s College Hospital. Hepatocytes were purified by low-speed centrifugation at 50 g for 5 min at 4°C, and corresponded to >90% of the isolated cells by morphological analysis. Once isolated, the cells were plated at a density of 1.5 × 10⁶ viable cells/well in collagen-coated six-well plates and cultured in Williams’ E medium (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FCS) (Invitrogen), 10 mM HEPES (Cambrex), 2 mM L-glutamine (Invitrogen), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.1 μM dexamethasone, and 0.1 μM insulin (Sigma-Aldrich) at 37°C in 95% O₂-5% CO₂. HepG2 cells and HuH7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Isolated human primary hepatocytes were cultured for at least 16 h before experiments. HepG2 and HuH7 cells were seeded at 1 × 10⁶ cells/well in six-well plates and used 24–48 h postseeding. All cells were treated for 24 h with 100 μM iron supplemented to the growth media in the form of hemin, ferric ammonium citrate, or holotransferrin (Sigma-Aldrich).

RNA isolation and quantitative real-time PCR. Following treatment, total RNA was isolated from the cells by using Trizol reagent (Invitrogen) according to the manufacturer protocol. First-strand cDNA synthesis was performed in a total volume of 20 μl with 1 μg RNA primed with anchored oligo(dT)18 by use of a Transcriptor high-fidelity cDNA synthesis kit (Roche).

Quantification of cDNA was performed by real-time PCR by using an ABI Prism 7000HT PCR cycler and a Quanti-Tect SYBR Green PCR kit (Qiagen) according to the manufacturer’s protocol. For each gene a standard curve was generated with 10-fold serial dilutions of a pool of cDNA samples. The genes analyzed and the primers used are listed in Table 1. Gene expression levels were normalized to levels of...
β-actin in the same sample. Data are from at least three independent experiments.

Western blotting. Primary hepatocytes were lysed with ice-cold lysis buffer (phosphate-buffered saline, containing 1% sodium dodecyl sulfate, 10 mg/l protease inhibitor cocktail). Lysates were precleared of insoluble membrane material and nuclei by centrifugation (13,000 g for 10 min), and 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 TfR2 antibody (Santa Cruz Biotechnology). Cross-reactivity was observed by use of a horseradish peroxidase-linked secondary antibody (Dako) and ECL Plus (GE Healthcare). Band densities were semiquantified by use of Scion Image software (Scion). At the end of the experiment, the nitrocellulose membranes were stripped (Western Stripping Buffer, Perbio Science) and reprobed with an anti-actin antibody (1:2,000 dilution, Sigma-Aldrich) that acted as a loading control.

Statistical analysis. Statistical analyses were performed by one-way ANOVA and Dunnett’s post hoc test to compare control and Sigma-Aldrich) that acted as a loading control.

RESULTS

Basal expression of hepcidin is elevated in primary human hepatocytes compared with hepatoma cell lines. Basal levels of hepcidin mRNA were measured and quantified in primary human hepatocytes and in HepG2 and HuH7 hepatoma cells by real-time PCR (Fig. 1A). Hepcidin expression was some three-fold higher in primary hepatocyte cultures compared with HuH7 cells (P < 0.05). HepG2 cell hepcidin levels were not significantly different from those observed in primary hepatocytes.

TfR2, HFE, and HJV are candidate iron-sensing genes in hepatocytes that might act as regulators of hepcidin expression (1, 2, 18, 21, 22). To determine whether the aberrant hepcidin response of the immortalized cell lines to holotransferrin could be explained by expression levels of any of these genes, we measured the mRNA levels in primary hepatocytes and HepG2 and HuH7 cells. Candidate iron-sensing genes were also differentially expressed in the three cell lines. TfR2 mRNA was significantly lower in both hepatoma cell lines compared with primary hepatocytes (Fig. 1B). Relative HFE expression was higher in primary hepatocytes compared with hepatoma cells; however, these differences were not statistically significant (Fig. 1C). In contrast, HJV levels were significantly higher in HuH7 cells than in primary hepatocytes (Fig. 1D).

Hepcidin expression correlates with TfR2 levels in primary human hepatocytes. Next we determined whether the relative levels of TfR2 in primary hepatocytes, HepG2 cells and HuH7 cells influenced hepcidin expression. In primary human hepatocytes, preliminary analysis (n = 3 samples) using a multiple linear regression model, incorporating TfR2, HFE, and HJV as predictive variables for hepcidin expression, only TfR2 mRNA significantly correlated with hepcidin mRNA (adjusted R² = 0.997; P = 0.026). Hepcidin did not correlate significantly with either HFE (R² = 0.845; P = 0.25) or HJV (R² = 0.787; P = 0.31) in primary hepatocytes. Analysis of further samples of primary hepatocytes confirmed that there was a significant positive correlation between hepcidin expression and the mRNA levels of TfR2 (Fig. 2A; P = 0.001). In contrast, TfR2 expression did not significantly correlate with hepcidin expression in either HepG2 (Fig. 2B; P = 0.09) or HuH7

![Fig. 1. Basal mRNA expression of hepcidin (A), transferrin receptor 2 (TfR2; B), HFE (C), and hemojuvelin (HJV; D) in primary human hepatocytes, HepG2 cells, and HuH7 cells. Data are means ± SE of 3–6 observations in each group. *P < 0.05 (ANOVA and Dunnett’s post hoc test), significant difference compared with primary hepatocytes.](http://ajpgi.physiology.org/)

**Table 1. PCR primers used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>ACTB</td>
<td>CCAACC667AAGAG667667AAAT667GGA</td>
<td>CCAAGGGGCT667TAC667GGA667TGA667AG667GAG667GAA</td>
</tr>
<tr>
<td>TfR2</td>
<td>TATA667CTGGAG667TCT667GGG</td>
<td>C667GAGGTT667AG667TAC667GCT667CT667TA</td>
</tr>
<tr>
<td>HFE</td>
<td>AG667AAC667G667GCT667AC667TT667CAC667AAC667AAG667GAG667GAA</td>
<td>AT667GTTAG667GCT667CCC667TG667TG</td>
</tr>
<tr>
<td>HFE2</td>
<td>GG667AG667CT667GG667CT667ACT667GGA</td>
<td>AT667GTTAG667GCT667CCC667TG667TG</td>
</tr>
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</table>
To explore the possibility that iron treatment in primary hepatocytes influenced Tfr2 expression and thereby regulated hepcidin, we measured Tfr2 protein and mRNA in primary hepatocytes. There was no effect of hemin or ferric ammonium citrate (FAC) on Tfr2 protein levels; however, incubation with holo-transferrin significantly increased (P < 0.05) Tfr2 expression (Fig. 4A). There was no significant effect of iron treatment of Tfr2 mRNA levels in primary hepatocytes (Fig. 4B).

cells (Fig. 2C; P = 0.26). There was no significant correlation between hepcidin and either HFE or HJV in hepatoma cell lines (data not shown).

Holo-transferrin increases hepcidin expression in primary human hepatocytes. Hepcidin mRNA was significantly increased in cultures of primary human hepatocytes incubated with holo-transferrin (Fig. 3A). In contrast, transferrin-bound iron significantly decreased hepcidin expression in both HepG2 (Fig. 3B) and HuH7 (Fig. 3C) hepatoma cells. Hepcidin expression was not significantly altered by nonheme iron (ferric ammonium citrate) in any of the cell lines, but incubation with hemin significantly decreased hepcidin levels in primary hepatocytes.

Fig. 2. Correlation between Tfr2 mRNA and hepcidin levels in primary human hepatocytes (n = 7) (A), HepG2 cells (n = 9) (B), and HuH7 cells (n = 6) (C). Tfr2 levels positively correlated with hepcidin. This relationship was statistically significant in primary hepatocytes but not in either of the hepatoma cell lines.

Fig. 3. Effects of iron on hepcidin expression in primary human hepatocytes (A), HepG2 cells (B), and HuH7 cells (C). Hepcidin mRNA levels were significantly increased by transferrin (Tf)-Fe in primary human hepatocytes. In contrast, Tf-Fe resulted in a significant decrease in hepcidin mRNA levels in both HepG2 and HuH7 hepatoma cells. Data are means ± SE of 4–9 observations in each group. *P < 0.001; **P < 0.05 compared with untreated control cells.
DISCUSSION

Hepcidin is mainly produced by hepatocytes and is the major circulating regulator of iron homeostasis (14, 23, 24). Hepcidin expression is decreased following prolonged consumption of an iron-deficient diet and is inversely correlated with intestinal iron transporter expression (6). In contrast, feeding a high-iron diet is associated with hepatic iron loading and elevated hepcidin expression (24). However, these findings have not been recapitulated with in vitro cell culture models, in which iron loading is associated with a decrease in hepcidin mRNA (9, 19). In this study we investigated the possibility that there are molecular differences in the iron-sensing pathways in primary hepatocytes and hepatoma cell lines that might explain the anomalous data from in vivo and in vitro studies.

Previous studies have shown that hepcidin mRNA levels in whole human liver are some 140 times higher than HepG2 cells and 160 times higher than HuH7 cells (4). In contrast, hepcidin mRNA in our isolated human primary hepatocyte preparations was only modestly increased (30%) compared with the levels in HepG2 hepatoma cells but was significant greater (3-fold) than in HuH7 cells. Interestingly, low hepcidin expression in HuH7 cells may in part be due to a recently identified mutation in the HFE gene in these cells (29).

A number of putative iron-sensing genes have been described in hepatocytes that might act as regulators of hepcidin expression, including TfR2, HFE, and HJV (1, 2, 18, 21, 22). We investigated whether differential expression of these genes might explain the variations in hepcidin expression in primary hepatocytes and hepatoma cell lines. Compared with levels in primary hepatocytes, it was evident that TfR2 was downregulated in both HepG2 cells and Huh7 cells by almost an order of magnitude. Moreover, in primary hepatocytes there was a positive and significant correlation between TfR2 mRNA levels and hepcidin expression. This correlation was not present in either HepG2 or HuH7 cells. This latter finding is consistent with recent work in HuH7 cells showing that hepcidin mRNA levels are not significantly affected following either overexpression or small interfering RNA-mediated knockdown of TfR2 (11).

Knockout mouse studies have shed some light on the relative roles of TfR2 and HFE in the regulation of hepcidin expression (30). This report demonstrated that liver hepcidin mRNA levels in HFE knockout mice were significantly decreased compared with wild-type controls when normalized for liver iron content. Expression of hepcidin was further decreased in TfR2 knockout mice and was lower still in dual HFE/TfR2 knockout mice. Importantly, these data demonstrate that both HFE and TfR2 are able to sense and signal changes in iron status (30); if that were not the case, hepcidin expression would be expected to be the same in all three knockout models. HFE knockout mice (1) and HFE-related hemochromatosis patients (25) are still able to regulate hepcidin in response to changes in iron stores although the response is impaired (i.e., inappropriately low) compared with control subjects. Nonetheless, recent studies highlight the requirement for the expression of both HFE and TfR2 for full iron-sensing activity (7, 8). In our studies, although HFE levels and hepcidin expression were positively correlated, this association did not reach statistical significance, and this finding is consistent with the data from Wallace et al. (30) suggesting that HFE is lower in the hierarchy of iron sensors than TfR2.

Recent work has revealed that hepcidin mRNA expression is increased in primary mouse hepatocytes in response to holotransferrin (15, 26). In primary human hepatocytes we also observed a significant increase in hepcidin following exposure to holotransferrin. This is, to our knowledge, the first demonstration of a positive link between transferrin-bound iron and hepcidin mRNA in human hepatocytes, in contrast with previ-
ous work that reported a decrease in hepcidin following exposure to transferrin (19). Isolated hepatocytes are notoriously difficult to work with and there is evidence that cryopreservation (28) or prolonged culture periods significantly alters hepatocyte cell function. Thus the differences between our study and previous work (19) may well reflect the difference in the hepatocyte preparations used.

Iron has been shown to increase the stability of TfR2 protein levels in hepatocytes and hepatoma cells (11, 12, 27). In our study we observed an increase in TfR2 protein in primary human hepatocytes following exposure to holo-transferrin; TfR2 mRNA levels were increased but not significantly. There was no effect of either FAC or hemin on TfR2 expression. The perceived role of TfR2 in iron sensing is complex. Under high-iron conditions, holo-transferrin is thought to displace HFE from TfR1, allowing it to bind to TfR2 (3, 10). It has been suggested that the TfR2/HFE complex interacts with HJV initiating the BMP/SMAD signaling pathway (15), which leads to an increase in hepcidin expression. Recently, direct activation of SMAD signaling in response to an iron challenge has been demonstrated in mouse liver (13). Our data in primary human hepatocytes are consistent with this model.

In conclusion, our present studies suggest that TfR2 is a key gene linking iron sensing and the regulation of hepcidin expression in primary human hepatocytes. Furthermore, low expression of TfR2 in human hepatoma cell lines may contribute to inappropriate downregulation of hepcidin by holo-Tf.

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GRANTS

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DISCLOSURES

The authors declare that there are no conflicts of interest.

REFERENCES