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Serum-induced up-regulation of hepcidin expression involves the bone morphogenetic protein signaling pathway

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ABSTRACT

Hepcidin is a peptide hormone that is secreted by the liver and that functions as the central regulator of systemic iron metabolism in mammals. Its expression is regulated at the transcriptional level by changes in iron status and iron requirements, and by inflammatory cues. There is considerable interest in understanding the mechanisms that influence hepcidin expression because dysregulation of hepcidin production is associated with a number of disease states and can lead to iron overload or iron-restricted anemia. In order to shed light on the factors that alter hepcidin expression, we carried out experiments with HepG2 and HuH7, human hepatoma cell lines that are widely used for this purpose. We found that the addition of heat-inactivated fetal calf serum to these cells resulted in a significant dose- and time-dependent up-regulation of hepcidin expression. Serum also activated signaling events known to be downstream of bone morphogenetic proteins (BMPs), a group of molecules that have been implicated previously in hepcidin regulation. Inhibition of these signals with dorsomorphin significantly suppressed serum-induced hepcidin up-regulation. Our results indicate that a BMP or BMP-like molecule present in serum may play an important role in regulating hepcidin expression.

1. Introduction

Iron metabolism has to be stringently controlled in order to avoid the adverse effects of both iron deficiency and iron excess. A major player in the regulation of systemic iron homeostasis is the peptide hormone hepcidin, which is secreted by hepatocytes [1]. Hepcidin expression is induced by elevated circulating or tissue iron levels and by inflammatory mediators, whereas it is suppressed by states of iron deficiency or increased iron requirements. Hepcidin, in turn, controls the amount of iron entering the circulation by binding to the plasma membrane iron transporter ferroportin, thereby promoting degradation of the latter protein. Ferroportin is the sole means by which iron absorbed from the diet or recycled from effete erythrocytes, the two major sources of the metal, can be exported to the serum from enterocytes and phagocytes, respectively [2]. When tissue or serum iron is elevated, hepcidin expression is increased, leading to ferroportin down-regulation and a consequent decrease in iron entering the system. Conversely, when tissue/serum iron is low or when there is an increased demand for iron, hepcidin expression is inhibited, leading to ferroportin up-regulation and a consequent increase in the movement of iron into the circulation. Thus, the hepcidin–ferroportin axis is a key component of a negative feedback loop that maintains serum iron concentrations within a narrow physiologic range. Inherited or acquired derangements of hepcidin expression can lead to significant clinical problems. Inappropriately low levels of hepcidin produce systemic iron overload, with pathologic tissue deposition of iron and impairment of organ function [3,4]. On the other hand, when hepcidin levels are inappropriately high, an abnormality that is often associated with chronic inflammatory disorders, an iron-restricted anemia can develop [4,5].

Because of the key role played by hepcidin in iron metabolism, there is a great deal of interest in the mechanisms that regulate expression of the hormone. Hepcidin expression is regulated exclusively at the level of transcription, and two major signaling pathways are known to influence this process. Bone morphogenetic proteins (BMPs) can induce up-regulation of hepcidin by activating the SMAD (similar to mothers against decapentaplegic) signaling pathway [1,6]. Interaction of BMPs with their cognate receptors leads to activation of the receptor-associated kinase and increased phosphorylation of members of the SMAD family of signal

Abbreviations: BMP, bone morphogenetic protein; DMSO, dimethylsulfoxide; qRT-PCR, quantitative RT-PCR; RIPA, radioimmunoprecipitation assay; SMAD, similar to mothers against decapentaplegic; STAT3, signal transducer and activator of transcription 3.

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transducing proteins, specifically SMADs 1, 5 and 8. Each of these phosphorylated SMADs can then form a heterodimer with SMAD4, another member of the family, and the complex is translocated to the nucleus to transcriptionally activate the hepcidin gene. Several different BMPs have been shown to induce hepcidin expression in vitro. However, BMP6, which is produced mainly by non-parenchymal cells of the liver such as sinusoidal endothelial cells and stellate cells, is believed to be the most important inducer of hepcidin in vivo [7–9]. BMP-activated signals are modulated in a poorly understood fashion by the interaction of the type 2 transferrin receptor with HFE, the hereditary hemochromatosis protein, which acts as the major sensor of circulating iron levels. [10,11]. The second important signaling pathway that affects hepcidin transcription is activated by inflammatory cytokines, mainly IL-6, and leads to the phosphorylation and increased activity of the transcription factor STAT3 (signal transducer and activator of transcription 3) [12–14]. STAT3-dependent up-regulation of hepcidin requires a functionally intact BMP6–SMAD signaling pathway [7]. Additional signals, for instance, those activated by endoplasmic reticulum stress, have also been shown to influence hepcidin transcription [15,16].

In order to further characterize the factors that influence hepcidin expression, we carried out studies with the HepG2 and HuH7 human hepatocellular carcinoma cell lines, which have been used widely in the field for this purpose. Our results, described herein, suggest that a BMP or BMP-like molecule present in serum may play an important role in regulating hepcidin expression.

2. Materials and methods

2.1. Cells and stimulations

The HepG2 human hepatoma cell line was obtained from Dr. Sunitha Nagrath, BioMEMS Resource Center, Massachusetts General Hospital, and the HuH7 human hepatoma cell line from Dr. Raymond Chung, Division of Gastroenterology, Massachusetts General Hospital. Both cell lines were maintained in Eagle's Minimal Essential Medium containing 10% heat-inactivated fetal calf serum (Life Technologies, Grand Island, NY). Prior to stimulation, they were trypsinized and distributed into 24-well tissue culture plates at a density of 5×10^5 cells per well in 0.5 ml of serum-free medium, followed by overnight incubation at 37 °C. The next morning, the cells were stimulated with heat-inactivated (56 °C, 30 min) fetal calf serum at the concentrations and for time periods indicated in the individual experiments. In some experiments, the cells were stimulated with serum in the presence of 5 μM dorsomorphin (Sigma–Aldrich, St. Louis, MO), a small molecule inhibitor of the BMP–SMAD pathway [17], or equivalent volumes of the vehicle dimethylsulfoxide (DMSO).

2.2. Quantitative RT-PCR (qRT-PCR) measurement of hepcidin mRNA

After stimulation, total RNA was prepared from the cells using Trizol reagent (Life Technologies, Grand Island, NY) and following manufacturer's recommendations. Reverse transcription of the RNA, followed by PCR amplification of hepcidin and actin transcripts was carried out as previously described using primers specified earlier [18]. The relative expression of hepcidin was normalized to actin and calculated by the $2^{-\Delta\Delta Ct}$ method.

2.3. Western blotting

Following serum stimulation for the specified time periods, the cells were washed with phosphate-buffered saline, and then lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) containing a cocktail of protease inhibitors (10 μg/ml of aprotinin and leupeptin, 1 mM phenylmethylsulfonylfluoride) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate). After estimating the protein concentrations of the lysates, equal amounts of total protein were loaded in the lanes of a 10% acrylamide gel, subjected to denaturing electrophoresis and transferred to a nitrocellulose membrane, all as described in detail earlier [18]. The membrane was blocked and then incubated with primary antibodies against phosphorylated SMAD1/5/8, total SMAD 1, phosphorylated STAT3, or total STAT3 (all from Cell Signaling Technology, Beverly, MA). The blots were then developed with the appropriate fluorescently-labeled secondary antibodies and visualized on an Odyssey infra-red fluorescence imaging system (Li-COR Biosciences, Lincoln, NE).

2.4. Statistical analysis

Means and standard deviations of relative hepcidin expression are displayed. Differences between experimental groups were analyzed by the Student's t test, with $p < 0.05$ being considered significant. All experiments were carried out at least twice to confirm reproducibility.

3. Results and discussion

When HepG2 human hepatoma cells cultured in serum-free medium were treated with heat-inactivated fetal calf serum, the expression of hepcidin mRNA was significantly up-regulated in a dose- and time-dependent manner (Figs. 1A and B). After 6 h of treatment, the increase in hepcidin expression was observed with as little as 0.1% serum and reached a level of 4–5-fold above baseline in the presence of 10% serum (Fig. 1A). With addition of 10% serum, hepcidin expression was elevated 4–5-fold after 2–6 h of treatment (Fig. 1B). Similar results were obtained with HuH7, a second human hepatoma cell line, and the observations held true with two different lots of fetal calf serum (data not shown). Six hours of treatment with 10% serum was used for all subsequent experiments except where otherwise indicated.

To elucidate the mechanism of serum-induced hepcidin up-regulation, we examined the 2 major signaling pathways that are known to influence hepcidin expression, viz., the BMP-SMAD pathway and the STAT3 pathway. To investigate the former, we carried out western blotting experiments with an antibody recognizing the phosphorylated forms of the 3 receptor SMADs – SMADs 1, 5 and 8 – that are proximal signal transducers in this pathway. As shown in Fig. 2A, the addition of 10% fetal calf serum to serum-starved HepG2 cells led to a clear increase in phosphorylation of SMAD 1/5/8 within 30 min, with a maximum at about 2 h. There was no change in the amount of total SMAD 1 protein levels. In contrast, when the same cell lysates were immunoblotted with antibodies to phosphorylated and total STAT3, no change in phosphorylation of this protein was detected (Fig. 2B). The results thus indicate that serum activates the BMP–SMAD pathway but not the STAT3 pathway in HepG2 cells.

To determine the functional importance of SMAD pathway activation in serum-induced hepcidin up-regulation, we made use of the small molecule inhibitor dorsomorphin, which has been shown to inhibit BMP-SMAD signals [17]. We found that dorsomorphin treatment of the cells suppressed serum-dependent SMAD 1/5/8 phosphorylation, confirming its efficacy in blocking this pathway, whereas the vehicle DMSO had no effect (Fig. 3). orsomorphin treatment, but not DMSO, also significantly inhibited serum-induced up-regulation of hepcidin expression (Fig. 4). These results clearly support the idea that the effect of serum on hepcidin

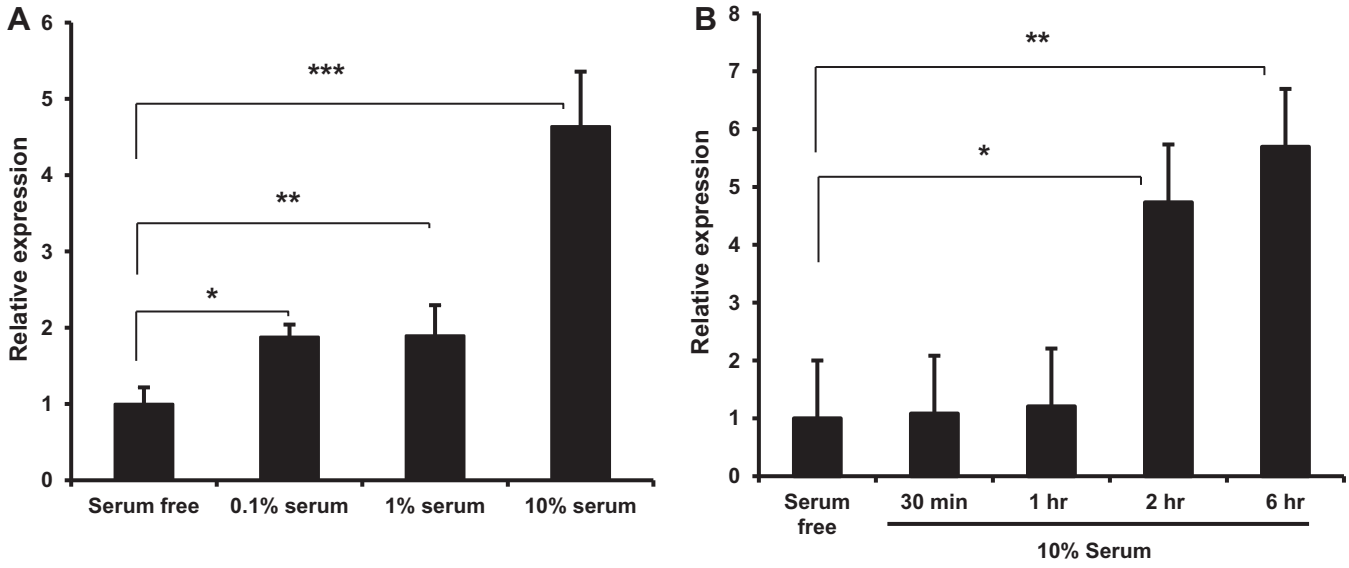

Fig. 1. Effect of serum on hepcidin mRNA expression in HepG2 cells. (A) HepG2 cells were stimulated with the indicated concentrations of heat-inactivated fetal calf serum for 6 h and qRT-PCR was used to quantify hepcidin mRNA levels. $^{*}p = 0.008$, $^{**}p = 0.018$, $^{***}p = 0.0006$, $n = 5$ or 6 independent stimulations in each group. (B) HepG2 cells were stimulated with 10% heat-inactivated fetal calf serum for the indicated time periods and qRT-PCR was used to quantify hepcidin mRNA levels. $^{*}p = 0.004$, $^{**}p = 0.006$, $n = 4$ or 5 independent stimulations in each group.

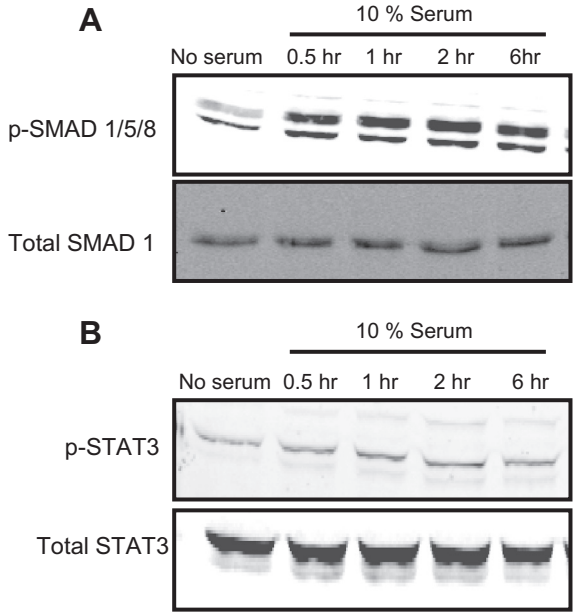

Fig. 2. Effect of serum on activation of the SMAD and STAT3 signaling pathways in HepG2 cells. HepG2 cells were treated with 10% heat-inactivated fetal calf serum for the indicated times and cell lysates were subjected to electrophoresis and immunoblotting with antibodies to phosphorylated SMAD (p-SMAD) 1/5/8 and total SMAD 1 (A) or phosphorylated STAT3 (p-STAT3) and total STAT3 (B).

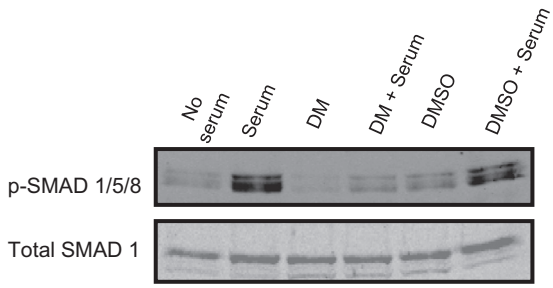

Fig. 3. Effect of dorsomorphin on serum-induced activation of the SMAD signaling pathway in HepG2 cells. HepG2 cells were stimulated with 10% fetal calf serum for 6 h in the presence or absence of 5 μM dorsomorphin (DM) or an equivalent volume of the vehicle DMSO. As additional controls, the cells were also treated with either dorsomorphin or DMSO in the absence of serum.

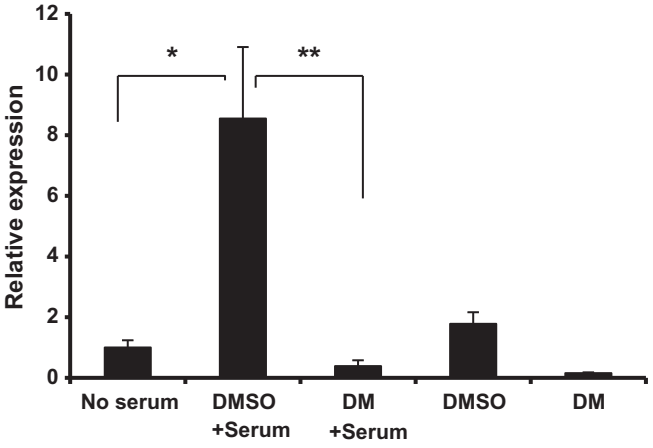

Fig. 4. Effect of dorsomorphin on serum-induced hepcidin expression in HepG2 cells. HepG2 cells were stimulated with 10% fetal calf serum for 6 h in the presence or absence of 5 μM dorsomorphin (DM) or an equivalent volume of the vehicle DMSO. As additional controls, the cells were also treated with either dorsomorphin or DMSO in the absence of serum. $^{*}p = 0.01$, $^{**}p = 0.029$, $n = 4$ independent stimulations for each group.

expression is dependent on signals transduced by the SMAD 1/5/8 proteins, and suggest that a BMP is responsible for this effect.

The results described in this manuscript demonstrate that fetal calf serum contains a factor that is able to induce significant hepcidin up-regulation in HepG2 and HuH7 cells. Since these cells are widely used to characterize hepcidin regulating molecules, our findings highlight the importance of carrying out such experiments under serum-free or low-serum conditions in order to avoid the confounding effects of hepcidin induction by bovine serum. Our data also suggest that the hepcidin-inducing factor in fetal calf serum is a BMP or BMP-like molecule. We have not yet identified

the relevant BMP or BMP-like molecule, but both bovine and human sera are known to contain BMPs 4, 6 and 9 [19], so it is plausible that these proteins may contribute to the serum-induced hepcidin expression seen in our experiments. Interestingly, when we tested a commercial preparation of pooled human plasma (Sigma–Aldrich), we were not able to demonstrate up-regulation of hepcidin in HepG2 cells (unpublished data). However, other investigators have found that the addition of normal human serum to HuH7 cells resulted in a modest increase in hepcidin promoter activity as measured by a luciferase reporter assay [20]. Therefore, it is possible that the failure of pooled human plasma to up-regulate hepcidin in our experiments may be related to the properties of the specific preparation that we used. Nevertheless, we cannot rule out a real biological difference between fetal calf serum and human plasma with respect to hepcidin induction. This issue will require further study.

The exact role played by the putative serum BMP in regulating hepcidin expression and iron metabolism is not clear. BMP6 is the only member of the family whose expression is influenced by iron concentrations, particularly in the liver [21–24], and it is possible that fluctuations in serum concentrations of BMP6 or other BMPs could link tissue iron to hepcidin expression. BMPs in serum may also contribute to the changes in hepcidin expression associated with inflammation and other pathologic conditions [4,5]. Serum BMP2 levels have been found to be elevated in patients with multiple myeloma, an abnormality that has been linked to increased hepcidin and the development of anemia in this disease [20]. Alternatively, serum BMPs could simply influence basal hepcidin production, with further modulation occurring in response to other inputs such as tissue and circulating iron levels and inflammatory signals. The exact role of serum BMPs in the in vivo regulation of hepcidin and iron metabolism merits further investigation.

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