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Blood Cells Mol Dis. Author manuscript; available in PMC 2008 May 1.

Published in final edited form as:
Blood Cells Mol Dis. 2007 ; 38(3): 253–257.

In Vivo Imaging of Hepcidin Promoter Stimulation by Iron and Inflammation

Jonathan M. Flanagan, **Jaroslav Truksa**, **Hongfan Peng**, **Pauline Lee**, and **Ernest Beutler**
Department of Molecular & Experimental Medicine, The Scripps Research Institute, MEM-215, 10550 Torrey Pines, La Jolla, CA, USA

Abstract

Hepcidin is an acute-phase response antimicrobial peptide that has emerged as a central regulator of iron absorption. Circulating hepcidin levels have been shown to affect iron uptake, release and storage. Hepcidin is mainly liver-derived and regulated, at least in part, transcriptionally. Hypoxia, erythroid demand, iron content and inflammation each have been shown to influence hepcidin mRNA expression in intact animals. *In vitro*, regulation of hepcidin by cytokines and by hypoxia is readily demonstrated in primary hepatocytes or in hepatocyte lines, but incubating the same cell lines with iron does not increase transcription of hepcidin. Thus, how iron excess stimulates hepcidin production in hepatocytes remains unknown. In addition, there is no current technique available that can investigate how iron induces hepcidin expression. To provide a better understanding of hepcidin gene expression in response to these regulatory stimuli, we have established a whole animal *in vivo* bioluminescence imaging assay to measure the activity of hepcidin promoter constructs in the animals liver after hydrodynamic transfection of hepcidin promoter/luciferase constructs into mice. Transfected hepcidin promoter constructs were shown to respond to both inflammatory and increasing iron stimuli *in vivo*. This work highlights the ability of this new imaging technique to investigate the key regions of the hepcidin promoter involved in iron induction of hepcidin expression.

Keywords

Hepcidin; IVIS; Luciferase; Iron Stimulation

Introduction

Hepcidin is an iron-regulatory hormone produced by the liver. The function of hepcidin is to regulate extracellular iron concentration by inhibiting intestinal iron absorption and iron release from macrophages [1,2]. Low or high plasma hepcidin levels result in increased or decreased iron absorption respectively. Hepcidin performs this function by directly binding to the iron transporter ferroportin, causing internalization and subsequent degradation of ferroportin [3]. The search is now on for the molecular signals that directly or indirectly stimulate the activity of hepcidin, thereby affecting iron homeostasis.

The regulation of hepcidin expression is thought to occur predominantly at the transcriptional level [4-6]. It is becoming apparent from the variety of signals that induce hepcidin that the transcriptional controls regulating its expression may be varied and multifaceted. Hypoxia,

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erythroid demand, iron content and inflammation have all been shown to influence hepcidin mRNA expression in intact animals [7,8]. Regulation of hepcidin by cytokines or by hypoxia is readily demonstrated in primary hepatocytes or in hepatocyte cell lines. However, direct exposure of hepatocytes to ferric iron or iron-saturated transferrin, with or without serum, does not induce hepcidin mRNA expression [7,9]. Thus, how iron excess stimulates hepcidin production in hepatocytes remains undetermined.

To provide a better understanding of hepcidin gene expression in response to these regulatory stimuli, we took advantage of the fact that 2 hepcidin genes exist in mice. Hepcidin-1 is homologous to human hepcidin while hepcidin-2 is the result of a tandem duplication of murine hepcidin-1. *In vivo*, murine hepcidin-1 mRNA expression responds to iron and to inflammation, but hepcidin-2 responds only to iron. As little information is presently available about promoter elements and transcription factors that control hepcidin expression, we have established a whole animal *in vivo* bioluminescence imaging assay to measure the activity of hepcidin promoter constructs in the animals' liver after hydrodynamically transfecting hepcidin promoter/luciferase constructs into mice. Through this method, various murine hepcidin-1 and hepcidin-2 promoter constructs have been shown to have basal luciferase activity. When transfected mice were stimulated with iron or endotoxin, the murine hepcidin-1 constructs were found to respond to both of these stimuli. In contrast, the murine hepcidin-2 transfected mice only showed a weak response to iron stimulation while there was no stimulation by endotoxin.

These findings validate, in an *in vivo* system, what is known about these promoters from other studies. In addition, the development of this *in vivo* imaging technique provides a mechanism to investigate the key regions of the hepcidin promoter involved in iron induction of hepcidin expression. Future experiments will focus on testing other hepcidin promoter constructs using this strategy to identify the iron response site of the hepcidin promoter

Materials and Methods

Materials

Lipopolysaccharide (LPS) was obtained as a preparation from *Escherichia coli* 055:B5 from Sigma (St. Louis, MO). Three mouse chow diets were used: a high iron diet containing 2% carbonyl iron (2×10^4 ppm iron), a low iron diet (50 ppm iron) and an iron deficient diet (2-5 ppm), all from Harlan Teklad (Madison, WI). Mice were kept on the low iron diet after weaning and placed on the iron deficient diet at least two weeks prior to a planned experiment.

Luciferase Constructs

Four murine hepcidin-1 promoter reporter constructs were made by cloning proximal 1.0kb, 530bp, 260bp and 140bp fragments of murine hepcidin-1 promoter into Promega pGL3 basic vector containing *firefly* luciferase. The mouse hepcidin-1 1.0 Kb clone was made by amplifying the PCR product using forward tgtaggctgggtaccaatctc (*Kpn*I) and reverse tgccttcagatctgctgtgc (*Bgl*II) primers, cutting with the restriction enzymes *Kpn*I and *Bgl*II and inserting it into pGL3 basic using these sites. The mouse hepcidin-1 530 bp clone was obtained by cleavage of the 1.0 Kb clone by *Nde*I and blunt ligation after filling the overhangs with Klenow fragment. Mouse hepcidin-1 260 bp was cloned by cutting the 1.0 Kb construct with *Kpn*I and *Pst*I and connecting the overhangs by using linker containing *Kpn*I and *Pst*I overhangs on each side. Mouse hepcidin-1 140 bp was cloned by amplifying the PCR product using forward gagctcttacgcgtgttcttggaaatgagtcagagca (*Mlu*I) and reverse cttagatcgcagatctctgtgtggtggctgt-ctaggagc (*Bgl*II) primers, cutting with the restriction enzymes *Mlu*I and *Bgl*II and inserting it into pGL3 basic using these sites. We also cloned mouse hepcidin-2 1.2Kb fragment by amplifying the PCR product using murine hepcidin-2 specific forward gagctcttacgcgtgtcttgagaaccctgtctttgg (*Mlu*I) and reverse

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atcgcagatctcgagcttgtgtggtggctgtctaggt (*Xho*I) primers, cutting with the restriction enzymes *Mlu*I and *Xho*I and inserting it into pGL3 basic using these sites. All cloned fragments were originally amplified from genomic C57BL/6J DNA. The sequences of all promoter constructs were verified by sequencing.

Hydrodynamic Transfection

C57BL/6J, 129×1/SvJ or *Hfe* −/− mice were used for all experiments and were maintained in accordance with the National Institutes of Health Guidelines for Animal Care. The rapid tail vein injection of a large volume of plasmid DNA was performed using an established method [10]. Animals were injected via the tail vein with a volume equivalent to 7% of the total body weight. The average injection was delivered within 5-8 seconds, at a final plasmid delivery concentration of 10μg/mouse.

Imaging bioluminescence using In vivo Imaging System (IVIS)

Mice were hydrodynamically transfected with the hepcidin promoter luciferase constructs. Three days later, the mice were anesthetized by isoflurane and then injected intraperitoneally with 50μl of luciferin (30μg/ml). After five minutes, the mice were placed ventral side up in an IVIS Live image instrument (Xenogen, Hopkinton, MA). While still under anesthesia, images of luciferase activity were continuously acquired until maximal activity was detected. The acquired images were analyzed using the Live Image 2.5 software) to provide a quantitative amount of luciferase activity. For iron stimulation experiments, iron deficient mice were hydrodynamically transfected and baseline luciferase activity was determined. The mice were then randomized and separated into two groups, one remaining on the iron deficient diet as a control group while the second group was stimulated with iron by placing the mice on a high iron diet. Luciferin was injected intraperitoneally and luciferase activity remeasured at the indicated times and expressed as a percentage of baseline activity. Similarly for inflammation stimulation experiments, iron deficient mice were hydrodynamically transfected with the hepcidin promoter luciferase constructs. Baseline luciferase activity was determined, followed by intraperitoneal injection of LPS (0.5μg/g body weight). After 8 hours, luciferase activity was remeasured and expressed as a percentage of baseline activity.

Results

To investigate how iron levels or inflammatory signals induce hepcidin mRNA expression, four luciferase reporter vectors were generated containing the 1.0kb, 530bp, 260bp or 140bp upstream of the transcription start site of the murine hepcidin-1 promoter. A promoter vector containing the 1.25kb fragment upstream of the murine hepcidin-2 promoter was also made. The luciferase reporter constructs were all functionally active when transfected into the human hepatocyte cell line HepG2 (data not shown). Following hydrodynamic transfection of the hepcidin promoter/luciferase constructs, IVIS imaging of mice showed the constructs produced quantifiable baseline bioluminescence *in vivo* (Fig. 1). Since the baseline bioluminescence could vary by one order of magnitude between animals, all data were calculated using each animal as their own control.

The effect of inflammatory cytokines was assessed on the hepcidin-1 1.0kb construct in 129×1/SvJ and C57BL/6J mice; and on the hepcidin-1 530bp, hepcidin-1 260bp, hepcidin-1 140bp and hepcidin-2 1.25kb constructs in C57BL/6J mice. Treatment of transfected mice with endotoxin revealed that only the hepcidin-1 constructs showed a response (Fig. 2). The minimal hepcidin-1 140bp construct showed an endotoxin response. In contrast, the mice transfected with the murine hepcidin-2 construct showed no stimulation by endotoxin. To assess the effect of iron on the hepcidin promoter constructs in vivo, mice transfected with the murine hepcidin-1.0kb and hepcidin-2 1.25kb constructs were assayed. When these transfected mice

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were stimulated with iron, the murine hepcidin-1 and hepcidin-2 constructs were both found to respond to this stimulus (Fig. 3). The response to iron stimulation was not as great as that caused by endotoxin stimulation. The effect of iron on the hepcidin-1 1.0kb construct was shown in three mice strains; C57BL/6J, 129×1/SvJ and *Hfe* −/−. The effect of iron on the hepcidin-2 1.25kb construct was shown in C57BL/6J mice.

Discussion

Hepcidin expression increases in response to iron overload and inflammatory stimuli to reduce duodenal iron absorption and to increase iron retention in the reticuloendothelial system. Recent findings have provided information regarding the part of the promoter required for IL-6 to induce hepcidin expression through the mediation of STAT3 [11]. However, the mechanism by which iron stimulates hepcidin expression still remains unclear. A barrier to answering the iron induction question is that treatment of hepatocytes or mixtures of hepatocytes and macrophages or marrow cells with iron does not increase transcription of hepcidin (Lee, P., Unpublished data). Thus, there is no *in vitro* method to assess the effect of iron on the hepcidin promoter. To circumvent this problem, we have established a whole animal *in vivo* bioluminescence imaging assay to measure the activity of hepcidin promoter constructs in the animals liver after hydrodynamic transfection of hepcidin promoter/luciferase constructs into mice

From the data, the findings have been shown to validate, in an *in vivo* system, what is known about these promoters from other studies. In mice, the endotoxin responsive region is in the proximal 140bp promoter region of hepcidin-1 but not in the hepcidin-2 promoter (Fig. 2). Both the expression of hepcidin-1 and hepcidin-2 mRNA have been shown to respond to iron (Fig. 3). However, while the endotoxin response was consistently robust in the *in vivo* bioluminescence assay, the iron response was found to be more modest and variable between mice and experiments. In mice that had not been placed on a low iron diet after weaning it was barely perceptible. It seems likely that initial iron status of the mice plays a significant role in determining the degree of iron stimulation of the hepcidin promoter. In addition, there may be major iron responsive regulatory regions of the hepcidin gene outside of the current promoter region of study.

The development of this *in vivo* imaging technique provides a mechanism to investigate the key regions of the hepcidin promoter involved in iron induction of hepcidin expression. Future experiments will focus on testing other hepcidin promoter constructs using this strategy to identify the iron response site of the hepcidin promoter.

Acknowledgments

This is manuscript number 18702-MEM from The Scripps Research Institute. Supported by the National Institutes of Health grant DK53505-09, the Skaggs Clinical Scholars Program and the Stein Endowment Fund.

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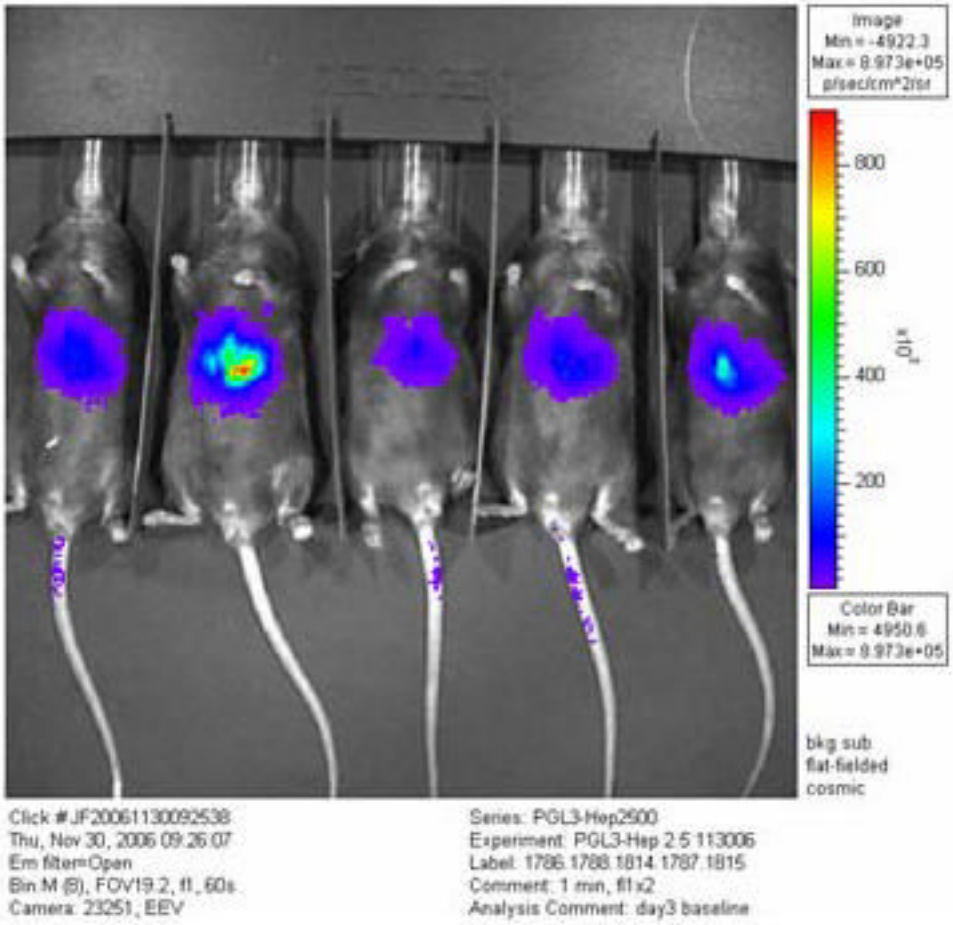

Fig 1.

In *vivo* bioluminescence imaging using a Xenogen IVIS instrument. Four mice were transfected hydrodynamically with 10 μg of the 1.0 kb hepcidin-1 luciferase promoter construct. After three days, baseline luciferase bioluminescence was measured using the Xenogen IVIS instrument.

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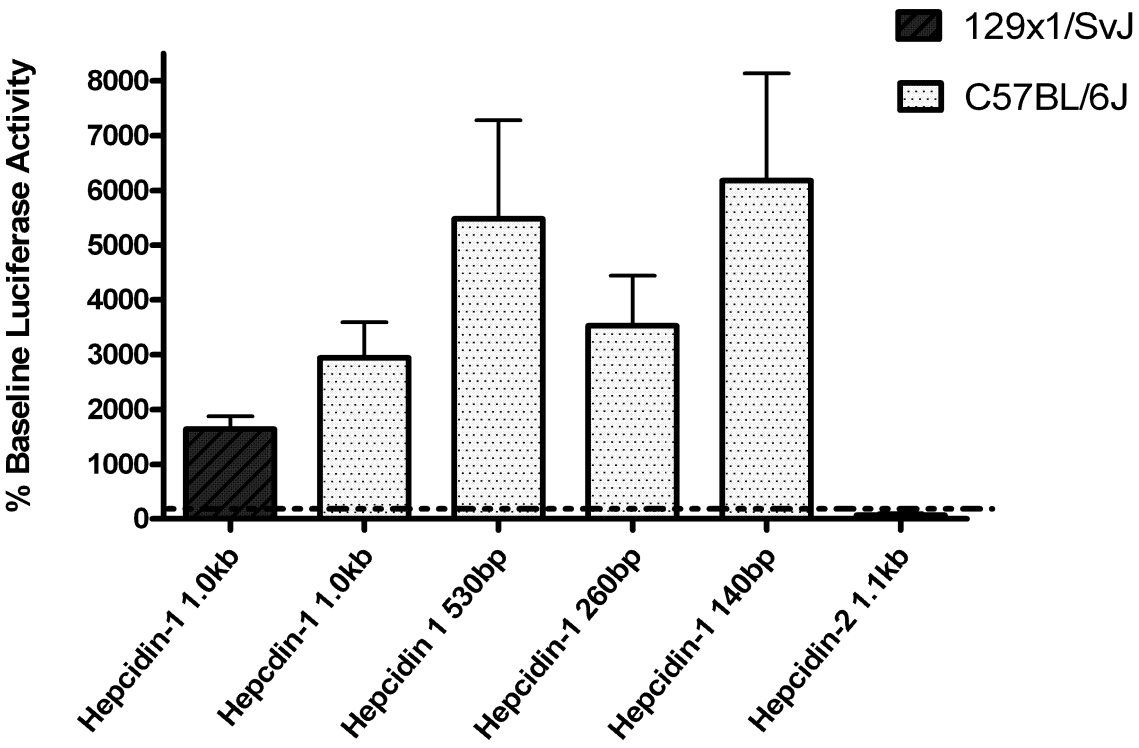

Fig 2.

Endotoxin stimulation of hepcidin promoter constructs. Baseline luciferase activity was determined, followed by intraperitoneal injection of LPS (0.5 μg/g). After 8 hours, luciferase activity was remeasured and expressed as a percentage of baseline activity. The effect of endotoxin was assessed on the Hepcidin-1 1.0kb construct in 129×1/SvJ and C57BL/6J mice, and on the hepcidin-1 530bp, hepcidin-1 260bp, hepcidin-1 140bp and hepcidin-2 1.25kb constructs in C57BL/6J mice. The dashed line in the figure denotes % baseline activity of control mice (no LPS). Each bar represents the mean and 1 standard error.

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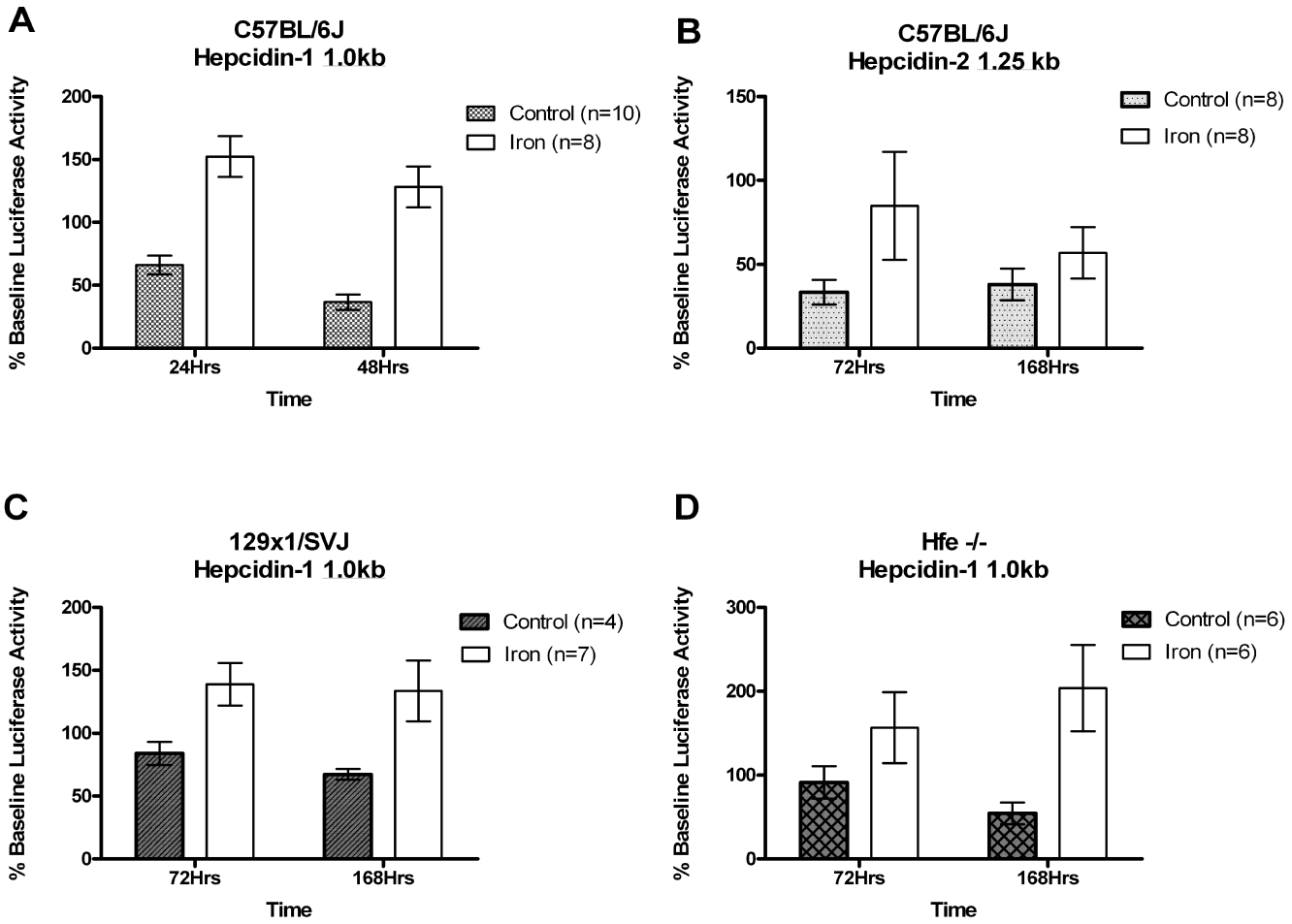

Fig 3.

Iron stimulation of hepcidin promoter constructs. Iron deficient mice were hydrodynamically transfected and baseline luciferase activity was determined. The mice were randomized and separated into two groups, one remaining on the iron deficient diet (2-5 ppm carbonyl iron) as a control group while the second group was stimulated with iron by placing the mice on a high iron diet (2×10^4 ppm iron). Luciferase activity was remeasured at the indicated times and expressed as a percentage of baseline activity. The effect of iron on the hepcidin-1 1.0kb construct is shown in three mice strains; C57BL/6J **(A)**, 129×1/SvJ **(C)** and *Hfe* −/− **(D)**. The effect of iron on the hepcidin-2 1.25kb construct is shown in C57BL/6J mice **(B)**. Each bar represents the mean and 1 standard error.