Hepcidin regulation of ferroportin 1 expression in the liver and intestine of the rat

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Yeh, Kwo-yih, Mary Yeh, and Jonathan Glass. Hepcidin regulation of ferroportin 1 expression in the liver and intestine of the rat. Am J Physiol Gastrointest Liver Physiol 286: G385–G394, 2004. First published October 30, 2003; 10.1152/ajpgi.00246.2003.—Hepcidin has been implicated as the iron stores regulator: a hepatic signaling molecule that regulates intestinal iron absorption by undefined mechanisms. The possibility that hepcidin regulates the expression of ferroportin 1 (FPT1), the basolateral iron transporter, was examined in rats after administration of LPS, an iron chelator, or His-tagged recombinant hepcidin (His-rHepc). In the liver, LPS stimulated a biphasic increase of hepcidin mRNA with peaks of mRNA at 6 and 36 h. Concurrently, hepatic FPT1 mRNA expression decreased to minimal level at 6 h and then increased with a peak at 24–36 h. LPS also induced biphasic changes in intestinal FPT1 mRNA expression, with decreased levels at 6 h and increased expression at 48 h. Whereas the initial decrease of FPT1 coincides with an LPS-induced decrease in serum iron, both intestinal and hepatic FPT1 expression recovered, whereas serum iron concentration continued to decrease for at least 24 h. Dietary iron ingestion increased intestinal ferritin protein production but did not reduce intestinal FPT1 mRNA expression. The iron chelator pyrrolidinedithiocarbamate (PDTc) stimulated hepatic hepcidin without suppressing intestinal FPT1 expression. In PDTc-treated rats, LPS stimulated no additional hepatic hepcidin expression but did increase intestinal FPT1 expression. Administration of His-rHepc induced significant reduction of intestinal FPT1 expression. Taken together, these data suggest that hepcidin mediates LPS-induced downregulation of intestinal FPT1 expression and that the hepcidin signaling pathway involves a PDTc-sensitive step.

INTRODUCTION

Iron is an essential component of numerous proteins and enzymes that are critical for cell respiration, proliferation, and signal transduction (4, 25). Hence, iron deficiency affects the function of multiple tissues. In excess, iron is a prooxidant and toxic, because labile iron catalyzes the formation of hydroxyl radicals resulting in the oxidation of surrounding macromolecules (9). Thus nearly all organisms possess mechanisms to modulate iron acquisition and maintain iron homeostasis. In mammals, iron homeostasis is maintained by the regulation of intestinal iron absorption, because there is no regulatory mechanism for the excretion of excess iron. Intestinal iron absorption is regulated both by an iron stores regulator and by an erythroid regulator. The former responds to low body iron stores by upregulating intestinal iron transports, and the latter acts similarly in response to increased erythropoietic activity. The regulatory mechanisms for both the stores and erythroid regulators are largely unknown but presumably involve signals sent from the sites of iron storage and erythropoietic activity to the intestine, which then modulate intestinal iron absorption. In the intestine, the divalent metal transporter (DMT1; also known as DCT1 and NRAMP2) and ferroportin 1 (FPT1; also known as IREG1 and MTP1) are thought to be iron transporters critical for intestinal iron absorption (1, 10, 14, 20, 26). It is likely then that DMT1 and FPT1 are the molecules that respond to the regulatory signals from the iron stores and/or erythropoietic activity, and indeed appropriate changes in both transporters occur with iron deficiency and with enhanced erythroid activity.

Recently, hepcidin, a small antimicrobial peptide specifically produced in the liver, has been suggested to be the signal of hepatic iron stores that regulates intestinal iron absorption. The lack of hepatic hepcidin expression in upstream stimulation factor 2 (USF2) knockout mice leads to iron overload in liver, pancreas, and heart (30). Conversely, overexpression of hepcidin in transgenic mice causes severe iron deficiency (31). Moreover, in normal mice, iron overload increases and iron deficiency decreases hepatic expression of hepcidin; the changes in hepcidin expression are accompanied by inverse changes of intestinal iron absorption (34). The mechanism(s) underlying the inverse relationship between hepcidin and intestinal iron absorption remains undefined. In USF2 knockout mice, liver iron overload occurs without changes in the expression of the hemochromatosis gene (HFE), transferrin receptor, or DMT1 (30), implying that these molecules are not involved in the iron overload that occurs in the absence of hepcidin. In normal rats, decreased dietary iron intake leads to a decrease of hepcidin expression and increases of intestinal iron absorption and FPT1 expression (2, 17), suggesting that there is a causal relationship between the expression of hepatic hepcidin and intestinal FPT1 expression. However, iron alone could alter the expression of hepatic hepcidin and intestinal DMT1 and FPT1 independently (1, 10, 20, 26, 34), and hence the changes of hepcidin and intestinal iron transport might be independent events.

The possibility that hepcidin regulates intestinal iron absorption by modulating FPT1 expression could be explored using conditions that alter hepatic hepcidin expression with parallel determinations of intestinal FPT1 expression. Bacterial infection provokes intense increase of hepatic hepcidin expression in fish (39). Gram-negative bacterial endotoxin LPS and other inflammatory agents, such as turpentine and Freund’s complete adjuvant, stimulate hepatic hepcidin expression in mice and rats (2, 32, 34). LPS is known to decrease intestinal iron absorptive uptake in mouse intestine (26, 34).

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absorption by blocking enterocyte iron efflux (7) and by reducing FPT1 expression in the intestine and in reticuloendothelial cells (42). It is possible that hepcidin mediates the LPS-induced decrease of intestinal FPT1 expression. We hypothesize that if hepcidin plays a hormonal role in the modulation of FPT1 expression, then after the administration of LPS, the inverse changes in hepcidin and intestinal FPT1 expression should be temporally related. We used various rat models to determine whether there is an inverse and temporally related expression of hepatic hepcidin and intestinal FPT1 expression. First, we examined the time-dependent changes in hepatic hepcidin and FPT1 and intestinal FPT1 expression in rats after LPS administration. We then examined the inverse relationship between hepcidin and FPT1 expression in early postnatal rats, because the basal level of hepcidin expression is undetectable and intestinal iron absorption is high and probably immaturely regulated (3, 8). Subsequently, the possibility that iron mediates the effect of LPS was examined by altering the iron status with bolus iron feeding or by administration of the iron chelator pyrrolidinedithiocarbamate (PDTC). Finally, we determined whether His-tagged recombinant hepcidin (His-rHepc) would suppress intestinal FPT1 expression in rats. The present study supports the hypothesis that there is an inverse causal relationship between hepcidin and FPT1 expression and that this relationship is a complex one involving redox signaling mechanisms sensitive to PDTC.

MATERIALS AND METHODS

Materials. LPS (Salmonella abortus, cat L-5886), PDTC, and monoclonal anti-His antibody were purchased from Sigma (St. Louis, MO). Nitrocellulose and Nylon membranes and ECL Western blotting system were obtained from Amersham (Arlington Heights, IL). RT-PCR cloning kits were obtained from Stratagene (La Jolla, CA). B-Per bacterial protein extraction solution was obtained from Pierce (Rockford, IL). The pET30 vectors and S-Tag purification kits were purchased from Novagen (Madison, WI).

Animals. Sprague-Dawley rats from Charles River Breeding Laboratories (Wilmington, MA) were bred and raised in our animal quarters with a 12:12-h light-dark cycle. Rats were fed Harlan Teklad 22/5 rodent chows and water ad libitum. Litters were reduced to 10 at day 1, the second day after birth. Male rats were used for the present study unless otherwise indicated. Rats at 40–60 days of age were fasted overnight and then administered normal saline (as control, t = 0) or defined doses of LPS (0.25–2 μg/g body wt ip) and killed at indicated times after LPS by pentobarbital sodium injection. The rats designated to be killed at 24 h or later time intervals were allowed free access to chows. Heart blood was collected for assay of serum iron. The major hepatic lobe was removed, infused with ice-cold PBS to minimize circulating red blood cells, and used for analyses as described. The duodenum was quickly collected, rinsed with ice-cold PBS, and blotted dry, and the mucosa was scraped and used for analyses as described previously (44). For studies of developmental changes in LPS-stimulated hepcidin and FPT1 expression, rats (both male and female) were fasted overnight, administered LPS (1 μg/g body wt ip) at the age of days 6, 12, and 24, and killed 6 h later. In studies of the effect of the iron chelator, PDTC (100 μg/g body wt) was administered intraperitoneally 1 h before LPS. Belgrade rats, at 6 mo old, were used as an animal model of severe iron deficiency to determine whether the expression of hepcidin affected FPT1 expression. The Belgrade rats are defective in intestinal absorption as a result in a mutation in DMT1. The genotype for the DMT1 G185R mutation in Belgrade rats was assured by PCR using allele-specific primers. For studies of the effect of His-rHepc on hepatic FPT1 expression, days 19–20 rats were administered intraperitoneal saline (control), His-peptide (4 μg/g or 500 nmol/g), His-rHepc (8 μg/g or 500 nmol/g), or LPS (0.5 μg/g), and were killed 10 h later.

Northern blot analysis. Hepatic and duodenal mucosal RNA were size-fractionated by gel electrophoresis on a formaldehyde gel and vacuum transferred to a Nylon membrane. After RNA was blotted, it was ultraviolet (UV) cross-linked to the membrane with a Gene Linker UV Chamber (Bio-Rad, Hercules, CA). Hybridization was performed using 32P-labeled probes prepared from cDNA clones according to the standard method with high-stringency washing. The hybridization signals were detected by a PhosphorImager and quantitated with the ImageQuant software. For the quantitation of tissue FPT1 mRNA levels, the 2.5- and 4.5-kb bands were combined. Hepcidin, FPT1, hephaestin (Heph), and H-ferritin (H-f) cDNAs were labeled with [32P]dATP (New England Nuclear, Boston, MA) by the random primer labeling method. The cDNAs were isolated from rat ferritin H (H-f) (28), DMT1 (44), hepcidin, FPT1, and Heph cDNA clones. The complete coding sequences of rat hepcidin, FPT1, and Heph cDNA were cloned using standard RT-PCR cloning techniques with the pCR-Script SK(+) cloning kits (Stratagene). The primer pairs for hepcidin [5′ primer: 5′-GAGATATCCATGGCACCTAAGCACTC-3′ (underlined is a EcoRV site) and 3′ primer: 5′-CTTGGGATCCATAGTTATGCAACAG-3′ (underlined is a BamH1 site, and CTA in bold is the termination codon)], Heph [5′ primer: 5′-TGGAATTCATGAGCCAGGCCATC-3′ and 3′ primer: 5′-AGAATTCGGAAACTGGGATACTC-3′ (underlined are EcoRI sites)], and FPT1 [5′ primer: 5′-GAAGATATCTGAC-CAAGTCAGGATCATGAG-3′ and 3′ primer: 5′-GAAGATATTCA-CAACACAGGTTAGCTGAGAC-3′ (underlined are EcoRI sites)] were synthesized according to the sequence of rat hepcidin (GenBank accession no. NM-053469) (34), Heph (GenBank accession no. AF-246120) (15), and cell adhesion regulator 1 (GenBank accession no. U76714), respectively. The clones were sequence verified, and the complete coding sequence of FPT1 cDNA is listed in GenBank database (GenBank accession no. AF-394785).

Preparation of His-rHepc. The full-length rat hepcidin cDNA lacking the 5′-end coding sequence of the first six amino acids (upstream of the unique BamH1 site) was isolated after restriction digestion of the hepcidin cDNA clone with BamH1 and was inserted into the BamH1 site of pET30 expression plasmid (Novagen). The constructed pET30-Hepc plasmid was verified by sequencing to have an in-frame insertion of the hepcidin cDNA with the termination codon. The His-tagged peptide (His-peptide) and His-rHepc were expressed in Escherichia coli strain BL21(DE3) RIL transfected with pET30 and pET30-Hepc plasmid, respectively, and purified using Ni-NTA agarose beads according to the manufacturer’s instructions. Purified His-peptide and His-rHepc were dialyzed against Tris-buffered saline (pH 7.4) and subjected to Western blot analysis using either monoclonal anti-His antibody (Sigma) or polyclonal anti-His-rHepc antibodies produced by rabbits after immunization with His-rHepc. The purified His-peptide and His-rHepc exhibited a single peptide band with apparent molecular masses of 7.5 and 15 kDa, respectively.

Determination of serum iron concentrations. Serum iron concentrations were determined by a rapid colorimetric micromethod (12). Briefly, 200 μl of serum and a series of standards were mixed with the same volume of freshly prepared iron-releasing reagent (145 mM KMnO4 in 0.6 N HCl) and incubated at 60°C for 2 h. After incubation, the solution was mixed with 20 μl reducing and iron-chelating reagent (6.5 mM Ferrozine, 13 mM neocuproine, 2 M ascorbic acid, and 5 M ammonium acetate), allowed to stand at room temperature for 30 min for the formation of ferrous Ferrozine, and read at 562 nm.

Statistical Analysis. GraphPad Prism 4 software was used to determine the ANOVA from the experimental data for all different time points after the administration of LPS and for animal groups administered either LPS and/or PDTC. When the F-value obtained from ANOVA was significant, Bonferroni posttests were applied to deter-
mine differences between sample groups. *P* values <0.05 were considered significant.

**RESULTS**

Changes in hepatic hepcidin and FPT1 and intestinal FPT1 expression after LPS administration. LPS, at the dose of 1 μg/g body wt, induced a rapid increase in hepatic hepcidin mRNA expression. Before LPS, there was a low basal level of hepcidin expression in the liver. By 1 h after LPS, hepcidin expression increased markedly. The increase continued with a peak of expression at 6 h, and then the expression subsided (Fig. 1, A and B). An expansion of the study to 48 h showed that there was a second increase of hepcidin mRNA expression at 36 h after LPS administration but with a level of expression ~50% lower than that at 6 h (Figs. 1 and 2). Hepatic FPT1 mRNA also showed biphasic changes in expression, with a decrease to low levels at 6–8 h followed by a rise with increased expression at 24 and 48 h (Figs. 1 and 2). The similarity of biphasic temporal changes in hepatic hepcidin and FPT1 mRNA levels suggest either a close causal relationship or the same temporal response to LPS for both genes. FPT1 expression in the intestine showed a different pattern following LPS administration. LPS induced significant reduction of intestinal FPT1 mRNA at 6 h. The reduction was sustained at 24 h, at which time hepatic FPT1 had recovered (Figs. 1 and 2). By 36 and 48 h, intestinal FPT1 had recovered and increased to higher than pre-LPS levels (Figs. 1 and 2). The LPS-induced reduction was specific for FPT1, because the expression of Heph mRNA was increased in the intestine (Fig. 1A) and H-Ft mRNA showed no significant change either in the liver (Fig. 2) or intestine (data not shown). A dose-response curve (Fig. 3) demonstrated similar behavior of hepcidin and FPT1 at doses of LPS as low as 0.25 μg/g body wt.

Because intestinal iron absorption is highly efficient in suckling rats (3) and the regulation of iron absorption in the immature rat has not been defined, it seemed important to examine whether the inverse response of hepcidin and FPT1 also occurs in suckling rats. Indeed, hepatic hepcidin showed a developmental increase of susceptibility to LPS, with the expression of hepatic hepcidin exhibiting an age-dependent increase after LPS stimulation (Fig. 4). Although only a low level of hepcidin stimulation was seen at postnatal day 6, the earliest postnatal stage examined, the LPS-induced increase of hepcidin expression was accompanied by a decrease of FPT1 mRNA expression in both the liver and intestine (Fig. 4).

![Northern blot analysis of ferroportin 1 (FPT1), hepcidin (Hepc), and hephaestin (Heph) expression in the duodenum and liver of rats after LPS administration. A and B: rats were administered LPS (1 μg/g ip), then killed at the indicated times. Total RNA (20 μg) isolated from the duodenum and liver was size fractionated by gel electrophoresis on formaldehyde gels, vacuum transferred to a Nylon membrane, and hybridized sequentially with 32P-labeled cDNA probes for Heph, FPT1, and Hepc as described in MATERIALS AND METHODS. The figures shown are representative of 6 experiments. C-E: quantitation of hepatic Heph and FPT1 mRNA (C and D, respectively) and duodenal FPT1 mRNA (E) levels. The volume density of mRNA hybridization signals from Northern blots as shown in A was detected by a PhosphorImager, quantitated with the ImageQuant software and normalized to the 28s rRNA in each sample. Data shown are the means of %control ± SE (n = ~4–6 samples/time point). Significant (*P < 0.05) difference vs. the previous column.](http://ajpgi.physiology.org/)

![Fig. 1. Northern blot analysis of ferroportin 1 (FPT1), hepcidin (Hepc), and hephaestin (Heph) expression in the duodenum and liver of rats after LPS administration. A and B: rats were administered LPS (1 μg/g ip), then killed at the indicated times. Total RNA (20 μg) isolated from the duodenum and liver was size fractionated by gel electrophoresis on formaldehyde gels, vacuum transferred to a Nylon membrane, and hybridized sequentially with 32P-labeled cDNA probes for Heph, FPT1, and Hepc as described in MATERIALS AND METHODS. The figures shown are representative of 6 experiments. C-E: quantitation of hepatic Heph and FPT1 mRNA (C and D, respectively) and duodenal FPT1 mRNA (E) levels. The volume density of mRNA hybridization signals from Northern blots as shown in A was detected by a PhosphorImager, quantitated with the ImageQuant software and normalized to the 28s rRNA in each sample. Data shown are the means of %control ± SE (n = ~4–6 samples/time point). Significant (*P < 0.05) difference vs. the previous column.](http://ajpgi.physiology.org/)
We next examined whether changes in tissue iron distribution might mediate LPS-induced hepatic hepcidin and hepatic and intestinal FPT1 expression. After LPS administration, there was a steady decrease of serum iron concentration to 50 and ~30% of the control level at 6 and 12–24 h, respectively (Fig. 5). However, the prolonged time-dependent decrease in the serum iron concentration during the first 24 h after LPS did not correlate with the dynamic changes of hepcidin and FPT1 mRNA expression, which in the liver occurred rapidly and then recovered by 24 h and which in the intestine FPT1 decreased rapidly at 6 h and remained low at 24 h. To address the issue of the effect of body iron stores on the expression of hepcidin and FPT1 in rats, we used the Belgrade rats as a model of iron deficiency. Belgrade rats have a G185R mutation in DMT1, which in the homozygous state (bb) results in decreased intestinal iron uptake. As a result the bb rats have low hepatic iron stores and iron-deficiency anemia (13, 44). In the bb rats, hepatic hepcidin mRNA was undetectable compared with hepcidin expression in heterozygote (+/b) and wild-type (+/+) rats (Fig. 6). The undetectable levels of hepcidin mRNA in the bb rats were accompanied by an increase of intestinal FPT1, supporting an inverse relationship of hepatic hepcidin and intestinal FPT1. Interestingly, intestinal Heph expression was upregulated in bb rats. However, in the bb rats, the lack of hepcidin did not lead to an increase in hepatic FPT1 expression. The tissue-specific increase of FPT1 expression in the intestine of bb rats suggests a complex relationship in which local iron stores may be important in dictating gene expression.

Effect of PDTC on the expression of hepatic and intestinal FPT1 expression. To investigate further the possibility that iron mediates LPS-induced changes in hepcidin and FPT1 expression and the inverse relationship between hepcidin and intestinal FPT1 expression, we depleted cellular iron with the iron chelator PDTC before LPS administration. In the liver, PDTC induced a significant increase of hepcidin, but the increase of hepcidin did not lead to a decrease of FPT1 expression in either the liver or intestine (Fig. 8). Administration of LPS to PDTC-pretreated rats resulted in no further increase of hepatic hepcidin mRNA levels, and the decrease of hepatic FPT1 was similar to that seen with LPS alone. However, PDTC did perturb the response of intestinal FPT1 expression to LPS with the observation of significantly increased

![Fig. 2. Northern blot analysis of hepatic FPT1, H-ferritin (H-Ft), and hepatic and duodenal FPT1 mRNA levels. A: rats were administered LPS (1 μg/g ip) then killed at the indicated times from 0 to 48 h. Total RNA was used for analysis as described in Fig. 1 except that the membrane containing hepatic total RNA was hybridized with a mixture of 32P-labeled cDNA probes for hepcidin, FPT1, and H-Ft. B-D: quantitation of hepatic Hepc (B), FPT1 (C), and duodenal FPT1 (D) mRNAs. The hybridization signals from Northern blots as shown in Fig. 1A were detected by a PhosphorImager, quantitated with the ImageQuant software, and were normalized to the 28s rRNA in each sample. Data shown are the means of %control ± SE (n = 4). Significant (*P < 0.05) differences compared with the previous column.](http://ajpgi.physiology.org/).
rather than decreased FPT1 levels (Fig. 8). These results suggest that the effects of LPS on hepcidin and FPT1 expression are complex and might be modulated through tissue-specific cellular iron compartmentalization and changes in redox status.

Effect of His-rHepc on FPT1 expression. The effect of LPS to increase hepcidin expression in the liver with a lag in the decrease of intestinal FPT1 expression provides circumstantial evidence that in the rat, hepcidin suppresses FPT1 expression. To test this possibility directly, His-rHepc was prepared (Fig. 9) and intraperitoneally administered to rats to determine whether His-rHepc reduces intestinal FPT1 expression. Preliminary studies detected marginal decreases of intestinal FPT1 expression in rats administered 4 μg His-rHepc/g (250 nmol/g) but not with 1 and 2 μg/g His-rHepc or with 4 μg/g His-peptide/g (500 nmol/g). However, increasing the dose of His-rHepc to 8 μg/g (500 nmol/g) resulted in significant reduction of duodenal FPT1 expression compared with saline or His-peptide-treated controls (Fig. 10). The intraperitoneally administered His-rHepc did not suppress hepatic FPT1 expression, a result that differs from the significant reduction of hepatic FPT1 mRNA expression after LPS administration (Fig. 10, A and C).

DISCUSSION

Recent evidence suggests that hepcidin is the iron stores regulator: under conditions of low “iron stores,” hepcidin is expressed at low levels to increase intestinal iron absorption; under conditions of “high iron” stores, the converse occurs with increased hepcidin expression leading to decreased intestinal iron absorption (30, 31, 34). The mechanism by which hepcidin exerts its effect on intestinal iron absorption remains to be defined. Intestinal iron absorption is a complex process requiring transport of iron across the epithelium from the site of absorption at the brush-border membrane (BBM) to the site of exit at the basolateral membrane (BLM). Hepcidin may regulate iron absorption by modulating the expression of any one of the molecules involved in the intestinal iron absorption. Potential targets for hepcidin are DMT1, the iron transporter in
the BBM, and FPT1, the iron transporter in the BLM (1, 10, 14, 20, 26). Although DMT1 mRNA levels are unchanged in USF2 knockout mice (30), changes in DMT1, FPT1, and the ferric reductase DcytB expression have been noted to occur with changes in hepcidin activity (2, 17), suggesting that these proteins critical to intestinal iron absorption may be regulated by hepcidin. The present study was undertaken to test the possibility of FPT1 as a regulatory target of hepcidin using a rat model with various experimental treatments.

The presented data demonstrated that LPS induced biphasic changes in hepatic expression of hepcidin. Concurrent with the first rise in hepcidin, hepatic FPT1 mRNA levels decreased. These inverse changes also occurred in rats treated with lower

Fig. 4. Northern analysis of hepatic FPT1 and hepcidin and duodenal FPT1 mRNA levels in LPS-treated rats at different postnatal ages. A: rat littermates at the indicated ages were administered LPS (1 μg/g ip), then killed 6 h later. Hepatic and duodenal total RNA was isolated and used for Northern blots as described in Fig. 1A. B–D: quantitation of hepatic Hepc and FPT1 and of duodenal FPT1 mRNAs in days 6 (B), 12 (C), and 24 (D) rats. Hybridization signals from Northern blots were detected by a PhosphorImager, quantitated with the ImageQuant software, normalized to the 28s rRNA in each sample, and expressed as arbitrary units. Shown are the means ± SE (n = 3). Significant (*P < 0.05) difference vs. the previous column.

Fig. 5. Effect of LPS on serum iron concentration. Rats were administered LPS (1 μg/g ip) then killed at the indicated times. The serum iron concentration was measured as described in the MATERIALS AND METHODS. LPS induced a steady decrease of serum iron concentration to 50 and 30% of the control level at 6 and 12 h. Data are the means ± SE (n = 4–7). Significant (*P < 0.05) difference vs. the previous column.

Fig. 6. Northern blots of hepatic hepcidin (Hepc) and FPT1 and duodenal FPT1 and hephaestin mRNAs in Belgrade rats. Hepatic and intestinal mucosa RNA was isolated from Belgrade rats (6 mo old), which were either homozygous for the G185R DMT1 mutation (b/b), heterozygous (+/b), or wild-type (++/+). Northern blots were performed as described in Fig. 1A. In the b/b rats, hepatic hepcidin mRNA was undetectable but was present in ++/+ and +/+ animals. Hepatic FPT1 was expressed at similar levels in all 3 categories of animals. The duodenum of b/b rats expressed higher levels of FPT1 and hephaestin mRNAs than those of +/+ and +/+ rats. Figures shown are representative Northern blots from 3 sets of experiments using 5 b/b, 6 +/+b, and 4 ++/+ rats.
doses of LPS and in rats at different postnatal ages, consistent with a causal relationship between hepcidin and FPT1. It is possible that the observed reduction of FPT1 expression might result not from the action of hepcidin but from direct stimulation by LPS through Toll-like receptor 4 (TLR4)-dependent signaling processes (42). This possibility is based on the observation that in TLR4 knockout mice, LPS did not reduce FPT1 expression in reticuloendothelial cells (42); however, these results could not exclude hepcidin as the effector, because the TLR knockout mice would likely also fail to activate hepcidin in response to LPS. The issue could be addressed by examining the effects of LPS on FPT1 expression in the USF2 knockout mice.

In the intestine, we also observed a reduction of FPT1 mRNA, delayed in relationship to the hepatic changes. The delayed decrease of FPT1 might reflect the time for diffusion and transport of sufficient hepatic hepcidin to the intestinal epithelium. The reasons for the subsequent increases in hepatic and intestinal FPT1 are not clear. Events observed in the intestine at 36–48 h may reflect changes in gene expression from feedback reprogramming of crypt cells in response to LPS- and/or hepcidin-induced reduction of FPT1 in the villus. The reprogramming would result in new cells in the villus committed to an elevated FPT1 expression. In the liver, there is cross-talk between hepatocytes that express hepcidin and Kupffer cells that express FPT1. Hence, altered expression may reflect a variety of changes wrought by the initial signal from LPS (18). The temporal differences in the decrease of

**Fig. 7.** Effects of dietary iron ingestion on duodenal FPT1 and H-Ft mRNA and H-Ft protein analyzed by Northern blots and Ft immunoprecipitates. Rats were fed a bolus of iron-fortified chow, killed at 0, 2, 4, and 6 h, and the duodenal mucosal RNA was isolated and analyzed by Northern blots. Mucosal Ft protein was immunoprecipitated followed by gradient SDS-PAGE to separate H- and L-Ft subunits and visualized by Coomassie blue staining as previously described (43). Figures shown are representatives from 3 sets of experiments.

**Fig. 8.** Effects of pyrrolidinedithiocarbamate (PDTC) and/or LPS on hepatic hepcidin and FPT1 and intestinal FPT1 and H-Ft mRNA expression. A: Northern analysis of hepcidin, FPT1 and H-Ft expression. Adult Rats were separated into 4 groups (4 rats/group) and administered saline, PDTC (100 μg/g ip), LPS (1 μg/g ip), and PDTC + LPS, respectively. Rats were killed 6 h later, and the liver and duodenum were collected for total RNA isolation. RNA samples from duplicate rats were analyzed by Northern blots as described in Fig. 1. Figures shown are representative Northern blots from 2 sets of duplicate rats. B–D: quantitation of hepatic Hepc (B), FPT1 (C), and duodenal FPT1 (D) mRNA levels. The hybridization signals from Northern blots as shown in A were detected by a PhosphorImager, quantitated with the ImageQuant software, and were normalized to the 28S rRNA in each sample. Shown are the means ± SE (n = 4). Significant (*P < 0.05) difference compared with the previous column.
FPT1 expression may reflect the differences of hepcidin-signaling pathways in the liver and the intestine, the former by an auto- and/or paracrine signaling pathway and the latter by an endocrine signaling pathway. The reduction of FPT1 expression occurring in the first phase of response in both tissues is consistent with the hypothesis that FPT1 is the regulatory target of hepcidin. The presence of different signaling pathways is supported by our preliminary data that His-rHepc reduced FPT1 mRNA expression in the intestine but had no effect on FPT1 expression in the liver. The reason for the lack of effect in the liver may be related to the dose of rHepc being too low to mimic the auto- or paracrine pathway of regulation.

It was necessary to consider that the observed changes were the result of LPS affecting cellular iron metabolism directly or through cytokine stimulation. It is well documented, which the present study supports, that LPS induces a rapid reduction of iron in the systemic circulation, which persists at a low level for 24 h (7, 35, 42). LPS is a potent stimulator of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, capable of stimulating transferrin receptor-dependent iron uptake from the blood with sequestration of the iron in ferritin in RES cells and hepatocytes (7, 24, 35, 36, 40, 42). LPS and other proinflammatory cytokines may stimulate enterocyte iron uptake with intracellular iron retention in enterocytes (7). Therefore, iron...
and/or proinflammatory cytokines might play a role in the LPS-induced changes observed in this study. TNF-α can be excluded as an effector because LPS will decrease FPT1 in reticuloendothelial cells in mice lacking TNF-α (42). After LPS administration, cytokines including IL-1 and IL-6 transiently increase within 5–8 h (19, 37). This pattern of response might be related to the first but not the second phase of changes of hepcidin and FPT1 expression. In primary human hepatocyte culture, both IL-6 and LPS (in the presence of serum) but not iron stimulate hepcidin expression (29), indicating that LPS can activate hepcidin independent of IL-6. A direct effect of IL-6 on suppression of FPT1 expression could not be excluded in the present study without using an IL-6 knockout animal model. The lack of a direct effect of iron on hepatocyte hepcidin expression is also supported by the present data, because the reduction of serum iron concentration continued for 24 h after LPS, whereas the changes of expression of hepatic hepcidin and FPT1 had recovered by a 12-h period. The lack of changes in intestinal FPT1 mRNA expression in mice and rats after either parenteral iron loading or a large oral dose of iron has been reported previously (16, 26).

Interestingly, PDTC increased intestinal FPT1 expression in rats challenged with LPS, although hepcidin expression was increased. PDTC is an iron chelator, which can exert either prooxidant or antioxidant effects in a cell type-specific manner (6, 11, 22, 27, 38). The observation that PDTC or LPS alone activated hepatic hepcidin expression and together exhibited no additive effects suggests that PDTC and LPS share the same pathway to affect hepcidin expression. PDTC and LPS have been noted to be antagonistic, the former inhibiting and the latter activating NF-κB (38, 41). Our laboratory has also observed that PDTC blocks LPS-induced NF-κB activation in the liver (data not shown), suggesting that the activation of hepcidin is not through the NF-κB pathway. The signaling pathways that are activated by both PDTC and LPS are activator protein 1 (AP-1) and CCAAT/enhancer binding protein (C/EBP) (5, 21, 23). In mice and humans, C/EBPα has been reported to be a key regulator of the promoter activity of the hepcidin gene (8). In the rat, multiple C/EBP and AP-1/CREB sites are present in the 5′-flanking region of hepcidin, and the overlapping C/EBP and AP-1/CREB sites at −70 to −92 nucleotides from the transcription initiation site confer high levels of transcriptional activity (GenBank accession no. AY230877) (45). The precise role of C/EBP and AP-1 sites in the activation of hepcidin after PDTC and/or LPS administration in vivo remains to be defined.

The PDTC-induced hepatic hepcidin expression was not accompanied by a reduction of FPT1 in either the liver or the intestine. The reason for this dissociation of the inverse relationship between hepcidin and intestinal FPT1 is unknown. Hepcidin is synthesized in cytoplasm, transported into the nucleus of hepatocytes, and presumably secreted as the mature peptides containing COOH-terminal 25 and 20 amino acids (33, 34). PDTC may interfere with the posttranslational processing of hepcidin and prevent hepcidin from exerting both its autocrine effect in the liver and endocrine effect on the intestine to modulate FPT1 expression. Another possibility is that hepcidin modulation of FPT1 expression is iron and/or redox dependent. PDTC might sequester iron in the labile iron pool or exert its antioxidant effect to a threshold that allows activation of the hepcidin gene but not hepcidin-mediated inhibition of FPT1 expression. Hepcidin expression has recently been reported to be sensitive to hypoxia, turpentine and Freund’s complete adjuvant (2, 32). The induction of hepcidin expression by Freund’s complete adjuvant was not accompanied by the reduction of intestinal FPT1 expression (2). Hence, local factors may control the effects of hepcidin.

The inverse relationship between hepatic hepcidin and intestinal FPT1 expression was also observed in the Belgrade rat model. In the b/b rats, homozygous for the G185R mutation in DMT1 and therefore with chronic iron deficiency (13, 14), hepatic hepcidin mRNA was undetectable and FPT1 mRNA was markedly elevated compared with the +/b and +/- rats. Interestingly, intestinal Heph expression was increased in b/b rats in parallel to the increase of FPT1. This relationship is consistent with Heph being part of the regulated iron export mechanism for the increase of intestinal iron absorption with iron deficiency. However, because LPS increased intestinal expression of Heph, then presumably Heph is not a target of hepcidin regulation.

Our preliminary data with His-rHepc suggests that hepcidin directly affects intestinal FPT1 expression. Although we noted a response at the higher levels of administered recombinant protein, the results need to be interpreted with caution, because neither the physiological levels nor the active form(s) of hepcidin are known. In addition, we examined response only at one time point, and the kinetics of response in physiological situations are also not yet defined. Hepcidin is synthesized as an 84-amino acid propeptide with processed forms consisting of COOH-terminal 25- and 20-amino acid residues found in human serum and urine (33). We do not know whether the 126-amino acid His-rHepc recombinant protein is active or whether the His-rHepc protein requires additional processing to modulate intestinal FPT1 expression. In addition, we did not observe an effect of His-rHepc on hepatic FPT1 expression. This lack of an effect may reflect the inability of the recombinant protein to gain access to the hepatocyte or too low a concentration of the administrated protein. Regardless, our preliminary data with His-rHepc are consistent with the evidence that hepcidin regulates intestinal iron absorption as is well documented in mice and humans. Further studies are needed to define the molecular mechanisms by which hepcidin modulates intestinal FPT1 expression.

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REFERENCES

HEPCIDIN SIGNALING MODULATES FERROPORTIN 1 EXPRESSION


