Hepcidin and hemoujuvelin gene expression in rat liver damage: in vivo and in vitro studies

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Iron is vital for almost all living organisms because it participates in a wide variety of metabolic processes, including oxygen transport, DNA synthesis, electron transport, and mitochondrial function (27, 33). It is also an essential element required for growth and survival. The metal serves as a cofactor for many heme and nonheme iron proteins (5, 17). Iron balance is maintained by the tight regulation of absorption from the intestine. The intestinal iron absorption is modulated in response to the level of body iron stores and by the amount of iron needed for erythropoiesis (11). However, excess free iron is toxic for the cell because mammals lack a regulated pathway for iron excretion.

Hepcidin previously reported as liver-expressed antimicrobial peptide (22) is a recently discovered, circulating antimicrobial peptide mainly synthesized by hepatocytes in the liver. It regulates intestinal iron absorption as well as maternal fetal iron transport across the placenta (28). It affects the release of iron from hepatic stores and from macrophages involved in the recycling of iron from hemo-

globin (13, 29). This 25-amino acid, 2- to 3-kDa, cationic peptide (30) is an acute-phase protein. Its production is increased during inflammation and in iron-overload conditions (2). It is a major regulator of iron balance in the intestinal mucosa, which seems to have a significant role during inflammation, and it is a major contributor to the hypoferremia associated with inflammation (31).

Recently, several other genes involved in iron homeostasis have been cloned and characterized, including ferroportin 1 (Fpn-1), transferrin receptor 2 (TfR2), and hemoujuvelin (Hjv). Patients with pathogenic Hjv mutations as well as animals with such mutations produce low levels of hepcidin and subsequently develop hemochromatosis (18, 38). In vitro studies have suggested a stimulatory effect of cellular Hjv on hepcidin synthesis in hepatocytes (38). The regulation of Hjv gene expression is still unknown. Hepcidin and Hjv genes behave differently during inflammation, and it has been suggested that in humans, Hjv gene expression could be modulated by inflammation (23). Because the liver is considered a central organ for iron metabolism regulation (5, 14), it is of importance to evaluate the changes of the hepatic pathway of iron metabolism when the liver itself is the target of injuring noxae.

In the present study, we showed the changes of hepcidin and Hjv gene expression in the liver in two different models of liver injury [partial hepatectomy (PH) and CCl4 administration]. Furthermore, we investigated the regulation of the main proteins of iron metabolism induced in isolated hepatocytes by different cytokines whose gene expression was also analyzed in damaged livers. In one model (PH), IL-6 could be the main mediator of the changes observed. In the other model (CCl4 administration), mediators other than IL-6 could also be involved. We also showed that the same mediators may also regulate the expression of the intrinsic iron transport protein Fpn-1.

MATERIALS AND METHODS

Animals

Male Wistar rats of 170–200 g body weight were purchased from Harlan-Winkelmann (Brochen, Germany). The rats were kept under standard conditions with 12:12-h light-dark cycles, and they had ad libitum access to fresh water and food pellets. All animals were taken care of according to the institutional guidelines, the German convention for the protection of animals, and National Institutes of Health guidelines.

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Materials

All the chemicals used were of analytical grade and were purchased from commercial sources as described previously (4, 16, 36, 45).

Animal Models

For the present study, two different models of liver injury were used with a potential of regeneration after injury: PH and CCl4-induced liver injury.

PH. PH was performed under ether anesthesia by midventral laparotomy, ligation of the median anterior and left lateral hepatic lobes separately with a silk suture, and complete excision of ligated lobes. Control animals were subjected to sham operation (SO) by the same extent and gentle manipulation of the liver, followed by surgical closure of the abdomen similar to PH rats. Rats (n = 4) were killed 2, 4, 8, 16, 24, and 48 h after PH. Livers were snap frozen in liquid nitrogen and stored at −80°C.

CCl4-induced liver injury. Rats were orally administered 3 ml/kg (body wt) of a CCl4-corn oil mixture (1:1) by means of gastric tubes several hours after administration of a CCl4-corn oil mixture (1:1) by means of gastric tubes. Treated and control animals (n = 3) were killed 2, 4, 8, 16, 24, and 48 h after PH. Livers were snap frozen in liquid nitrogen and stored at −80°C.

Cell Culture Studies

Hepatocytes were isolated by a two-step enzymatic dissociation from the liver of male Wistar rats according to Seglen (44) or as described elsewhere (36, 40). Control animals were subjected to sham operation (SO) by the same extent and gentle manipulation of the liver, followed by surgical closure of the abdomen similar to PH rats. Rats (n = 4) were killed 3, 6, 12, 24, and 48 h after CCl4 administration. The livers from the animals were taken out, washed with saline, snap frozen in liquid nitrogen, and stored at −80°C until further use. Blood samples were collected from the inferior vena cava of the control and treated animals, allowed to clot overnight at 4°C, and centrifuged for 20 min at 2,000 g. Serum was removed and stored at −20°C.

RNA Isolation

Total RNA was isolated from different liver samples by means of guanidine isothiocyanate extraction, cesium chloride density gradient ultracentrifugation, and ethanol precipitation according to the method previously described (6) with some modifications as described elsewhere (40). The RNA obtained was quantified by measuring the absorbance at 260/280 nm. Total RNA from cultured hepatocytes was isolated using the Nucleospin II RNA isolation kit with DNase treatment (19).

Table 1. Primer sequences used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepcidin</td>
<td>5'-GAAGGCAAGATGGCACTAGCA-3'</td>
<td>5'-TCCTGCTCTGTTGCGGCGAGATAG-3'</td>
</tr>
<tr>
<td>TIR1</td>
<td>5'-ATAAGTGGGTTGGTGGAG-3'</td>
<td>5'-GGCGGAGACTGAGATGTTG-3'</td>
</tr>
<tr>
<td>Ferritin-H</td>
<td>5'-GCCCTGAAGAATCTTGGCAAAT-3'</td>
<td>5'-TCGAGGAAGATCTTGGCAATCT-3'</td>
</tr>
<tr>
<td>Fpn-1</td>
<td>5'-TCTGGACATTTTGCGAGATG-3'</td>
<td>5'-TACAGTGGAAAGCGGACTGTT-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-GTCAGCTTGGATGGCGGACTG-3'</td>
<td>5'-GCGCGGAGACTGCAACACGT-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-TACATCTGCTGGCGGAGATG-3'</td>
<td>5'-CTCCTGCTGATTGCGGAAATG-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-ACAGGTAAGGGCCGACTGAT-3'</td>
<td>5'-TTCGAGGAGGAGGAGGAGG-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-AGTCGCAAGAACTTCTTTAATTCTGACATG-3'</td>
<td>5'-TTCGAGGAGGAGGAGGAGG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TCTGGACATTTTGCGAGATG-3'</td>
<td>5'-TTCGAGGAGGAGGAGGAGG-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-TTCTGAGGAGGAGGAGGAGG-3'</td>
<td>5'-AAACAGGGCTGATGGCTAC-3'</td>
</tr>
</tbody>
</table>

TIR1, transferrin 1; FPN-1, ferroportin 1.
respectively, \(P < 0.05\)). \(Hjv\) gene expression was significantly downregulated at the same time to a maximum of 0.2 \(\pm\) 0.08-fold after the PH \(\(P < 0.05\); Fig. 1A\). A time-dependent significant downregulation of \(ferritin\ H\) and \(Fpn-1\) gene expression \(0.45 \pm 0.09-\text{fold}\) and \(0.5 \pm 0.01\)-fold, respectively, \(P < 0.05\) \(16\ h\) after PH was also observed (Fig. 1B).

In the case of cytokine gene expression, a strong and statistically significant upregulation of \(IL-6\) gene expression was observed \(15 \pm 0.84\)-fold at \(8\ h\), \(P < 0.05\) after the PH. A weak but significant upregulation of \(IL-1\beta\) gene expression \(2.06 \pm 0.36\)-fold at \(8\ h\), \(P < 0.05\) was found; on the other hand, \(IFN-\gamma\) gene expression was not significantly upregulated \(1.88 \pm 1.23\)-fold at \(8\ h\), \(P < 0.05\) compared with control values. \(TNF-\alpha\) gene expression \(1.9 \pm 0.39\)-fold was delayed \(24\ h\) after PH, although these changes were not significant (Fig. 1C).

Fig. 1. Real-time PCR analysis of rat liver RNA. Livers were obtained at different times after operation [partial hepatectomy (PH)]. Shown are fold changes of hepcidin (\(Hepc\)), transferrin (\(TfR1\)), and hemojuelin (\(Hjv\); A); \(ferritin\ H\) and \(ferroportin\ 1\) (\(Fpn-1\); B); and \(IL-6\), \(IL-1\beta\), \(TNF-\alpha\), and \(IFN-\gamma\) (C) gene expression after PH liver injury. Values represent the amount of target mRNA compared with GAPDH mRNA \(\(*P < 0.05\).\) Error bars represent SE \(\(n = 3\).\)

Fig. 2. Real-time PCR analysis of rat liver RNA. Livers were obtained at different times after intragastral administration of \(CCl_4\). Shown are fold changes of \(Hepc\), \(TfR1\), and \(Hjv\) (A); \(ferritin\ H\) and \(Fpn-1\) (B), and \(IL-6\), \(IL-1\beta\), \(TNF-\alpha\), and \(IFN-\gamma\) (C) gene expression after liver injury. Values represent the amount of target mRNA compared with GAPDH mRNA \(\(*P < 0.05\).\) Error bars represent SE \(\(n = 4\).\)
Differential gene expression was also studied in SO rats and was found to be slightly changed compared with control values (data not shown).

**CCl4-induced liver injury.** Oral administration of CCl4 to the rats induced strong and statistically significant upregulation of hepcidin and TfR1 gene expression (4.3 ± 0.73- and 9 ± 2-fold after 12 and 3 h, respectively, \( P < 0.05 \)) after CCl4 administration (Fig. 2A). Hjv and Fpn-1 gene expression was sharply and significantly downregulated (0.142 ± 0.08- and 0.5 ± 0.18-fold 12 and 6 h, respectively, \( P < 0.05 \)) after the injury.

Ferritin H gene expression was significantly upregulated (1.33 ± 0.15-fold) 12 h after the liver injury (Fig. 2, A and B). To study the role of specific acute-phase cytokines mediating the inflammation and hepcidin gene expression, we studied the expression of IL-6, IL-1β, TNF-α, and IFN-γ at different times after the liver injury. We found that CCl4 local damage, and a strong and statistically significant upregulation of IL-6 gene expression was observed 6 h after CCl4 administration, reaching a maximum (42 ± 18.1-fold, \( P < 0.05 \)) 12 h after the injury. Gene expression of other acute-phase cytokines such as IL-1β, TNF-α, and IFN-γ also attained the significant peak 12 h after the onset of the injury (6 ± 0.28-, 17 ± 2.22-, and 10 ± 0.378-fold, respectively, \( P < 0.05 \); Fig. 2C).

**Northern blot analysis (in vivo).** Real-time PCR results were confirmed by Northern blot analysis for hepcidin and Hjv mRNA. Our RT-PCR results were supported by Northern blot analysis in PH-induced liver injury. The mRNA level of hepcidin was increased to the maximum between 8 and 16 h after PH, which was in agreement with our quantitative real-time PCR results (Fig. 3A).

Hepcidin mRNA levels were found to rise early and reach a maximum at 12 h after the CCl4 administration, and Hjv gene

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**Fig. 3.** Northern blot analysis of Hepc and Hjv mRNA levels in the livers of PH and CCl4-treated animals. Livers were taken at different times (MATERIALS AND METHODS). A time-course increase of hepcidin gene expression is observed with a maximum expression between 8 and 16 h in PH (A; 1 of the experiments used for real-time PCR analysis) and 12 h in CCl4-induced liver injury (B). Filters of hepcidin and Hjv were exposed to the autoradiographic film for 5 days at −80°C before being developed. Note that the intensity of the signal does not mean difference of RNA amount because the probe used for the Hjv hybridization was 4 times longer than the hepcidin probe, and, for this reason, it better hybridized.

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**Fig. 4.** Real-time PCR analysis of rat hepatocyte RNA (in vitro). Rat hepatocytes were treated with different doses of different cytokines for 3 h. Experiment was started 24 h after cells were plated. Fold changes of Hepc (A), TIR1 (B), Hjv (C), ferritin H (D), and Fpn-1 (E) are shown. Values represent the amount of target mRNA compared with β-actin mRNA (* \( P < 0.05 \)). Error bars represent SE (\( n = 3 \)).
expression was downregulated at the same times (Fig. 3B). An oligonucleotide complementary to 28S rRNA was used to ensure equal loading of the RNA.

Changes of Specific mRNA of Iron Pathway Proteins In Vitro

Cell culture studies. Real-time analysis revealed dose-dependent statistically significant upregulation of hepcidin and ferritin H gene expression in hepatocytes stimulated with IL-6 (4.33 ± 0.87- and 1.38 ± 0.01-fold, respectively, P < 0.05) at 100 ng/ml. TIR1, Hjv, and Fpn-1 gene expression was significantly downregulated at 100-ng/ml (0.7 ± 0.05-fold)- and 500-ng/ml doses (0.38 ± 0.03- and 0.8 ± 0.03-fold, respectively, P < 0.05; Fig. 4, A-E).

Hepatocytes stimulated with IL-1β have shown a minor statistically nonsignificant upregulation of hepcidin gene expression (1.52 ± 0.55 fold, P > 0.05) at the 1,000-ng/ml dose. TIR1 gene expression was significantly upregulated (2 ± 0.2-fold, P < 0.05) at a dose of 500 ng/ml. However, Hjv and ferritin H gene expression was significantly downregulated (0.5 ± 0.005- and 0.78 ± 0.05-fold, respectively, P < 0.05), whereas the downregulation of Fpn-1 gene expression was not significant (0.6 ± 0.2-fold, P > 0.05), at a dose of 500 ng/ml (Fig. 4, A-E).

Hepatocytes stimulated with TNF-α have shown no significant changes in hepcidin, ferritin H, and Hjv gene expression. Fpn-1 and TIR1 gene expression was significantly downregulated (0.6 ± 0.02- and 0.4 ± 0.02-fold, P < 0.05) at a dose of 500 ng/ml, respectively (Fig. 4, A-E).

Hepatocytes treated with IFN-γ have shown no significant changes of hepcidin and Hjv gene expression. IFN-γ was not so potent as to induce the changes in the gene expression of other iron-regulatory proteins such as ferritin H, Fpn-1, and TIR1 (Fig. 5). The changes observed using other cytokines were not modified by adding IFN-γ (data not shown).

Northern blot analysis (in vitro). In vitro studies with hepatocytes stimulated with IL-6 showed dose-dependent upregulation of hepcidin to a maximum level at a dose of 100 ng/ml and dose-dependent downregulation of Hjv as found by quantitative real-time PCR (Fig. 6). 28S rRNA was used to show the equal loading of RNA (3). By this technique, we confirmed that hepcidin and Hjv gene expression are two oppositely regulated but timely related genes.

Serum Analysis

To elucidate the extent of liver damage and a possible relationship between hepatic hepcidin expression and serum Fe²⁺ levels, we measured serum transaminases, serum Fe²⁺, and prohepcidin levels in vivo.

Fig. 5. Fold changes of different gene expression in rat hepatocytes treated with different doses of IFN-γ for 3 h. Experimentation was started 24 h after cells were plated. Values represent the amount of target mRNA compared with β-actin mRNA (*P < 0.05). Error bars represent SE (n = 3).

Fig. 6. Northern blot analysis of RNA extracted from isolated rat hepatocytes. Hepatocytes were stimulated with different doses of IL-6. Total RNA was used for analysis as described in MATERIALS AND METHODS. Filters were exposed to autoradiographic film for the Hepc probe for 10 days and the Hjv probe for 1 day at −80°C before being developed.

Fig. 7. In vivo serum transaminase levels. The concentration was measured as described in MATERIALS AND METHODS. The strong increase in the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations in PH (A; n = 3) and CCl₄-induced liver injury (B; n = 4) indicate the extent of liver injury (*P < 0.05). Error bars represent SE.
Serum transaminases. We found statistically significant elevated levels of aspartate aminotransferase and alanine aminotransferase in the serum of both models of liver injury compared with the control (P < 0.05), and the ratio was quite high, up to 6, which indicated the existence of severe liver damage (Fig. 7, A and B).

Serum iron concentrations. Statistically significant abridged serum Fe²⁺ levels were found early after the liver injury. In PH-induced rats, iron levels were declined to 16.8 ± 1.03 μM compared with 61.9 ± 2.5 μM (controls) 8 h after the liver injury and to a concentration of 24.1 μM (P < 0.05) compared with 66 μM (controls) in CCl₄-treated animals (Fig. 8, A and B).

Serum prohepcidin levels. An ELISA was performed to analyze serum prohepcidin concentration. A statistically nonsignificant increase in serum prohepcidin concentration was found in PH and CCl₄-administered rats (Fig. 9, A and B).

Serum cytokine levels. Serum analyses have shown significant changes in the acute-phase cytokine concentration. In PH-induced liver injury, IL-6 and IL-1β concentrations were significantly increased. However, IFN-γ concentration was significantly decreased from 4 to 24 h after the injury (P < 0.05). In CCl₄-induced liver injury, we found a significant increase in the TNF-α and IL-1β concentrations (P < 0.05) 12 h after the liver injury. However, IL-6 and IFN-γ concentrations were not significantly changed (Fig. 10, A and B).

DISCUSSION

In this work, we reported the changes of the hepatic hepcidin and Hjv gene expression together with the changes of other genes involved in iron metabolism in response to direct liver injury. We found that hepcidin and TfR1 gene expression were upregulated after resection of 70% of the liver (2.6- and 4-fold at 8 and 4 h, respectively). An early upregulation of these genes (4.3- and 9-fold, respectively) was observed in the liver of CCl₄-treated animals. In both cases, Hjv gene expression was downregulated together with hepcidin upregulation. At the same time, we studied the expression of the ferritin H and Fpn-1 genes and found that ferritin H and Fpn-1 gene expression was downregulated 0.45- and 0.5-fold, respectively, 16 h after the operation. In the liver of CCl₄-treated animals, the expression of the ferritin H gene was slightly upregulated (1.33-fold after 12 h), whereas Fpn-1 gene expression was downregulated 0.5-fold after 6 h of liver injury.
The gene expression of the cytokines IL-6, IL-1β, TNF-α, and IFN-γ was found to be upregulated in the damaged livers. In partially hepatectomized livers, upregulation of IL-6, IL-1β, and IFN-γ (15-, 2.06-, and 1.88-fold, respectively) was found to start 2 h and reached a maximum 8 h after the PH. Upregulation of TNF-α gene expression was weak and slightly delayed (24 h after the PH).

In CCl₄-treated animals, upregulation of hepcidin gene expression was observed earlier than the upregulation of IL-6 gene expression. In contrast, the time kinetics for IFN-γ, IL-1β, and TNF-α gene expression were similar to that of hepcidin gene expression, although all of these cytokines reached a peak 12 h after the onset of injury. Upregulation of IL-6 gene expression was seen at 6 h, whereas significant upregulation of hepcidin gene expression was detectable already at 3 h after CCl₄ administration; this may suggest the presence of some other regulatory mechanisms for hepcidin and Hjv gene expression. The possible reason for downregulation of the hepcidin gene expression at a later time could mark the beginning of necrosis of hepatocytes in response to the toxin.

To study the effect of single cytokines on the hepcidin and Hjv gene expression, isolated rat hepatocytes were treated with single cytokines at different doses at 24 h after being isolated and plated. The data support the assumption that IL-6 may be sufficient to induce the changes of hepcidin gene expression observed in the rat liver after PH. The kinetics of the changes of hepcidin and Hjv gene expression on one side and those of IL-6 on the other side suggest that in CCl₄-induced liver injury, besides IL-6, some other factors could be involved in hepcidin and Hjv gene regulation.

IFN-γ could be one such factor, because IFN-γ gene expression was upregulated after CCl₄ treatment along with IL-1β and TNF-α. Therefore, we studied the effects of IFN-γ treatment on isolated rat hepatocytes alone or in combination with the other cytokines. No significant changes of hepcidin or Hjv gene expression were found in hepatocytes stimulated with IFN-γ; furthermore, IFN-γ did not modify the effect of the other cytokines when cells were treated with the combinations.

Several converging lines of evidence from recent work have established that TNF-α and IL-6 are important components of the early signaling pathways after local injury (32) induced by PH. Previous studies (8, 9) have suggested that endotoxin, one of the key stimulants leading to TNF-α production by Kupffer cells, may be involved in PH. During recovery after PH-induced liver injury, the role of TNF-α is to regulate secretion of IL-6. IL-6 is secreted by Kupffer cells, and this secretion is stimulated by TNF-α (15). Our data seem to support this hypothesis; in fact, an upregulation of IL-6 starting 2 h after the PH may have induced the expression of the hepcidin gene, which was maximally expressed 8–16 h after resection of 70% of the liver. Interestingly, upregulation of hepcidin-specific
transcripts of a similar order of magnitude as observed in our study has been found in the liver of mice 6 h after the PH (20).

Hepcidin regulates the iron absorption into the bloodstream by affecting an iron transport protein, Fpn-1. Hepcidin binds and internalizes Fpn-1 (14, 35). Consequently, the iron absorbed in the enterocytes can no more be transported in the blood and is stored in association with ferritin H. The same holds true for other cells, especially for macrophages and hepatocytes (12). In turn, the expression of hepcidin is influenced by plasma transferrin saturation via a pathway that involves HFE, TfR1, and Hjv (1).

By Northern blot and real-time PCR analyses of liver RNA, we found that Hjv gene expression is downregulated during hepatic injury at the same time when hepcidin gene expression is upregulated. Similar data have been shown at 6 h after treating the mice with LPS (23, 38). Furthermore, we could reproduce the changes observed in vivo by IL-6 treatment of the rat under certain acute-phase conditions (45). On the other hand, we found no significant increase of IL-6 or IFN-γ in the serum of CCL4-treated rats, whereas IL-1β and TNF-α concentrations were significantly elevated 12 h after the administration of the toxin. IL-6-specific mRNA was increased in the liver, but this increase was not as high as that observed in classic acute-phase models (21, 45). This could be one of the reasons why serum levels of IL-6 were not significantly elevated in CCL4-induced liver injury. Its local action, however, could be sufficient to regulate the gene expression of some proteins such as hepcidin and Hjv in hepatocytes.

In fact, the in vitro studies seem to suggest that IL-6 is more potent than IL-1β, TNF-α, or IFN-γ in inducing hepcidin gene expression (14, 37), which is contrary to the findings of Lee et al. (26), in which IL-1β was stated to be more promising to induce the hepcidin gene expression in cultured murine hepatocytes. Our results also confirm, at least in part, those of Nemeth et al. (34), that is to say that IL-6 may be sufficient to induce hepcidin gene expression in acute inflammation.

Taken together, these findings demonstrate that hepcidin and Hjv gene expression changes in parallel but in the opposite direction in two models of liver injury. Hepatic hepcidin gene expression is increased during liver injury, and more than one mediator may be involved to regulate its gene expression, whereby IL-6 could be one of the principle mediators. In parallel, IL-6 also modulates the expression of Hjv and Fpn-1 genes, which are known to act in concert with ferritin H and transferrin (Fig. 11) directly on hepatocytes.

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