Hepcidin Mitigates Renal Ischemia-Reperfusion Injury by Modulating Systemic Iron Homeostasis

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ABSTRACT
Iron-mediated oxidative stress is implicated in the pathogenesis of renal ischemia–reperfusion injury. Hepcidin is an endogenous acute phase hepatic hormone that prevents iron export from cells by inducing degradation of the only known iron export protein, ferroportin. In this study, we used a mouse model to investigate the effect of renal ischemia–reperfusion injury on systemic iron homeostasis and determine if dynamic modulation of iron homeostasis with hepcidin has therapeutic benefit in the treatment of AKI. Renal ischemia–reperfusion injury induced hepatosplenic iron export through increased ferroportin expression, which resulted in hepatosplenic iron depletion and an increase in serum and kidney nonheme iron levels. Exogenous hepcidin treatment prevented renal ischemia-reperfusion–induced changes in iron homeostasis. Hepcidin also decreased kidney ferroportin expression and increased the expression of cytoprotective H-ferritin. Hepcidin-induced restoration of iron homeostasis was accompanied by a significant reduction in ischemia-reperfusion–induced tubular injury, apoptosis, renal oxidative stress, and inflammatory cell infiltration. Hepcidin-deficient mice demonstrated increased susceptibility to ischemia-reperfusion injury compared with wild-type mice. Reconstituting hepcidin-deficient mice with exogenous hepcidin induced hepatic iron sequestration, attenuated the reduction in renal H-ferritin and reduced renal oxidative stress, apoptosis, inflammation, and tubular injury. Hepcidin-mediated protection was associated with reduced serum IL-6 levels. In summary, renal ischemia–reperfusion injury results in profound alterations in systemic iron homeostasis. Hepcidin treatment restores iron homeostasis and reduces inflammation to mediate protection in renal ischemia–reperfusion injury, suggesting that hepcidin-ferroportin pathway holds promise as a novel therapeutic target in the treatment of AKI.


Renal ischemia–reperfusion injury (IRI) is a major cause of AKI and is associated with increased morbidity, mortality, and prolonged hospitalization. Acute ischemia leads to ATP depletion, tubular epithelial injury, and hypoxic cell death. Reperfusion further amplifies injury by promoting the formation of reactive oxygen species (ROS) and inducing leukocyte activation, infiltration, and inflammation.

Iron and ROS are important mediators of cell death and the ensuing inflammatory response during the course of IRI. During ischemia, cytochromes translocate from the mitochondria into the cytosol. Heme protein catabolism results in increase in labile ferrous (Fe^{2+}) iron. Fe^{2+} catalyzes the generation of tissue damaging hydroxyl radical (\cdotOH) by interacting with superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), both of which are increased during IRI. Labile iron’s contribution to tissue damage has been demonstrated not only in renal IRI but also in other models of AKI. Iron chelation is protective in diverse animal models of AKI.

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IRON (Fe^{2+}) is exported from cells by ferroportin, the only known iron export protein. Ferroportin is significantly expressed on macrophages, hepatocytes, and proximal and distal renal tubular cells. To sustain the physiologic iron requirement but avoid iron toxicity, systemic iron balance is regulated by hepcidin (hepatic antimicrobial peptide [HAMP]), an endogenous peptide hormone produced by hepatocytes. Hepcidin in- duces covalently modification of ferroportin, which leads to its internalization and lysosomal degradation, and thereby prevents cellular iron egress. Hepcidin is acutely and positively regulated during iron imbalance and inflammation and has antibacterial properties, whereas hypoxia and erythropoiesis negatively regulate its expression. Although human studies have indicated a positive correlation between increased urinary hepcidin levels and protection against AKI, its direct role has not been examined in any models of AKI. Here, we present evidence that renal IRI induces dynamic changes in renal and extrarenal iron homeostasis. Hepcidin-deficient mice sustain more severe renal injury after IRI and synthetic hepcidin mitigates renal IRI.

**RESULTS**

**Hepcidin Prevents Renal Ischemia-Reperfusion–Induced Iron Dyshomeostasis**

There is a dynamic and continuous exchange of iron between hepatosplenic compartments and plasma. This process is regulated by hepcidin through its modulation of reticuloendothelial (splenic macrophages and hepatic kupffer cells) ferroportin expression. Renal IRI increases serum iron levels; however, no previous studies have characterized the dynamic changes in iron distribution between different organs after renal IRI. Here, we first confirmed the previous observations that renal IRI increases serum iron levels compared with sham-operated animals (Figure 1A). Hepcidin treatment significantly reduced the IRI-induced increase in serum iron (Figure 1A) and kidney nonheme iron content (Figure 1B). Hepatosplenic nonheme iron levels were significantly lower after IRI than in the sham group and hepcidin pretreated mice demonstrated significantly higher hepatosplenic nonheme iron content than IRI or sham groups (Figure 1, C and D).

To determine if increased serum iron levels after renal IRI could result in an iron-dependent increase in hepcidin synthesis, we measured hepcidin transcripts and protein in liver and serum, respectively. Renal IRI resulted in significant upregulation of liver hepcidin gene expression and increased serum hepcidin levels compared with sham mice, and these were prevented by hepcidin treatment before IRI (Figure 1, E and F). Collectively, our observations indicate that IRI induces dynamic changes in iron metabolism characterized by hepatosplenic iron depletion, systemic iron mobilization, kidney nonheme iron accumulation, and post-IRI hepcidin induction. Hepcidin treatment before IRI effectively prevents these changes.

**Hepcidin Mitigates IRI, Reduces Acute Tubular Necrosis, and Improves Renal Function**

We examined the functional significance of hepcidin-mediated modulation of iron homeostasis on IRI. Renal IRI produced a marked increase in plasma creatinine that was reduced by a single bolus of 50 or 100 μg hepcidin (intraperitoneally) given 24 hours before IRI (Figure 2B). Because there were no significant differences between the effects of 50 and 100 μg hepcidin, all further experiments were performed using the 50 μg hepcidin.

We next established the timing of hepcidin injection that would offer maximum protection against IRI (Figure 2A). Although treatment at both 2 and 8 hours before IRI significantly reduced kidney injury, the protection was less than that observed with hepcidin treatment 24 hours before IRI (Figure 2B). In a separate experiment, to examine if hepcidin has renoprotective effects after the onset of reperfusion injury, we injected hepcidin 30 minutes after IRI. We observed a small but significant decrease in plasma creatinine (3.1±0.09 IRI group versus 2.6±0.05 hepcidin injected 30 minutes post-IRI group; P<0.01). Therefore, hepcidin was most effective when injected 24 hours before IRI, and the benefit was partial when administered after the onset of reperfusion injury (Figure 2B).

Quantification of the tubular injury by stereological analysis of hematoxylin and eosin-stained kidney sections correlated well with renal function and histology (Figure 2C). As compared to sham operated mice (Figure 2D, Supplemental Figure 1A), the kidneys of untreated IRI mice showed extensive tubular necrosis in the S3 segment in the outer stripe of the outer medulla and extending to the S1 and S2 segment of the proximal tubule in the deep cortex (Figure 2E, Supplemental Figure 1B). However, hepcidin-treated mice had negligible necrosis and luminal debris in the S1 and S2 segments of the proximal tubes and substantially reduced tubular damage and casts that were restricted to the S3 segment in the outer medulla (Figure 2F, Supplemental Figure 1C). Hepcidin pretreatment also reduced the kidney injury marker neutrophil gelatinase–associated lipocalin (NGAL) (Supplemental Figure 1D). Collectively, these findings demonstrate a protective role of hepcidin in renal IRI, amounting to approximately 70% reduction in injury as measured by plasma creatinine.

**Hepcidin Decreases Ischemia-Induced Renal Epithelial Apoptosis**

Apoptosis is a major mediator of tubular cell death after renal IRI. To investigate if hepcidin-mediated protection in renal IRI is mediated through modulation of epithelial apoptosis, we measured apoptosis-related genes in kidneys of the three experimental groups. Compared with sham-operated mice, caspase-3 expression was significantly increased in the kidneys of untreated IRI mice, and this was prevented by hepcidin (Figure 3A). Expression of Bcl-2, an antiapoptotic gene with known protective properties in renal IRI, was significantly increased in the kidneys of hepcidin-treated mice.
reduced in kidneys after IRI and hepcidin treatment restored Bcl-2 expression to levels seen in sham-operated mice (Figure 3B). These observations were further supported by terminal-deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) assay. We did not detect any TUNEL-positive cells in kidney sections of sham-operated mice (data not shown). There was an increase in TUNEL reactivity in renal tubules after IRI (Figure 3, C and D), which was greatly reduced in hepcidin-treated kidneys (Figure 3, E and F).

**Hepcidin Treatment Reduces Renal IRI-Induced Oxidative Stress and Inflammation**

Oxidative stress-induced apoptotic cell death is believed to be one of the major pathways involved in the pathogenesis of kidney IRI.\(^35,36\) Iron is known to play a central role in oxidative tissue injury through its ability to catalyze the redox cycle. Iron chelation and antioxidants attenuate IRI.\(^37,38\) Because hepcidin treatment directly modulates IRI-induced iron dyshomeostasis, we examined whether the reduction in injury is associated with lowering of oxidative stress. 4-hydroxynonenal (4-HNE), an aldehyde product of membrane lipid peroxidation, is used as a tissue marker of oxidative stress.\(^39\) Kidneys of untreated IRI mice showed prominent 4-HNE immunoreactivity. The pattern of staining mirrored renal injury distribution, being most intense in the medulla with lesser intensity in the corticomedullary junction and deep cortex. In contrast, kidneys of hepcidin-treated mice showed significantly reduced 4-HNE immunoreactivity (Figure 3, A, Supplemental Figure 2, A and B).

Apoptosis and ROS generation are early events after renal IRI, and they are followed by the infiltration of immune cells, which leads to further amplification of kidney pathology after IRI.\(^6,40,41\) Therefore, we investigated whether hepcidin treatment is associated with decreased kidney infiltration of inflammatory cells. IRI resulted in a large increase in CD45\(^+\) cells in kidney compared with sham; however, hepcidin treatment reduced renal inflammatory cell infiltration by almost half (Figure 4, B and C). Consistent with prior studies, we found that neutrophils (CD45\(^+\)CD11b\(^{hi}\)Ly6G\(^+\)Ly6Chi cells) were the most abundant immune cells in the kidneys after IRI. Hepcidin treatment significantly reduced IRI-induced renal neutrophil infiltration (Figure 4C). Collectively, these observations demonstrate that hepcidin treatment reduces renal IRI-associated oxidative stress and inflammation.

**Hepcidin Decreases IRI-Induced Renal Ferroportin and Increases the Expression of Cytoprotective H-Ferritin**

The primary known function of hepcidin is to induce ferroportin degradation and increase intracellular iron stores. In turn, this results in the induction of H-ferritin, an iron binding ferroxidase with cytoprotective function.\(^42\) To determine the involvement of this pathway in hepcidin-mediated protection, we measured the

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**Figure 1.** Hepcidin pretreatment prevents renal IRI-induced dysregulation of systemic iron homeostasis. Mice were subjected to sham operation or 26 minutes of kidney ischemia followed by 24 hours of reperfusion. Hepcidin (50 μg, intraperitoneally) was administered 24 hours before IRI. Serum iron was measured by ELISA and normalized to sample volume (A). Nonheme iron was measured after acid digestion of the kidneys (B), spleen (C), and liver (D), normalized to tissue weight and expressed as micrograms per gram of tissue. Liver hepcidin expression was measured by quantitative real-time PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (E). Hepcidin content in the serum was measured by ELISA (F). □, Sham; ○, IRI; ▽, Hepcidin+IRI. *P<0.05; **P<0.01; ***P<0.005; ****P<0.001. Data points are plotted as mean±SEM (n=4–5 per group). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
expression of these proteins and found that increase in renal ferroportin after IRI was prevented by pretreatment with hepcidin (Figure 5, A and B). H-ferritin showed a decreasing trend after IRI in vehicle or hepcidin-injected (50 or 100 μg/mouse, intraperitoneally) mice and compared with sham-operated mice (B). Tubular injury score (semiquantitative) of hematoxylin and eosin–stained kidneys was calculated for each section as mentioned in the Concise Methods section (C). Representative morphology (by hematoxylin and eosin staining) of kidneys after sham operation (D), IRI (E), and hepcidin-treated IRI (50 μg/mouse, 24 hours before IRI) (F). Arrow denotes edge of the kidney section. IRI resulted in severe tubular injury originating from the medullary region (S3) and extending all the way to the deep cortex (S1 and S2), whereas with hepcidin treatment, injury was restricted to occasional tubules in the S3 segment of the medullary region. Scale bar is 100 μm. □, Sham; ○, IRI; ■, Hepcidin+IRI (-2Hr); △, Hepcidin+IRI (-8Hr); ▽, Hepcidin+IRI (-24Hr); ◊, 100 μg Hepcidin+IRI (-24Hr). Data are represented as mean±SEM. *P<0.05; **P<0.005; ****P<0.001 (n=4−5 per group).

**Hepcidin Deficiency Increases Susceptibility to AKI**

Given the significance of hepcidin and ferroportin regulation in renal IRI, we hypothesized that genetic hepcidin deficiency would aggravate IRI. To reveal exacerbation of injury, we subjected mice to a subthreshold ischemic injury (subIRI) (24 minutes ischemia, 24 hours reperfusion), which did not result in a rise in plasma creatinine in wild-type (WT) mice. However, plasma creatinine was significantly elevated in hepcidin knockout (Hamp<sup>−/−</sup>) mice after subIRI (Figure 6A). We next treated Hamp<sup>−/−</sup> mice with a single 50 μg bolus of hepcidin, 24 hours before renal ischemia, and found that hepcidin reconstitution rescued these mice from IRI (Figure 6A). Renal histology and the tubular injury scores paralleled creatinine (Figure 6B). There were a large number of casts and necrotic tubules in the S1, S2, and S3 segments in Hamp<sup>−/−</sup> kidneys after subIRI. In contrast, casts and necrotic tubules were remarkably less in hepcidin-treated Hamp<sup>−/−</sup> mice after subIRI, and the occasional necrotic tubules were restricted to the deep medullary region (Figure 6, C–E, Supplemental Figure 3, A–C). NGAL expression paralleled changes in creatinine and histology (Supplemental Figure 3D).

Because Hamp<sup>−/−</sup> mice exhibit chronic iron overload, we investigated if protection induced by reconstitution of hepcidin was associated with changes in systemic iron levels. Compared with sham mice, there was a paradoxic trend toward lowering of serum iron in Hamp<sup>−/−</sup> mice after IRI. However, hepcidin reconstitution significantly lowered iron even further (Figure 6F). Changes in serum iron were associated with increased hepatic sequestration of nonheme iron after IRI in Hamp<sup>−/−</sup> mice, and this was further amplified by hepcidin treatment (Figure 6G). Although Prussian blue staining demonstrated qualitative increases in splenic iron content in hepcidin-treated Hamp<sup>−/−</sup> IRI mice, we did not observe differences in the nonheme iron content of the spleen and kidneys of sham, untreated, and hepcidin-treated Hamp<sup>−/−</sup> mice after IRI (data not shown). Taken together, our data show that hepcidin deficiency significantly increases the kidney’s susceptibility to IRI and that reconstitution with hepcidin at least partially restores iron homeostasis and results in mitigation of IRI.

**Hepcidin Reconstitution Preserves H-Ferritin and Prevents Apoptosis**

Similar to the observations in WT mice, a decrease in kidney H-ferritin was observed after IRI in Hamp<sup>−/−</sup> mice, and this was partially restored by reconstitution with hepcidin (Figure 7, A and B). We next measured apoptosis-related genes and
Hepcidin Reconstitution Restores Protection against IRI-Induced Oxidative Stress and Inflammation in Hamp−/− Mice

To evaluate the role of ROS in mediating injury in Hamp−/− mice, we stained kidneys for 4-HNE. Immunoreactivity for 4-HNE, which was not observed in sham-operated Hamp−/− mice, increased dramatically in these mice after IRI, particularly in the papilla, inner medulla, and along the corticomedullary junction. However, hepcidin-reconstituted animals demonstrated greatly reduced immunoreactivity to 4-HNE (Figure 8, A–C). These observations suggest that protection mediated by hepcidin in Hamp−/− mice is associated with reduced IRI-induced oxidative stress.

Substantial neutrophil (7/4 antigen positive cells) and CD11b+ cell infiltration was detected in kidneys of Hamp−/− mice after IRI (Figure 8, D and F), but it was not detected in sham-operated Hamp−/− mice (data not shown); this was almost completely prevented by hepcidin-reconstitution in Hamp−/− IRI mice (Figure 8, E and G). The reduction in inflammation correlated well with the lack of apoptosis and ROS in the protected animals.

Hepcidin Prevents IRI-Induced Ferroportin Upregulation and Splenic Iron Depletion

The spleen plays an important role in the pathophysiology of renal IRI.43–45 Splenic macrophages play a sentinel role in systemic iron homeostasis. Because splenic iron content decreases after renal IRI, we examined the expression of ferroportin in splenic macrophages. Renal IRI resulted in an upregulation of ferroportin in the splenic macrophages in both WT and Hamp−/− mice, we stained kidneys for 4-HNE. Immunoreactivity for 4-HNE, which was not observed in sham-operated Hamp−/− mice, increased dramatically in these mice after IRI, particularly in the papilla, inner medulla, and along the corticomedullary junction. However, hepcidin-reconstituted animals demonstrated greatly reduced immunoreactivity to 4-HNE (Figure 8, A–C). These observations suggest that protection mediated by hepcidin in Hamp−/− mice is associated with reduced IRI-induced oxidative stress.

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indicators. Caspase-3 was significantly upregulated in Hamp−/− mice after IRI, but this was prevented by a single dose of hepcidin (Figure 7C). Expression of the antiapoptotic gene Bcl-2 was significantly lower in kidneys of Hamp−/− IRI mice, and hepcidin treatment prevented this change (Figure 7D). We observed a large number of TUNEL-positive cells, especially in the corticomedullary region of the kidneys of Hamp−/− mice after IRI. Hepcidin reconstitution dramatically reduced the number of TUNEL-positive cells (Figure 7, E–G, Supplemental Figure 4, A–C). Collectively, these findings demonstrate that renal IRI in the setting of hepcidin deficiency is associated with reduced cytoprotective H-ferritin levels and increased epithelial apoptosis. Hepcidin reconstitution preserves H-ferritin and prevents apoptosis.

Figure 3. Hepcidin reduces apoptosis in the kidneys of mice after IRI. Hepcidin treatment protects kidneys from IRI-induced apoptosis [(A)–(F)]. Mice receiving hepcidin (50 µg, intraperitoneally) 24 hours before IRI had a lower transcriptional activation of proapoptotic caspase-3 (A) and better preservation of antiapoptotic Bcl-2 (B); n=3–5. Data for panels (A) and (B) are represented as mean±SEM of ΔΔCT values normalized to glyceraldehyde-3-phosphate dehydrogenase. TUNEL reactivity was used to assay apoptosis after renal IRI; fluorescein-dUTP-labeled apoptotic cells. Untreated IRI mice showed severe apoptosis in the cells of the corticomedullary region ([C]–[D]) that was reduced by hepcidin pre-treatment ([E]–[F]); scale bar is 200 µm ([C] and [E]) and 100 µm ([D] and [F]); □, Sham; ○, IRI; △, Hepcidin+IRI. *P<0.05; **P<0.01; ***P<0.001. Data points are plotted as mean±SEM of triplicates. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
after IRI. However, hepcidin-treated WT and *Hamp*<sup>−/−</sup> IRI mice showed a significant increase in H-ferritin (Figure 9, B and C). Therefore, renal IRI results in an increase in splenic ferroportin expression. Hepcidin treatment prevents this to induce splenic iron accumulation and an iron-dependent increase in H-ferritin.

**Iron and Hepcidin Have Contrasting Effects on IL-6 Production**

IL-6 is a pleiotropic cytokine that has been linked with the pathogenesis of renal IRI.<sup>46,47</sup> To investigate if hepcidin-mediated protection in renal IRI was associated with changes
in IL-6, we measured the serum IL-6 levels by ELISA. Renal IRI resulted in a significant increase in serum IL-6 in both WT and Hamp−/− mice, which was prevented by hepcidin treatment (Figure 10, A and B). To explore the possibility that free iron might directly regulate IL-6 production in immune cells, we treated splenocytes from WT mice with ferrous ammonium chloride and measured secreted IL-6. Treatment with iron significantly increased splenocyte IL-6 secretion compared with untreated ones (Figure 10C). These findings suggest that excess splenocyte-free iron may increase the production of IL-6, a cytokine implicated in the pathogenesis of renal IRI.

**DISCUSSION**

In this study we present several novel observations related to dynamic changes in renal and extrarenal iron homeostasis after IR, and identify a novel renoprotective effect of exogenous hepcidin. We demonstrate that renal IRI results in a systemic iron mobilization from the hepatosplenic compartments and is associated with kidney iron accumulation. Hepcidin prevents these changes by inducing ferroportin degradation, H-ferritin induction, and effective hepatosplenic iron sequestration. Hepcidin-induced restoration of iron homeostasis is associated with reduced ROS, apoptosis, and inflammation and nearly 70% improvement of renal function after IRI. We also provide evidence that hepcidin deficiency in mice severely increases susceptibility to renal IRI and that hepcidin reconstitution restores protection.

The beneficial effect of modulating iron metabolism in AKI has been demonstrated in previous studies that used iron chelators and NGAL. Iron-chelating agents are thought to be beneficial in renal IRI because of their iron binding effects in intra- and extracellular spaces. Conversely, NGAL was shown to be of benefit in AKI through its ability to deliver iron to viable cells to enhance recovery. Our findings not only demonstrate the role of iron in IRI but also reveal a more profound role of iron export and extrarenal iron homeostasis in renal pathophysiology.

The implication of iron in pathogenesis of AKI is rapidly unfolding. A recent study demonstrated worse outcome of AKI after deletion of H-ferritin from renal proximal tubules. Similarly, in a liver ischemia model, H-ferritin suppressed inflammation and had a direct antiapoptotic effect on hepatocytes. Each molecule of H-ferritin can bind to 4500 Fe2+ ions, making it an important endogenous iron chelator. Sequestration of Fe2+ by H-ferritin is known to prevent iron-mediated formation of ROS and cell injury. In our study, hepcidin treatment increased renal H-ferritin levels after IRI. Therefore, by limiting the availability of free intracellular iron in renal cells, H-ferritin could potentially reduce renal ROS and the ensuing injury. This is supported by our finding that lipid peroxidation (a marker of iron-mediated injury/ferroptosis) is reduced in hepcidin-treated mice subjected to IRI.

Previous studies have suggested an important role of the spleen and liver in the pathophysiology of renal IRI. We demonstrate that IRI induces hepatosplenic iron export, which likely contributes to the pathogenesis of renal IRI. Splenic macrophages along with hepatocytes are the main storage sites and sources of iron in the body. Because hepcidin acts rapidly on splenic macrophages, under our experimental conditions, contributions from the spleen are likely to be more critical. Hepcidin induced splenic ferroportin degradation and H-ferritin induction, thereby causing splenic iron retention. These changes prevent systemic iron mobilization after IRI and limit its availability to participate in ROS generation in the kidney. Despite having high splenic ferroportin expression after IRI, both untreated WT and Hamp−/− mice did not demonstrate a decrease in H-ferritin. This could be attributed to an iron-independent, cytokine-mediated induction of H-ferritin. The mechanism of paradoxical increase in splenic ferroportin despite ongoing tissue injury after IRI is unclear. One could speculate that renal hypoxia after IRI triggers stress erythropoietic response, which in turn could result in signals that induce ferroportin-dependent macrophage iron export.

Another novel observation is the increase in endogenous hepatic and serum hepcidin after renal IRI. Hepatic hepcidin production increases after endotoxemia and inflammation. Similar to hepatic iron, hepcidin levels increase after liver IRI.
Our data in Hamp$^{-/-}$ mice does not completely mimic the findings in WT mice, where the serum iron levels decreased and liver iron content increased after renal IRI. However, these mice are constitutively iron overloaded because of the lack of hepcidin. It has been demonstrated that hepatic ferritin content in patients with hemochromatosis is higher than normal individuals. Hence, liver ferritin could potentially act as a sink for excess iron released after kidney injury. Treating these animals with hepcidin could further increase ferritin content in the liver to facilitate iron sequestration after renal injury.

CD45$^+$ immune cells infiltrate the kidney after reperfusion, with neutrophils playing a major role in ensuing pathologic response. Hepsidin treatment reduced the infiltration of total CD45$^+$ cells, most of which were neutrophils. Whether hepcidin affects the chemotaxis of immune cells to the kidney or the reduction in infiltration is a consequence of less epithelial injury needs further study.

Inflammatory cytokines, such as IL-6, are deleterious to the outcome of renal IRI. It is made locally by injured epithelial cells and by infiltrating macrophages. Interestingly, IL-6 is also a powerful inducer of hepcidin. Hepsidin treatment prevented the IRI-induced increase in systemic IL-6 levels. Furthermore, our in vitro data suggest an iron-dependent increase in splenic IL-6 secretion could be an important source. In support of our findings, De Domenico et al. showed that iron-loaded bone marrow macrophages treated with hepcidin produced less IL-6 and TNF-$\alpha$ on stimulation with LPS. Collectively, these observations suggest that hepcidin treatment reduces IRI-induced IL-6 production through modulation of iron metabolism.

Our studies demonstrate a novel renal protective effect of hepcidin. We propose that hepcidin mediates protection by acting not only on the renal cells but also through its effects on extrarenal iron homeostasis and inflammation. The protective effects of hepcidin are mediated through ferroportin degradation, subsequent H-ferritin induction, and sequestration of free iron. Further studies are required to investigate the relative contribution of renal and extrarenal ferroportin in mediating the protective effects of hepcidin.

under settings of both sterile (IRI) and nonsterile acute inflammation, hepcidin is induced to facilitate iron sequestration and thereby prevent iron-dependent oxidant injury.

Figure 6. Renal IRI is exacerbated in hepcidin-deficient mice and mitigated by reconstitution with exogenous hepcidin. Mice were subjected to subIRI (24 minutes ischemia), which was not sufficient to increase plasma creatinine significantly in WT mice after 24 hours of reperfusion but produced a large increase in creatinine in Hamp$^{-/-}$ mice. Hepcidin reconstitution (50 $\mu$g hepcidin, intraperitoneally, 24 hours before IRI) rescued the Hamp$^{-/-}$ mice from IRI (A). Tubular injury score of hematoxylin and eosin–stained kidney sections (B). Data are represented as mean±SEM. ***P<0.001 (n=3–5 per group). Representative morphology of Hamp$^{-/-}$ kidneys (by hematoxylin and eosin staining) in sham (C), IRI (D), and hepcidin plus IRI (E) mice. Arrow denotes edge of the kidney section. IRI resulted in extensive tubular necrosis and cast formation throughout the kidney, whereas hepcidin-treated mice showed remarkably few injured tubules. Scale bar is 100 $\mu$m. Serum iron was measured by ELISA and normalized to sample volume (F). Hepcidin reconstitution significantly reduced serum iron in Hamp$^{-/-}$ IRI mice compared with both untreated Hamp$^{-/-}$ IRI and Hamp$^{-/-}$ sham-operated mice. Nonheme iron was measured after acid digestion of liver and normalized to tissue weight (G). Hepcidin-treated Hamp$^{-/-}$ IRI mice had significantly higher hepatic nonheme iron compared with both untreated Hamp$^{-/-}$ IRI and Hamp$^{-/-}$ sham-operated mice. ○, WT; ●, Hamp$^{-/-}$ Sham; ■, Hamp$^{-/-}$–IRI; ▲, Hamp$^{-/-}$–Hepcidin–IRI. *P<0.05; **P<0.01; ***P<0.001. Data points are plotted as mean±SEM.
Figure 7. Hepcidin treatment mitigates renal H-ferritin degradation in hepcidin-deficient mice and is associated with reduced apoptosis. Expression of H-ferritin in whole kidney lysates was measured by quantitative western blots. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to confirm equal protein loading and normalize H-ferritin. IRI resulted in a significant reduction in H-ferritin in the kidneys of both untreated and hepcidin-treated Hamp−/− mice compared with sham Hamp−/− mice [A and B]. Hepcidin-treated Hamp−/− IRI mice had significantly higher H-ferritin levels compared with untreated Hamp−/− IRI mice. *P<0.05; **P<0.01; ***P<0.001. Hepcidin reconstitution protects Hamp−/− kidney from IRI-induced apoptosis. Hepcidin-treated Hamp−/− IRI mice had lower transcriptional activation of proapoptotic caspase-3 (C) and higher levels of antiapoptotic Bcl-2 (D); n=3–4. ▲, Hamp−/− Sham; ■, Hamp−/−–IRI; ▼, Hamp−/−–Hepcidin–IRI. *P<0.05; **P<0.01. Data are represented as mean±SEM of 2ΔΔCT normalized to GAPDH. TUNEL reactivity was used to assay apoptosis after renal IRI. Sham-operated Hamp−/− mice did not show signs of apoptosis (E). Untreated Hamp−/− IRI mice showed severe apoptosis in the cells of the corticomedullary region (F), which was markedly reduced in the hepcidin-treated Hamp−/− IRI mice (G). Representative images from two different experiments with four to five each are depicted. Scale bar is 200 μm.

CONCISE METHODS

Mice and Surgical Protocol
All experiments were performed in accordance with the National Institutes of Health and Institutional Animal Care and Use Guidelines. The Animal Care and Use Committee of the University of Virginia approved all procedures and protocols. Male 8- to 10-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) and hepcidin knockout mice (Hamp−/−), a kind gift from Dr. Sophie Vaulont (National Institute of Health and Medical Research, Paris, France) and Dr. Cindy Roy (Johns Hopkins University), were used for all experiments. Kidney ischemia–reperfusion surgery was performed as previously reported.45 The core temperature of the mice was maintained between 34°C and 36°C using a heating pad. After anesthetizing the mice with an intraperitoneal injection of ketamine (120 mg/kg), xylazine (12 mg/kg), and atropine (0.324 mg/kg), bilateral flank incisions were made, and both renal pedicles were exposed

In some experiments hepcidin was injected 30 minutes after IRI intraperitoneally; Peptide International) 2, 8, or 24 hours before IRI. Hamp in oxidative stress and results in decreased in J Am Soc Nephrol 30°C and 32°C during the entire reperfusion period. Sham-operated ani- mals underwent bilateral ank incisions without clamping of renal pedicles. Twenty-four hours later, mice were euthanized, and tissues were harvested. Animals were injected with dH2O (vehicle) or hepcidin (50–100 μg/mouse, intraperitoneally; Peptide International) 2, 8, or 24 hours before IRI. In some experiments hepcidin was injected 30 minutes after IRI.

Figure 8. Hepcidin reconstitution of hepcidin-deficient mice reduces renal IRI-induced oxidative stress and results in decreased infiltration of immune cells. 4-HNE immunoreactivity was used as an indicator of oxidative stress in the kidneys of Hamp–/– mice after IRI. Sham-operated Hamp–/– mice did not show reactivity to 4-HNE (A). Renal IRI in Hamp–/– mice resulted in increased oxidative stress as indicated by the extensive 4-HNE immunoreactivity in the papilla, deep medulla, and corticomedullary regions (B). Kidneys of hepcidin-treated Hamp–/– IRI mice showed very little 4-HNE immunoreactivity, indicative of reduced oxidative stress (C). Representative images from two different experiments with four to five mice each are depicted; brown, 4-HNE immunoperoxidase labeling; blue, methylene blue counterstain. Scale bar is 200 μm. Immunofluorescence labeling of kidneys showed large infiltration of neutrophils (D) (7/4; green) and CD11b cells (E) (CD11b; red) in untreated Hamp–/– IRI mice. Infiltration of neutrophils (E) and CD11b cells (G) was dramatically reduced in hepcidin-treated Hamp–/– IRI mice (arrows). Nuclei were stained with DAPI (blue). Scale bar is 100 μm.

Blood and Tissue Samples
Before euthanasia, animals were anesthetized and blood was drawn from the axilla, after which tissues were harvested. All the tissue slices were fixed with 10% neutral-buffered formalin for paraffin embedding and periodate-lysine-paraformaldehyde fixative (PLP) to be frozen in optimal cutting temperature compound or snap frozen in liquid nitrogen for subsequent mRNA extraction.

Renal Function
Plasma creatinine, determined using a modified Jaffe colorimetric assay, was used as an indicator of renal function.

Histology and Stereologic Analysis of Tissue Morphology
Kidneys were fixed in buffered formalin for 48 hours, embedded in paraffin, sectioned (3-μm thickness), and stained with hematoxylin and eosin. The extent of acute tubular necrosis was assessed in an unbiased, systematic manner using design-based stereology to achieve statistically accurate random sampling of kidney sections. The investigator was blinded to the experimental identity of the sections. Sections were imaged by using a Zeiss Axio Imager Z1/ApoTome Microscope fitted with motorized focus drives and motorized XYZ microscope stage and integrated to a workstation running Stereo Investigator software (MBF Bioscience, Williston, VT). The area fraction fractoron probe was used for stereologic analysis of the fractional area of the medulla (the region of the kidney most susceptible to ischemic injury) occupied by tubular necrosis. The following parameters were defined: counting frame (400×400 μm), sample grid (800×800 μm), and grid spacing (85 μm). These values were determined empirically such that adequate numbers of sample sites were visited and adequate numbers of markers (indicating injured tubules) were acquired, in keeping with accepted counting rules for stereology. A total of 16.0±0.6 (mean±SEM) grid sites were evaluated per section; the sampling fraction was 25% of a total average area of 5.15×10^6±0.33 μm^2 for each kidney section. Injured tubules were identified on the basis of the presence of cast formation, tubule dilation, and/or tubular epithelial denucleation. To better represent the degree of injury, which was extensive in some groups and not adequately reflected in the fractional area of injury, a weighted injury score was calculated for each section as follows: [(the fraction of area occupied by casts and severely degenerated tubules)×2]+(the fraction of area occupied by mild to moderately injured tubules).
Serum Iron, Hepcidin, and IL-6 Measurement

Serum iron (Abcam, Inc.), hepcidin (Intrinsic LifeSciences), and IL-6 levels (eBioscience) were measured using commercial ELISA kits, as per manufacturers’ instructions.

Nonheme Iron Assay

Nonheme iron content (microgram of iron per gram of tissue) was measured as described previously, with slight modifications. Briefly, tissues were weighed, finely cut, and incubated for 20 hours at 65°C in a solution of 3 M HCl/0.61 M TCA. After cooling to room temperature, the acid extract was spun at 12,000 rpm, 0.1 ml of the supernatant was incubated with 5 chromogen solution (1.86 mM bathophenanthroline sulfonate/143 mM thioglycolic acid) for 20 minutes, and OD was measured at 535 nm. A standard curve was generated using an iron standard solution (Ricca Iron AA standard) against water as the blank. Nonheme iron content was calculated using the equation described.

Flow Cytometry

Single-cell suspensions from the kidney were prepared using standard methods. Briefly, kidney was cut into small pieces and digested with

Figure 9. Hepcidin treatment prevents renal IRI-induced upregulation of splenic ferroportin and increased expression of cytoprotective H-ferritin. Immunofluorescence labeling of spleen indicated upregulation of ferroportin (green) in F4/80+ macrophages (red) after renal IRI (A: middle panel). Hepcidin treatment resulted in degradation of ferroportin, which could be detected in only a few scattered F4/80+ cells in the red pulp region (A: right panel). Scale bar is 100 μm. Ferroportin expression is constitutively high in spleens of Hamp−/− mice compared with WT mice (A: left panel). Expression of H-ferritin in spleen lysates was measured by quantitative western blots using β-actin as the loading control. Hepcidin increased splenic H-ferritin levels in both WT (B) and Hamp−/− (C) mice after renal IRI. Representative blots from three independent experiments are shown. B (WT) and C (Hamp−/−). Sham; ■, IRI; ▲, Hepcidin+IRI. Quantitation of H-ferritin was carried out using densitometry software and is expressed as mean±SEM. *P < 0.05 (B); **P < 0.005 (C).
collagenase (type 2; Worthington) for 20 minutes at 37°C. The digested kidney was then passed serially through a 100- and 50-
μm sieve to collect the cell suspension. The cells were then incubated with anti-CD16/32 (Fc block, clone 93; eBioscience, La Jolla, CA) and stained with APC-eFlour 780-conjugated anti-CD45 (30-F11), PE-conjugated anti-CD11b (M1/70), APC-conjugated anti-Ly6G (RB6-8C5), and eFlour 450-conjugated anti-Ly6C (HK1.4) (eBioscience). Flow cytometry data were acquired using BD FACSCalibur (BD Biosciences, San Jose, CA) with Cytek eight-color flow cytometry upgrade (Cytek Development, Fremont, CA) and analyzed with FlowJo software 9.0 (TreeStar, Inc., Ashland, OR). There were 100–200,000 events/samples acquired. Neutrophils were identified as CD11b^+Ly6G^+Ly6C^- cells within the CD45^+ gate.

### TUNEL Assay

Apoptotic cells in the kidney were detected by TUNEL assay following the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Briefly, cryostat sections (3 μm) of PLP-fixed kidneys were immersed in 3% hydrogen peroxide-methanol mixture (10 minutes) and permeabilized with cold 0.1% Triton-X100 and 0.1% sodium acetate (2 minutes). DNA fragments in apoptotic cells were then labeled with fluorescein-conjugated dUTP by terminal transferase (Roche Diagnostics) for 60 minutes at room temperature in dark. The fluorescein-labeled apoptotic cells were imaged using a Zeiss Axiovert 200 microscope with ApoTome imaging and AxioVision 4.6 software (Carl Zeiss GmbH).

### Immunohistochemistry

Paraffin-embedded kidney sections (3 μm) were stained for 4-HNE using standard protocols. Briefly, sections were deparaffinized in xylenes, dehydrated in a series of ethanol rinses (100%–70%), and then washed in distilled water. Sections were then incubated in 3% H2O2 in methanol for 20 minutes. After treating with avidin and biotin for 15 minutes each (Avidin/Biotin Blocking Kit; Vector Laboratories) the sections were incubated in blocking buffer containing 10% donkey serum in 0.1 M sodium phosphate buffer pH 7.4 (phosphate buffer) at room temperature for 30 minutes. Sections were incubated in 4-HNE antibody (1:1000 in 1% BSA/phosphate buffer; Abcam, Inc.) overnight at 4°C. Sections were washed three times with phosphate buffer for 5 minutes each and incubated with biotinylated donkey anti-goat secondary antibody (1:400 dilution; Vector Laboratories) for 1 hour. After three washes in phosphate buffer, sections were incubated with ABC ready-to-use reagent (VECTASTAIN Elite ABC Kit; Vector Laboratories) for 30 minutes. After another three washes with phosphate buffer, the sections were incubated with 3,3'-diaminobenzidine for 5 minutes followed by washing with distilled water. The sections were counter-stained with 1% methylene blue solution, washed in water, and dehydrated with xylene. Sections were imaged by using a Zeiss Axio Imager Z2/ApoTome Microscope fitted with motorized focus drives and motorized XYZ microscope stage and integrated to a workstation running Stereo Investigator software, version 10.51 (MBF Bioscience, Williston, VT).

### Immunofluorescence

Three-micron PLP-fixed cryostat-cut kidney sections were used for the immunofluorescence detection of neutrophils and CD11b^- cells. Briefly, the kidney sections were air dried and incubated with 0.3% TritonX100/10% horse serum in PBS for 30 minutes. After washing the sections with PBS, anti-CD16/32 antibody was added to block Fc receptors. This was followed by incubation with FITC-labeled antineutrophil antibody (7/4, 1:30; Cedarlane, Burlington, NC) and PE-labeled CD11b (M1/70, 1:10; eBioscience) in 10% horse serum/PBS for 1.5 hours. The sections were then washed three times in PBS and mounted with ProLong Gold antifade agent with DAPI (Life Technologies).

### Iron Stimulation of Splenocytes

Splenocytes were harvested from 10-week-old B6 male mice. After excising the spleen, red blood cells were lysed, and the remaining splenocytes were seeded at 1 × 10^6 cells/ml in 24-well tissue culture plates and cultured under 5% CO2 at 37°C for 24 hours. The culture medium...
consisted of RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids (Gibco/Life Technologies, Foster City, CA), 2 mM L-glutamine (Sigma-Aldrich), and 50 μM 2-mercaptoethanol. After resting for 24 hours, the cells were treated with 10 μM ferric ammonium chloride for 4 hours. The cell culture supernatants were harvested, and IL-6 levels were measured by ELISA (eBioscience).

Western Blot Analysis
Snap-frozen tissue sections were homogenized in Tris-Triton tissue lysis buffer containing complete protease inhibitor cocktail (Halt Protease and Phosphatase Inhibitor Cocktail; Thermo Fisher Scientific, Rockford, IL) using a Dounce Homogenizer. Ferroportin was measured in membrane fractions isolated from the whole kidney lysate using a membrane isolation kit (Thermo Fisher Scientific). H-ferritin was measured in whole kidney and spleen lysates. Protein content in the homogenate was estimated using the Pierce BCA protein estimation kit (Thermo Fisher Scientific). Twenty to thirty micrograms of protein per sample were loaded on a 10% NuPage Bis-Tris gel under reducing conditions. The resolved proteins were transferred onto a nitrocellulose membrane (LI-COR Biosciences, Lincoln, NE) and probed with goat anti-mouse H-ferritin (Santa Cruz Biotechnology) and rabbit anti-mouse ferroportin (Novus Biologics) antibodies (concentrations). The primary antibodies were detected with donkey anti-goat Alexa 800 and goat anti-rabbit Alexa 800 antibodies (LI-COR). Mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase and β-actin (Abcam, Inc.) were used as the loading controls and detected using donkey anti-mouse Alexa 680 antibody (LI-COR). Quantitation of data was performed using densitometry software (LI-COR).

Real-Time PCR
For RNA isolation, frozen tissues were resuspended in RLT buffer (Qiagen, Valencia, CA) and homogenized using the TissueLyser system (Qiagen). Total RNA from tissue homogenates was purified using the RNeasy mini kit (Qiagen) following the manufacturer’s instructions. Then 1 μg of RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The cDNA template was mixed with iTAQ SYBR green universal super mix (Bio-Rad), and quantitative PCR was carried out on a CFX Connect system (Bio-Rad). Predesigned primers for NGAL, caspase-3, and Bcl-2 were purchased from Bio-Rad. Glyceraldehyde-3-phosphate dehydrogenase and β-actin were purchased from Bio-Rad. Glyceraldehyde-3-phosphate dehydrogenase was amplified in parallel and used as the reference gene in quantification. Data are expressed as relative gene expression and were calculated using the 2−ΔΔC(T) method.

Statistical Analyses
GraphPad Prism 6 (GraphPad) was used to analyze and present the data. Data were analyzed, after transformation if needed to generate a normal distribution, by t test, one- or two-way ANOVA with post hoc analysis as appropriate. P<0.05 was used to indicate significance.

REFERENCES


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Supplemental Figure 1

Hepcidin pretreatment mitigates kidney injury following renal IRI

Representative morphology (by H&E staining) of kidneys 24 hours after sham operation (a), IRI (b) and Hepcidin-treated IRI (50 μg/mouse, i.p., 24 hrs before IRI) (c). Scale bar = 100 μm. Relative NGAL mRNA expression in kidneys measured by RT-PCR and normalized to GAPDH (d). Data are represented as mean ± SEM. ***P < 0.001, ****P < 0.0001 (n = 4-5 per group).
Hepcidin reduces oxidative stress following renal IRI

Higher magnification images of 4-Hydroxynonenal (4-HNE) staining in the corticomedullary region of the kidney. Untreated WT IRI mice showed strong 4-HNE immunoperoxidase labeling (brown) (a) that was markedly reduced in the hepcidin treated WT-IRI mice (b). Blue; methylene blue counter stain. Representative images from two different experiments with 4-5 mice each are depicted. Scale bar = 100 µm.
Renal IRI is exacerbated in hepcidin-deficient mice and mitigated by reconstitution with exogenous hepcidin

Representative morphology of Hamp−/− kidneys (by H&E staining) 24 hours after sham operation (a), IRI (b) and IRI with hepcidin pretreatment 24 hrs before IRI (c). Scale bar = 100 µm. Relative NGAL mRNA expression in kidneys measured by RT-PCR and normalized to GAPDH (d). Data are represented as mean ± SEM. *P < 0.05, **P < 0.005 (n = 3-4 per group)
Hepcidin treatment is associated with reduced apoptosis

Higher magnification images of TUNEL staining. Sham-operated *Hamp<sup>-</sup>* mice did not show signs of apoptosis (a). Untreated *Hamp<sup>-/-</sup>*IRI mice showed severe apoptosis (dense TUNEL staining) in the cells of the corticomedullary region (b), which was markedly reduced in the hepcidin-treated *Hamp<sup>-/-</sup>*IRI mice (c). Representative images from two different experiments with 4-5 mice each are depicted. Scale bar = 100 µM.