Renal Handling of Circulating and Renal-Synthesized Hepcidin and Its Protective Effects against Hemoglobin–Mediated Kidney Injury

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ABSTRACT
Urinary hepcidin may have protective effects against AKI. However, renal handling and the potential protective mechanisms of hepcidin are not fully understood. By measuring hepcidin levels in plasma and urine using mass spectrometry and the kidney using immunohistochemistry after intraperitoneal administration of human hepcidin-25 (hhep25) in C57Bl/6N mice, we showed that circulating hepcidin is filtered by the glomerulus and degraded to smaller isoforms detected in urine but not plasma. Moreover, hepcidin colocalized with the endocytic receptor megalin in proximal tubules, and compared with wild-type mice, megalin-deficient mice showed higher urinary excretion of injected hhep25 and no hepcidin staining in proximal tubules that lack megalin. This indicates that hepcidin is reabsorbed in the proximal tubules by megalin dependent endocytosis. Administration of hhep25 concomitant with or 4 hours after a single intravenous dose of hemoglobin abolished hemoglobin-induced upregulation of urinary kidney injury markers (NGAL and KIM-1) and renal Interleukin-6 and Ngal mRNA observed 24 hours after administration but did not affect renal ferroportin expression at this point. Notably, coadministration of hhep25 and hemoglobin but not administration of either alone greatly increased renal mRNA expression of hepcidin-encoding Hamp1 and hepcidin staining in distal tubules. These findings suggest a role for locally synthesized hepcidin in renal protection. Our observations did not support a role for ferroportin in hhep25-mediated protection against hemoglobin-mediated early injury, but other mechanisms of cellular iron handling may be involved. In conclusion, our data suggest that both systemically delivered and locally produced hepcidin protect against hemoglobin-induced AKI.

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The iron–regulating peptide hormone, hepcidin, has recently been proposed as a potential biomarker to predict AKI in patients undergoing coronary artery bypass grafting (CABG). Diverse clinical studies showed an association between high urinary hepcidin concentration and reduced risk of AKI in these patients.1–3 AKI after CABG can be induced or mediated through iron released from increased amounts of heme in the kidney via hemolysis and/or ischemia-reperfusion injury (IRI).4 Because of its important role in iron homeostasis,5 these observations prompted the hypothesis that hepcidin may have protective effects against iron-mediated AKI. Its mechanisms of action remain elusive, but may include (1) binding of luminal iron,6–8 thus promoting iron
excretion, or (2) increased sequestration of renal iron in ferritin in the epithelial cells, thereby reducing serum and luminal iron levels. Both mechanisms would reduce the formation of iron–induced oxidative stress and subsequent cell damage.

A better understanding of the renal handling of hepcidin is needed to fully understand these findings and guide the introduction of hepcidin in the diagnosis and/or treatment of AKI. For instance, the source of urinary hepcidin during CABB is not completely understood. Hepcidin is reported to be present in the plasma in a protein-bound form (specifically bound to α2-macroglobulin and nonspecifically bound to albumin) and a freely circulating form; the ratio between the bound and unbound forms of hepcidin in plasma is still under debate. The (unbound) hepcidin is freely filtered across the glomerular membrane, which was shown in two murine studies in which radiolabeled human hepcidin (hhep) was administered intraperitoneally and intravenously and measured in urine. Indeed, plasma hepcidin levels were increased compared with baseline after CABB, which may have contributed to the elevated urinary hepcidin levels observed in these patients. However, plasma hepcidin levels were not associated with development of AKI. Therefore, it has been postulated that local renal production may contribute to the high urinary hepcidin levels and ultimately, the renoprotective effect observed. Hepcidin has been shown to be synthesized in the mouse kidney, specifically in the distal nephron segment. However, it is unclear whether this locally synthesized hepcidin is excreted in the urine. Other than uncertainties concerning the origin of urinary hepcidin during CABB, gaps exist in our common knowledge on renal hepcidin handling. On the basis of the low fractional excretion of hepcidin assessed in healthy humans, it is proposed that hepcidin may be reabsorbed in the proximal tubule, possibly via megalin-mediated endocytosis. However, solid evidence for such a mechanism is lacking. Moreover, it is known that full-length hhep25 can be degraded into smaller isoforms, which can be detected in human urine under physiologic conditions. These smaller hepcidin forms do not elicit a hypoferremic response, but it is currently unknown whether, in vivo, they retain other biologic functions that have been identified for hepcidin-25 (e.g., host defense or metal binding).

To further substantiate tubular reabsorption and degradation of hhep25, we administered hhep25 to wild-type and megalin-deficient mice. Here, we show, for the first time, reabsorption of hepcidin in the proximal tubules via megalin and breakdown of hepcidin into the smaller isoforms in the kidney. Subsequently, we show a protective effect of administered hhep25 against early hemoglobin-mediated kidney injury, which does not seem to comprise ferroportin degradation. Interestingly, we also show that locally synthesized hepcidin may be involved in renal protection.

RESULTS

Circulating Hepcidin Is Reabsorbed in the Renal Proximal Tubule Via Megalin but Also Degraded in the Tubular Lumen

To determine renal handling of circulating hepcidin, wild-type C57Bl/6 mice were administered a single intraperitoneal injection of hhep25. We confirmed rapid plasma clearance of hhep25, because hhep25 was detectable in plasma 1 hour but not 24 hours after administration (data not shown). Plasma levels of endogenous hepcidin-1 and hepatic Hampl mRNA expression levels were significantly reduced compared with baseline or control 1 hour after hhep25 administration but returned to baseline or control values after 24 hours (Figure 1, A and B). The negative feedback of hhep25 on endogenous hepcidin-1 levels suggests that hhep25 is biologically active, which is in agreement with the findings by Rivera et al. Administered hhep25 was also biologically active in the kidney, which was shown by a reduction in renal ferroportin protein levels (Figure 1C), the known target of hepcidin.

To investigate the contribution of megalin to proximal tubular reabsorption of filtered hepcidin, we applied two approaches by studying renal handling of hhep25 in (1) megalin-deficient mice (megalinf/f,Cre) and (2) wild-type mice in which megalin was pharmacologically blocked using succinylated gelatin, a plasma expander (Gelofusin: Braun Medical). Reduced Megalin mRNA expression and increased urinary hepcidin-1 excretion in megalin/f,Cre mice compared with their nondeficient transgenic littermates (megalinf/f,wt mice) was confirmed as previously reported (Supplemental Figure 1, A and B). In addition, increased hepcidin-1 excretion was also observed in wild-type mice after treatment with succinylated gelatin compared with baseline (Supplemental Figure 1C).

Urinary concentration of hhep25 was highest 1 hour after administration (Figure 2A), suggesting that the excretion rate of hhep25 in urine is relatively fast. Reduction in megalin-mediated protein reabsorption via genetic knockdown (megalinf/f,Cre mice) or succinylated gelatin treatment resulted in increased urinary concentrations of hhep25, which was significant in megalin/f,Cre mice (P<0.05) compared with their controls (Figure 2A). Interestingly, in addition to hhep25, we also detected hhep22 and to a lesser extent, hhep20, cleavage products of hhep25, which followed the same urinary excretion pattern as hhep25 (Figure 2, B and C). These N–truncated hepcidin isoforms could not be detected in plasma at any time point, suggesting that the isoforms are formed in the renal tubular lumen. This corroborates observations in humans, where under physiologic conditions, hepcidin isoforms are present in the urine but are not present or are present at very low concentrations in plasma.

Double staining for hepcidin and megalin by immunohistochemistry revealed hepcidin and megalin colocalization in endocytic vesicles at the apical site of the proximal tubules
To investigate a protective effect of urinary hepcidin against hemoglobin–mediated kidney injury, we administered hhep25 to wild-type mice at the same time or 4 hours after a single intravenous injection of human hemoglobin. Urine was collected in two time slots after hemoglobin injection: 0–8 and 9–24 hours. The single injection of hemoglobin did not lead to overt kidney injury 24 hours after administration as determined by histology (Figure 4A). Furthermore, terminal deoxynucleotidyl transferase–mediated dideoxynucleotide–dideoxynucleotide nick end labeling did not reveal evidence for apoptosis (data not shown). However, we could observe significantly increased renal HO-1 protein expression (Figure 4B) \( (P<0.05) \), which is indicative of intracellular hemoglobin catabolism.\(^{26}\) In addition, hemoglobin injection resulted in significantly increased concentrations of the early urinary marker for kidney injury neutrophil gelatinase–associated lipocalin\(^{27}\) (NGAL; \( P<0.001 \) for 0–8 hours) (Figure 4C) and to a lesser extent, kidney injury molecule 1\(^{28}\) (KIM-1) (Figure 4C) as well as significantly increased renal mRNA expression levels of interleukin-6 \((IL-6)\) (Figure 4D) \( (P<0.001) \), another early indicator of kidney injury,\(^{29}\) and a minor increase in renal Ngal mRNA expression (Figure 4D). Administration of hhep25 simultaneously with or 4 hours after hemoglobin significantly reduced 0–8-hour urine NGAL \( (P<0.001) \) and KIM-1 concentrations \( (P<0.05) \), renal IL-6 mRNA expression levels \( (P<0.001) \), and renal Ngal mRNA expression levels \( (P<0.05) \) compared with hemoglobin injection alone. In addition, renal HO-1 protein expression was reduced, albeit not significantly, in mice treated with hemoglobin and hhep25 compared with hemoglobin treatment alone (Figure 4B). In conclusion, addition of hhep25 was able to abolish early kidney injury induced by hemoglobin.

Although a single injection of hhep25 was able to reduce renal ferroportin protein concentrations at 24 hours after injection (Figure 1C), there was no reduction in renal ferroportin protein concentration or Ferroportin mRNA expression after 24 hours when hhep25 was administered during hemoglobin–mediated kidney injury (Figure 5A). This suggests that hhep25 may exert its protective effects via other mechanisms. Possible mechanisms for hepcidin–mediated protection against AKI include cellular sequestration of reactive iron and/or enhanced excretion of luminal iron through binding to hepcidin.\(^{1,3,6–9}\) Urinary non-heme iron levels (Figure 5B) were significantly increased by hemoglobin treatment at 0–8 hours \( (P<0.05) \) but reduced again by additional hhep25 administration (not significantly). A nonsignificant trend toward an increase in renal non-heme iron levels was observed in single intravenous injection of human hemoglobin administered 4 hours after hhep25–treated mice, whereas plasma non-heme iron levels remained unaffected by any treatment (Figure 5B). Together, these data, although not significant, point in the direction that addition of hhep25 leads to renal iron sequestration rather than enhanced iron excretion.
Strikingly, combined administration of hhep25 and hemo-
globin resulted in hepcidin staining in nephron segments
lacking megalin (Figure 6A, arrows), which was not observed
in control, hemoglobin-treated, and hhep25-treated mice
(Figure 3, Supplemental Figure 2). Costaining with markers
for distal tubule segments showed that most of the hepcidin
colocalized with calbindin-D28K (distal convoluted tubule,
connecting tubule, and the first part of the collecting duct)
and parvalbumin (distal convoluted tubule) and to a lesser
extent, Tamm–Horsfall protein (thick ascending loop of
Henle). Moreover, renal Hamp1 mRNA expression levels
were significantly increased in hemoglobin groups treated
with hhep25 ($P_{0.05}$ and $P_{0.001}$) compared with control
(Figure 6B), suggesting hepcidin-1 synthesis. Urinary hepcidin-1
concentrations were also increased in mice given hhep25
4 hours after hemoglobin injection compared with control,
which was significant for the 9- to 24-hour urine ($P_{0.05}$)
(Figure 6C). However, it cannot be concluded that the

![Figure 2](image1)

**Figure 2.** Increased urinary excretion of hhep25 and isoforms hhep22 and hhep20 in succinylated gelatin-treated and megalin deficient mice. Urinary concentration per millimolar creatinine of (A) hhep25, (B) hhep22, and (C) hhep20 after 1 and 24 hours in wild-type mice (white bars), mice pretreated with succinylated gelatin (Gelo) (black bars), and megalin<sup>−/−</sup> mice and megalin<sup>−/−,Cre</sup> mice (checkered bars; n=4–5 per group). *$P_{0.05}$ using t test; **$P_{0.01}$ using t test.

![Figure 3](image2)

**Figure 3.** Filtered hepcidin is reabsorbed in proximal tubules via megalin-mediated endocytosis. Double staining of (A) megalin and hepcidin (indicated by arrows) and (B) megalin and Lotus Tetragonolobus Agglutinin (LTA)-FITC in wild-type and megalin<sup>−/−,Cre</sup> mice treated with hhep25 for 1 hour. D, distal tubule; G, glomeruli. Scalebar, 20 μm. *Megalin-deficient tubules without hepcidin.
increased urinary hepcidin excretion originated from the increased synthesis in the kidney, because plasma hepcidin-1 levels were also increased in these mice (Figure 6D). The increased level of systemically circulating hepcidin-1, however, may explain the hepcidin staining observed in the proximal tubules in the hemoglobin and hhep25-treated mice. Because administered hhep25 was shown before to be undetectable in renal sections by immunohistochemistry at 24 hours after injection (data not shown), the proximal hepcidin staining (Figure 6A) probably reflects reabsorption of increased hepcidin-1 levels in the ultrafiltrate. Taken together, it seems that both proximal reabsorption of systemic hepcidin and distal hepcidin synthesis may be involved in protection against hemoglobin-mediated AKI.

We hypothesize that the observed upregulation of distal nephron hepcidin-1 in the hemoglobin groups treated with hhep25 is caused by a competition of both hemoglobin and hhep25 for uptake by the proximal tubule via megalin, which causes more hemoglobin to pass through the nephron to the distal tubules, where it could induce Hamp1 mRNA expression. To test this hypothesis, we exploited two in vitro models. Using human conditionally immortalized proximal tubular epithelial cells (ciPTECs)30 that express megalin,31 we showed intracellular uptake of both hhep25 (20 nM) and Alexa546–labeled hemoglobin (Alexa-Hb; 5 nM) during 1 hour of incubation (Figure 7A). Incubation of ciPTECs with both compounds simultaneously for 1 hour resulted in significantly less uptake of both (P<0.001) compared with incubation with either one alone (Figure 7B), which indicates competition for uptake between hhep25 and hemoglobin. Alexa-Hb (1 nM) was also taken up by mouse cortical collecting duct cells (mCCDcl1s) (Figure 7C) by active transport as indicated by the significant increase in uptake compared with Alexa-Hb uptake at 4°C (Figure 7D). Moreover, incubation of mCCDcl1s with hemoglobin for 24 hours significantly increased mCCDcl1 Ho-1 mRNA expression (P<0.001) (Figure 7E), indicating intracellular metabolism of hemoglobin and increased Hamp1 mRNA expression (P<0.05).

DISCUSSION

Urinary hepcidin is suggested to have protective effects against AKI. This study aimed to get more insight in renal hepcidin handling and assess its protective potential against hemoglobin-induced AKI. Collectively, our results show, for the first time, that circulating hepcidin is filtered by the glomerulus and subsequently, reabsorbed in the proximal tubule cells via megalin but also, degraded in the tubular lumen. Furthermore, a protective effect of hhep25 against hemoglobin–induced early...
Kidney injury was shown, which may be mediated by mechanisms other than degradation of ferroportin. Moreover, our data suggest that both circulating filtered hepcidin that is reabsorbed in the proximal tubules and hepcidin synthesized in the distal parts of the nephron play a role in the defense against iron–mediated kidney injury. 

Administered hhep25 was rapidly excreted in urine and partly reabsorbed through megalin in the proximal tubules. Other than hhep25, we also detected its smaller isoforms hhep22 and hhep20 in urine. Because excretion of hhep22 and to a lesser extent, hhep20 was increased in mice treated with succinylated gelatin and megalin/f,Cre mice, it can be argued that hepcidin isoforms are also reabsorbed by megalin. Alternatively, the increase of the isoforms may be attributed to breakdown of the increased urinary hepcidin-25. Our previous observations support the notion that degradation of hepcidin into smaller isoforms is an active process, because ex vivo incubation of spiked hhep25 in urine did not yield any isoforms, whereas others have shown that liver and pancreas extracts were able to induce the formation of hhep20 and hhep22 from hhep25 through enzymatic reactions. 

Because the brush border membrane of the tubular epithelial cells contains many enzymes, including dipeptidylpeptidase IV, which has been shown to degrade hhep22 to hhep20, breakdown of hhep25 into isoforms may be facilitated within the tubular lumen. Moreover, because these isoforms retain the iron-binding properties in vivo, they could participate in protection of the kidney.

Twenty-four hours after administration, a single dose of hemoglobin resulted in early kidney injury as indicated by the rise in urinary markers NGAL and KIM-1 and renal mRNA expression levels of IL-6 and Ngal. Overt histologic damage was absent in our model, which is in line with the majority of AKI observed in humans. Hhep25 administered together with or 4 hours after hemoglobin injection ameliorated this early kidney injury. Protective effects of hepcidin administration in a mouse model of IRI have recently been described by Scindia et al. In their study, renal injury was more pronounced compared with that in our study, and higher concentrations of hepcidin were administered (50 versus 10 µg in our study). Scindia et al. observed a reduction in IRI-induced apoptosis, oxidative stress, and inflammatory cell infiltration when hepcidin was administered 24 hours before IRI, possibly mediated by hepatic iron sequestration, a reduction in renal ferroportin protein levels, and concomitant increased renal H-ferritin protein concentration. In contrast, although we could show decreased renal ferroportin expression 24 hours after injection of hhep25 alone, coexposure or hhep25 treatment shortly after hemoglobin had no effect on renal ferroportin expression. These differences may be explained by the disparity in severity of kidney injury, the timing of hepcidin administration, and the experimental model used. Future studies should explore whether hepcidin can act via distinct mechanisms to protect the kidney from injury depending on the timing of treatment and type of iron–mediated kidney injury.

A possible mechanism underlying our observations is that hhep25 may bind reactive iron during hemoglobin-induced AKI, which can be released from hemoglobin in the kidney after internalization in proximal tubule cells by catabolism, the urine because of acidic conditions, or the plasma by peroxidases. During hemoglobin-induced AKI, circulating and filtered hepcidin may bind iron, which is, subsequently, internalized by the proximal tubules and as such, promotes renal cell sequestration. This is supported by the decrease of hemoglobin-induced elevation of urinary non-heme iron concentration. Moreover, ferroportin protein expression is upregulated by intracellular iron concentrations through a post-translational mechanism that comprises binding of the iron-regulatory protein to the iron-responsive element present on ferroportin mRNA. Therefore, increased intracellular iron concentrations as a result of hemoglobin catabolism may counteract the hhep25-mediated degradation

Figure 5. Administration of hhep25 in hemoglobin-treated mice does not affect renal ferroportin expression and reduces urinary non-heme iron concentration. (A) Renal protein and mRNA expression of ferroportin and (B) non-heme iron levels in 0- to 8- and 9- to 24-hour urine, plasma, and kidney tissue. Representative blots are shown in A. Hb, hemoglobin. *P<0.05 compared with control by one-way ANOVA with Bonferroni multiple comparisons test.
of ferroportin. We did not observe any effect on renal ferroportin expression or non-heme iron levels by hemoglobin injection alone at 24 hours and may, therefore, have missed a transient upregulation of renal ferroportin early after hemoglobin injection. Overall, our data show that hhep25 blunts the hemoglobin-induced effects, including induction of kidney injury markers, renal HO-1 protein expression, and urinary non-heme iron concentrations. More studies are needed to elucidate the molecular mechanisms involved in hepcidin-mediated renal protection against hemoglobin and at multiple time points after hemoglobin administration.

Proximal tubules virtually do not synthesize hepcidin\textsuperscript{15} but reabsorb filtered circulating hepcidin, whereas the distal tubules can synthesize hepcidin\textsuperscript{14,15} but did not show uptake of the administered hhep25 in our studies. Interestingly, we observed that hhep25 injection in hemoglobin-treated mice led to a dramatic increase in renal Hamp1 mRNA expression and hepcidin immunostaining in the distal tubules. It has been postulated by Ho et al.\textsuperscript{1} that patients who cannot effectively upregulate their hepcidin response during IRI are more likely to develop clinically relevant AKI. In agreement with this clinical observation, it is possible that high urinary hepcidin levels

**Figure 6.** Increased renal hepcidin synthesis, urinary excretion and plasma concentration after hhep25 administration in hemoglobin-treated mice. (A) Representative images of distal tubule hepcidin staining (indicated by arrows) of a mouse treated simultaneously with hhep25 and hemoglobin (hhep25+hb) in colocalization with megalin, Tamm–Horsfall protein, parvalbumin, and calbindin-D28K. Scalebar, 20 μm. *Proximal tubule hepcidin staining. (B) Renal Hamp1 mRNA expression, (C) urinary hepcidin-1 excretion, and (D) plasma hepcidin-1 levels in control, hemoglobin-treated, hhep25+hb-treated, and hhep25 injection 4 hours after hemoglobin–treated (hb+hhep25) mice.*\textsuperscript{P}<0.05 compared with control by one-way ANOVA with Bonferroni multiple comparisons test; \textsuperscript{***}P<0.001 compared with control by one-way ANOVA with Bonferroni multiple comparisons test. \textsuperscript{#}P<0.05 compared with hemoglobin by one-way ANOVA with Bonferroni multiple comparisons test.
Figure 7. Competition between hhep25 and hemoglobin uptake in ciPTECs and hemoglobin-mediated induction of Ho-1 and Hamp1 in mCCDcl1s. (A) Uptake of hepcidin and Alexa-Hb in ciPTECs. Control indicates hepcidin staining in untreated ciPTECs. Scalebar, 50 μm. (B) Quantification of hepcidin and/or Alexa-Hb uptake in ciPTECs (n=3). (C) Intracellular uptake of Alexa-Hb in mCCDcl1s after 1, 4, 16, and 24 hours of incubation quantified in (D). mCCDcl1 mRNA expression of Ho-1 and Hamp1 (E) in controls (n=5) and after 24 hours of hemoglobin incubation (n=3). Hb, hemoglobin; hb+hhep25, simultaneous incubation with hemoglobin and hhep25. Scalebar, 30 μm. *P<0.05 compared with control or as indicated by one-way ANOVA with Bonferroni multiple comparisons test; **P<0.01 compared with control or as indicated by one-way ANOVA with Bonferroni multiple comparisons test; ***P<0.001 compared with control or as indicated by one-way ANOVA with Bonferroni multiple comparisons test.
originating from the circulation prevent injury in the first hours after the damaging insult, but subsequently, local hepcidin synthesis is required to maintain protection along the entire nephron. Indeed, we showed uptake of hemoglobin as well as hhep25 by proximal tubular epithelial cells and competition in uptake between both compounds. Moreover, we showed that the distal tubular epithelial cells of the cortical collecting duct take up and catabolize hemoglobin, which induces hepcidin synthesis. Our in vitro studies provide preliminary evidence for a potential coordinated interaction between proximal and distal tubules mediated by hepcidin during hemoglobin–mediated kidney injury, but future studies are warranted to further unravel such dynamics during AKI.

Overall, our observations serve as a basis for improved insights of the dynamics of hepcidin during CABG and AKI. The next step is to investigate the protective molecular mechanism of hepcidin in iron–mediated kidney injury and determine the role of locally synthesized hepcidin in kidney injury by unraveling the driving forces behind its transcription. Together, these insights will provide novel leads for protection against iron–mediated kidney injury.

**CONCISE METHODS**

**Animal Experiments**

All experiments were approved by the local Animal Welfare Committee of the Radboudumc (DEC 2012–293) in accordance with the guidelines of the Principles of Laboratory Animal Care (National Institutes of Health). Male C57Bl/6N mice (8–11 weeks of age; Charles River Laboratories, Wilmington, MA) were housed under controlled conditions and randomly assigned to a treatment group. Unless stated otherwise, all experiments were carried out with five mice per group. Heterozygous megalin–deficient mice (ApoE Cre /gp330 fl/fl) were obtained from the Max Delbrück Center for Molecular Medicine (Berlin, Germany) and bred with C57Bl/6N mice to obtain homozygous megalin–deficient mice (megalin- ...

Synthetic hhep25 (Peptide International) was dissolved in saline, and 10 μg were administered to each animal via intraperitoneal injection. To investigate megalin–mediated renal hhep25 reabsorption, succinylated gelatin (Gelofusin; B.Braun Medical) was used to block the megalin receptor by injection of 4 mg in the tail vein 5 minutes before hhep25 injection in two additional groups. Succinylated gelatin alone (4×10 mg during 24 hours) was injected via intraperitoneal injection. Human hemoglobin (Sigma-Aldrich, St. Louis, MO) was dissolved in saline (20 mg/ml) and injected via the tail vein (250 μl per mouse).

Twenty-four-hour urine samples were collected at baseline or immediately after treatment by means of metabolic cages (Techniplast GmbH) with pulverized standard chow and water ad libitum. Urine samples collected 1 hour after treatment were directly taken from the bladder. Protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN) were added to urine samples, which were centrifuged for 10 minutes at 3000×g to remove debris. Mice were anesthetized with isoflurane and O₂, and blood was taken directly from the heart. Kidney and liver tissues were collected in liquid nitrogen and stored at −80°C for protein and mRNA isolation and in 4% formalin O/N before embedding in paraffin for histochemistry.

**Genotyping Megalin-Deficient Mice**

Mouse ear biopsies were taken after weaning, and HotShot DNA extraction was performed as described elsewhere. Primers for ApoE C57Bl/6N included forward 5′-CCCAAGAAGAGGAGGGTG-3′ and reverse 5′-GCTGGCCCAAATGTGGCTG-3′. The ApoE Cre PCR protocol was as follows: 4 minutes at 95°C; 32 cycles of 30 seconds at 95°C, 30 seconds at 56°C, and 30 seconds at 72°C; and 10 minutes at 72°C. The ApoE Cre PCR resulted in a product of approximately 300 bp, which was visualized on a 1.5% agarose gel. For gp330 fl/fl, we used three primers: F4a (5′-AACCTGGGACGACCCGTGGATGTC-3′), B4 (5′-GCAAGAAGATGTGGTGAAGACCAAC-3′), and TKP2 (5′-TGAAAACACCTGCTGATCGGGAAC-3′). The gp330 fl/fl Cre PCR protocol was as follows: 3 minutes at 95°C; 36 cycles of 30 seconds at 95°C, 40 seconds at 60°C, and 25 seconds at 72°C; and 10 minutes at 72°C. On a 1.5% agarose gel, two products can be visualized: a 480-bp band for the wild-type allele and/or a 550-bp band for the floxed allele.

**Creatinine Determination**

Urinary creatinine concentration was determined using the assay kit on the basis of the Jaffé method from Labor & Technik (LT-SYS 0251).

**Hepcidin Analyses**

The concentrations of hhep25 and also, endogenous mouse hepcidin-1 were determined in urine, plasma, and kidney homogenate using mass spectrometry. Briefly, 50 μl acetonitril (ACN) was added to a 25-μl sample, to which 5 μl internal standard (0.1 μM synthetic hhep-24; custom made; Peptide International) was added. The solution was mixed and centrifuged at 27,500×g for 5 minutes; 50 μl supernatant, 25 μl weak cation exchange beads (Macro-Prep Support Beads; Bio-Rad, Hercules, CA), and 150 μl binding buffer were combined and mixed thoroughly, and hepcidin was allowed to bind to the beads by incubation for 15 minutes on the rollerbank at RT. Beads were washed three times with 150 μl wash buffer before hepcidin was eluted from the beads using 50 μl elution buffer (50% ACN and 2% TFA) for 15 minutes on the rollerbank. Of the prepared sample, 1.5 μl were spotted onto an MSP 96 Polished Steel Target Plate (Bruker Daltonics) followed by 1.5 μl energy-absorbing matrix (5 μg/ml molybdenum-4-hydroxy cinnamic acid; Bruker Daltonics). Plasma samples were pretreated with ACN but without beads. The supernatant obtained after centrifugation was spotted directly on the target plate. The sample and matrix were dried in N₂ atmosphere and measured using matrix–assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics). Hepcidin was measured using the following settings: positive linear ion mode; 500 laser shot; initial laser power, 55%; offset, 30%; range, 30%; pulsed ion extraction, 250 ns; and mass range, 2000–10,000 m/z.

The concentration of hhep25 was calculated by comparing its mass peak height with that of the internal standard, which had a final concentration of 10 nM. Because the internal standard is of human
RNA Isolation and Quantitative PCR
Frozen kidney and liver tissues were homogenized using a Mikro Dismembrator U (Sartorius Stedim) before RNA isolation by means of TRIzol (Life Technologies, Carlsbad, CA) according to manufacturer’s instructions.

Quantitative PCR was performed on a CFX96 (Bio-Rad) using 4 μl cDNA (10 ng/μl), 10 μl SYBR Green Mastermix (2×; Applied Biosystems, Foster City, CA), and 6 μl Primersmix (containing 5 μl 100 μM forward primer, 5 μl 100 μM reversed primer, and 490 μl sterile water). Primers used are β-actin (housekeeping gene) forward 5′-GCTATGCTTCCCT- CAGCCA-3′; β-actin reversed 5′-CTCTTGTAGTTCAGCAGAT-3′; Hamp1 forward 5′-TTGCGATACCAATGCGACAAT-3′; Hamp1 reversed 5′-GGATGTGGCTCTAGGCTATGTT-3′; Megalin forward 5′-CCCTCTCCACCTGCGACAAT-3′; Megalin reversed 5′-TCCCGGAGTGTCAGCATCTC-3′; Ngal forward 5′-GCCTCAAG- GACGACAACTCA-3′; Ngal reversed 5′-TTCTCTGTCCCCACCGA- CCAAATGC-3′; IL-6 forward 5′-GAGATACACTCCTCACCAGACC-3′; IL-6 reversed 5′-AAGTGACATCTGTTGCATACA-3′; Ferroportin forward 5′-TCATACCAGGAGATGATGATCC-3′; and Ferroportin reversed 5′-CAAATGTCAATCTGGC-3′. The PCR protocol was as follows: 7 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with a measurement at the end of each cycle. Fold change values compared with control or baseline were calculated with the 2−ΔΔCT formula.

Protein Isolation and Western Blot
Frozen kidney tissue were homogenized using a Mikro Dismembrator U (Sartorius Stedim) before western blot analysis by means of ImageJ software.

Immunohistochemistry
Tissue sections were embedded in paraffin, and 4-μm sections were mounted on APES-coated glass slides. Periodic acid—Schiff staining was used for kidney sections for gross histology. Images were taken using the VisionTekDigital Microscope (Sakura). For double staining of hepcidin and the various markers, kidney sections were deparaffinized, and an antigen retrieval step was performed using citrate buffer (pH6) followed by washing with 0.1% PBS-Tween. Sections were then incubated with 1% BSA in PBS-Tween for 1 hour followed by the first primary antibody, a rabbit polyclonal antihepcidin antibody (ab30760; Abcam, Inc., Cambridge, MA), in a 1:200, 1:100, or 1:75 dilution of O/N at 4°C or 1 hour at RT. The first secondary antibody, a goat anti–rabbit Alexa Fluor 568 (Invitrogen), was added in 1:200 dilution for 30 minutes. Lotus Tetragonolobus Agglutinin FITC (Vector Laboratories, Burlingame, CA) was added at this step at a 1:100 dilution. Sections were incubated O/N at 4°C or for 1 hour at RT with the second primary antibody, namely goat polyclonal antimegalin antibody (sc16478; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500, sheep anti–human Tamm–Horsfall (BT85–9500–54; Bio Trend) diluted 1:2000, goat anti–human parvalbumin (7449; Santa Cruz Biotechnology) diluted 1:2500, or mouse anti–bovine calbindin-D28K (C9848; Sigma-Aldrich) diluted 1:8000. The following secondary antibodies were used diluted 1:200: donkey anti–goat Alexa Fluor 488 antibody (A-11055; Invitrogen), biotin–SP–conjugated goat anti–mouse (115–066–003; The Jackson Laboratory), biotin–SP–conjugated donkey anti–goat (705–065–147; The Jackson Laboratory), and biotin–SP–conjugated donkey anti–sheep (713–066–147; The Jackson Laboratory). The signal of biotin–conjugated secondary antibodies was amplified with a fluorescent label using the Renaissance TSA Kit (PerkinElmer, Waltham, MA) according to the manufacturer’s instructions. Finally, for nuclear staining, DAPI (1:1000) was added to the sections for 5 minutes. Sections were fixed with fluorescent mounting medium (DAKO), dried, and stored at 4°C in the dark. Fluorescent staining was visualized using an Apotome.2 FL Microscope (Carl Zeiss).
ELISA
The concentrations of NGAL and KIM-1 were determined in mouse urine samples using the DuoSet ELISA Development Kits from R&D Systems (Minneapolis, MN; DY1857 for NGAL and DY1817 for KIM-1) according to the manufacturer’s protocol.

Cell Culture Experiments
The ciPTEC line was generated and cultured as described previously.30,49 For each experiment, cells were cultured at 33°C to 40% confluency followed by maturation for 7 days at 37°C in a 5% (vol/vol) CO₂ atmosphere. Experiments were performed on cells with passage number between 33 and 50.

The mCCDcl1 line was established by Rossier and coworkers50 and cultured as described. Cells were used for experiments between passage 26 and 44.

To study uptake of hemoglobin in ciPTECs and mCCDcl1s, human hemoglobin (Sigma-Aldrich) was labeled with an Alexa Fluor 546 Protein Labeling Kit (Invitrogen) according to the manufacturer’s instructions. Cells were grown on glass coverslides and incubated with hhep25 (Peptide International), Alexa-Hb, or vehicle in serum free medium for the indicated time points at 37°C or 4°C. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% TritonX-100 (ciPTEC) or 1% SDS (mCCDcl1). For detection of hhep25, a rabbit polyclonal antihemoglobin antibody (ab30760; Abcam, Inc.) diluted 1:100 was applied for 2 hours at RT followed by 1:500 rabbit Alexa Fluor 488 (A11008; Invitrogen) for 1 hour at RT. Cells were counterstained with 300 nM DAPI (Invitrogen; for ciPTEC) or 0.8 µM Hoechst 33342 (mCCDcl1) for 5 minutes at RT. Uptake of hhep25 and/or Alexa-Hb was quantified using ImageJ (ciPTEC) or Metamorph (mCCDcl1) software as described previously.31

To determine whether hemoglobin exposure induced HO-1 and hepcidin mRNA synthesis, mCCDcl1s were incubated with 1 and 10 µM hemoglobin for 24 hours in serum free medium at 37°C. Quantitative PCR was performed as described above using the following primers: β-actin forward 5′-GCTATGCTTCCCCAGGCA-3′; β-actin reversed 5′-CTCTTTGATGTCACGCAGAT-3′; Hamp1 forward 5′-TTGGCATACCAATGCGAAG-3′; Hamp1 reversed 5′-GGATGTTGCTCTAGGCTATGT-3′; Ho-1 forward 5′-CTTGTGAGTGGAGAGGAT-3′; and Ho-1 reversed 5′-CCAGAGTGGTCAATCCAGACA-3′.

Statistical Analyses
Data were statistically analyzed using GraphPad Prism 5.03 software (GraphPad Software, La Jolla, CA) and presented as means ± SEMs. Results were analyzed for statistically significant differences using the t test or one-way ANOVA with post hoc analysis wherever appropriate. A P value of <0.05 was considered statistically significant.

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DISCLOSURES
R.P.L.v.S., C.M.M.L., and D.W.S. are managing director, technician, and medical director, respectively, of the Hepcidinanalysis,cominitiative, which aims to serve the scientific and medical communities with high-quality hepcidin measurements (www.hepcidinanalysis.com).

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