The iron-regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney

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Abstract

It is generally accepted that iron homeostasis is mainly controlled in the gastrointestinal tract by absorption of dietary iron. However, recent studies have shown that the kidneys are also involved in iron metabolism. Since the iron-regulatory and antimicrobial peptide hormone hepcidin was originally isolated from human urine we have investigated the expression as well as the zonal and cellular localization of hepcidin in the mammalian kidney and developed an ELISA assay to analyze hepcidin concentrations in serum and urine. The expression of hepcidin was shown by RT-PCR and immunoblot experiments; its cellular localization was studied by immunocytochemistry in human, mouse and rat kidney, which revealed similar patterns of immunoreactivity. Hepcidin expression was absent from the proximal tubule and descending and ascending thin limbs. There was strong expression in the thick ascending limb of the cortex and in connecting tubules. Moderate expression was noted in the thick

ascending limb and collecting ducts of the medulla and in collecting ducts of the papilla. Importantly, the cells of the macula densa were unstained. At the cellular level, hepcidin was localized to the apical cell pole of the renal epithelial cells. Based on its presence in urine, hepcidin may be released apically into the urine. Enhanced levels of hepcidin were determined in patients with chronic renal insufficiency (156.8 ng/ml, controls 104.2 ng/ml) indicating that the kidneys may metabolize and/or eliminate the circulating peptide. From the expression of hepcidin in the mammalian kidney, we have concluded that the iron-regulatory hormone is an intrinsic renal peptide which is not only eliminated by the kidney but is also synthesized in the kidney tubular system. Localization of hepcidin in the kidney implicates an iron-regulatory role of this peptide hormone in the renal tubular system, possibly in connection with the iron transporter divalent metal transporter-1.

Journal of Endocrinology (2005) **184**, 361–370

Introduction

Hepcidin is a recently discovered peptide hormone that was isolated from human urine (Park *et al.* 2001) and blood (Krause *et al.* 2000) and was found to exhibit antibacterial and antifungal activity. From human urine, two predominant forms, hepcidin-20 and hepcidin-25, differing by amino-terminal truncation, were characterized as 20 and 25 amino acid (aa) residues with 8 cysteines connected by intramolecular disulfide bonds (Park *et al.* 2001). A hepcidin cDNA encoding an 83 aa precursor in mice and an 84 aa precursor in rat and man, including a putative 24 aa signal peptide, was subsequently identified when searching for liver-specific genes that were regulated by iron (Pigeon *et al.* 2001). Hepcidin expression is abolished in mice exhibiting iron overload due to the targeted

disruption of the upstream stimulatory factor 2 gene resembling the phenotype of *hfe* – / – mice (Nicolas *et al.* 2001). In contrast, overexpression of hepcidin was shown to result in severe iron deficiency anemia in transgenic mice (Nicolas *et al.* 2002a), indicating that hepcidin plays a pivotal role in iron metabolism. Recent studies have found abnormal hepcidin expression and disrupted hepcidin regulation (Bridle *et al.* 2003, Kulaksiz *et al.* 2004) in hemochromatosis gene (HFE)-associated hemochromatosis and association of hepcidin mutations with severe juvenile hemochromatosis (Roetto *et al.* 2003). Based on these observations, it has been suggested that hepcidin is a key component of iron homeostasis that acts as a negative regulator of iron absorption in the small intestine and of iron release from macrophages (Nicolas *et al.* 2001, 2002a).

While the majority of studies have concentrated on the regulation and function of this peptide hormone in the liver as the major site of hepcidin production (Park *et al.* 2001, Kulaksiz *et al.* 2004), accumulating data suggest that this bioactive peptide may also play a role in the kidney and urinary tract (Ferguson *et al.* 2003, Wareing *et al.* 2003, Kulaksiz *et al.* 2004). It is widely believed that iron homeostasis is mainly controlled in the gastrointestinal tract at the level of uptake from the diet. The current dogma is that there is no excretory route for iron in the organism. However, recent studies have shown that the kidney plays an important role in iron transport (Gunshin *et al.* 1997, Ferguson *et al.* 2003, Wareing *et al.* 2003); a significant proportion of iron in serum is available for ultrafiltration by the glomerulus and the majority of iron filtered at the glomerulus is reabsorbed (Wareing *et al.* 2000).

Hence, it is reasonable to analyze whether hepcidin is also present in the kidney as a local peptide. We have raised antisera against various epitopes of the hepcidin precursor molecule and investigated the expression of hepcidin in the kidney of three mammalian species at RNA and protein level. Our findings indicate that, beside its elimination in the kidney, hepcidin is also produced as an intrinsic peptide in the tubular cells of the mammalian kidney. Its presence at the apical cell pole suggests that it might be released lumenally into the urine, implicating a regulatory role for hepcidin in the kidney and/or urinary tract.

Materials and Methods

Tissues and tissue preparation

Human kidney samples ($n=5$) were obtained after kidney resection in adult patients with hypernephroma. Human liver samples ($n=7$) were obtained after hemihepatectomy in adult patients with liver metastases (Kulaksiz *et al.* 2004). The ethics committee of University Hospital Heidelberg approved the study, and experiments were undertaken in accordance with the Helsinki Declaration of 1975. All patients had given written permission for the use of the tissue for research purposes. Healthy tissues were fixed in 4% paraformaldehyde or in Bouin's fixative for immunohistochemistry or immediately frozen in liquid nitrogen for RT-PCR and immunoblot.

For RT-PCR and immunoblot analysis, rats ($n=5$) and mice ($n=5$) were anesthetized and killed by cervical dislocation. For biochemical analysis, tissue specimens from kidney and liver were obtained and immediately frozen in liquid nitrogen. For immunohistochemical analysis, kidneys of rats and mice were perfused retrogradely through the abdominal aorta using phosphate-buffered saline (PBS) adjusted to 330 mosmol/kg H₂O with sucrose, pH 7.4, for 20 s. Next, a solution of 3% paraformaldehyde in PBS was infused for 5 min, followed

by PBS adjusted to 330 mosmol/kg H₂O for 1 min. Kidneys were removed and protected from freezing artifacts by subsequent overnight immersion in PBS adjusted to 800 mosmol/kg H₂O by sucrose (pH 7.3), shock-frozen and stored at -80°C for subsequent cryostat sectioning.

Peptide synthesis, immunization procedure and antibodies

From the published pro-hepcidin sequence (Krause *et al.* 2000, Pigeon *et al.* 2001), the peptides hepcidin-(28–47) and hepcidin-(70–84) were synthesized as C-terminal amides using a standard Fmoc (N- α -(9-fluorenylmethyloxycarbonyl)) protocol (Kulaksiz *et al.* 2002a,b). Peptides were coupled to keyhole limpet hemocyanin using m-maleimidobenzoyl-N-hydroxy-succinimide ester, and two SPF rabbits (Charles River-Iffa Credo, Wilmington, MA, USA) were immunized with each peptide conjugate (Eurogentec, Seraing, Belgium). The antibodies EG(1)-HepC, EG(2)-HepC (each directed against pro-hepcidin-(70–84)) and EG(1)-HepN and EG(2)-HepN (each directed against pro-hepcidin-(28–47)) have been generated and characterized elsewhere (Kulaksiz *et al.* 2004).

The antibodies employed for identification of kidney structures have been characterized and used previously (Tian *et al.* 2004).

Expression analyses in the kidney

Based on the GenBank cDNA sequences, the following primers were constructed and used: human hepcidin (database accession number NM021175) given in 5'–3' orientation, 5'–CTG CAA CCC CAG GAC AGA G–3' and 5'–GGA ATA AAT AAG GAA GGG AGG GG–3'; rat hepcidin (number NM 053469), 5'–ACA GAA GGC AAG ATG GCA CT–3' and 5'–GAA GTT GGT GTC TCG CTT CC–3', mouse hepcidin-1 (number NM 032541), 5'–CGA TAC CAA TGC AGA AGA GAA GG–3' and 5'–TTC AAG GTC ATT GGT GGG GA–3'. The primers displayed no homology to any previously reported sequences.

RNA isolation was performed using Qiagen (Valencia, CA, USA) RNeasy kit including DNA digestion. RT-PCR analysis was performed as described previously (Kulaksiz *et al.* 2002a,b). After an initial denaturation of 94°C for 4 min, reactions were subjected to 30 cycles of the following thermal program: 94°C for 30 s, 60°C for 30 s and 72°C for 30 s; this program was followed by a final 5-min elongation step at 72°C . Amplification products were run on an ethidium bromide-stained 1.8% 89 mM Tris/89 mM boric acid/2 mM EDTA (pH 8.3) agarose gel. As a control for specificity, the amplified PCR products were sequenced by MWG-Biotech (Ebersberg, Germany).

Table 1 Precision of the ELISA

	Intra-assay			Interassay		
	<i>n</i>	Mean $\pm$ S.D. (ng/ml)	C.V. (%)	<i>n</i>	Mean $\pm$ S.D. (ng/ml)	C.V. (%)
Serum						
1	12	426.7 $\pm$ 20.2	4.69	23	431.96 $\pm$ 20.8	4.82
2	12	210.7 $\pm$ 8.58	4.07	23	216.17 $\pm$ 14.44	6.68
3	12	110.7 $\pm$ 4.74	4.28	23	109.8 $\pm$ 10.72	9.76

Immunoblot analysis

Immunoblot experiments were performed on 16.5% tricine–SDS–polyacrylamide gels. Proteins from human, mouse and rat kidney and liver, as well as from human urine (50 ml for each experiment) were extracted according to the published protocols (Kulaksiz *et al.* 2002a,b, 2004). Following electrophoresis, proteins were transferred onto hydrophobic polyvinylidene fluoride-based membranes (Pall, Portsmouth, Hants, UK) by semidry blotting. The membranes were incubated overnight with hepcidin antibodies diluted 1:1000. After washing in Tris–buffered saline containing 10 mM Tris–HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20, immunoreactive proteins were visualized after incubation with alkaline phosphatase-conjugated goat anti-rabbit antibody (diluted 1:50 000; Sigma) using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogens (Sigma). The immunoreaction on the immunoblot was specifically blocked after preincubation of the antibodies with the corresponding peptide immunogens. Cross-reactivity with the second goat anti-rabbit antibody was excluded by appropriate controls (Kulaksiz *et al.* 2002a,b).

Immunocytochemical protocol

Five micrometer thick cryostat sections were used. After blocking with 5% skim milk in PBS (pH 7.4) for 1 h at room temperature, sections were incubated with primary antibody for 2 h at room temperature and then overnight at 4 °C, followed by incubation with fluorochrome-coupled secondary antibody. For double-labeling, suitable secondary antibodies coupled to different fluorochromes were applied (Dianova, Hamburg, Germany), or serial sections were used. Slides were evaluated with a digital camera (Spot; Diagnostic Instruments), processed with MetaVue software (Universal Imaging; Visitron, Puchheim, Germany) and viewed with a Leica light microscope equipped with an high pressure fluorescence lamp.

Immunoperoxidase staining Tissues were fixed in 4% paraformaldehyde for 18 h at 4 °C and embedded in paraffin. Paraffin sections (4–5 μ m) were immunostained for hepcidin (antibodies EG(1)–HepN, EG(2)–HepN,

EG(1)–HepC and EG(2)–HepC, each diluted 1:2000) by the avidin–biotin–peroxidase complex technique as detailed elsewhere (Kulaksiz *et al.* 2002a,b, 2004).

With respect to the immunoreactivities obtained, non-specificities in the immunohistochemical protocol were excluded by running the following controls: (a) omission of single steps in the immunohistochemical protocol, (b) addition of polylysine (M_r 15 000; 2 mg/ml; Sigma) to the first antibody and (c) use of high molar (0.5 M) PBS as a rinsing solution between the various steps of the immunohistochemical protocol. Antibody specificities were tested by preadsorption of the antibodies with homologous and heterologous antigenic peptides (6.25–100 μ g/ml of the antiserum) (Kulaksiz *et al.* 2002a,b). Preadsorption of the antibodies with homologous antigens at concentrations as low as 6.25 μ g/ml completely blocked immunostaining in the kidney, while preadsorption of the antibodies with heterologous antigens at concentrations up to 100 μ g/ml had no effect on immunostaining.

Hepcidin ELISA competitive binding assay

The ELISA has been characterized and used previously (Kulaksiz *et al.* 2004).

The analytical sensitivity was calculated from the mean minus 2 S.D. (S.D.=0.055) of 21 replicates ($n=21$) analysis of zero standard. The sensitivity of the assay is 3.95 ng/ml. Precision, recovery tests and dilution tests are described in Tables 1–3 respectively.

Serum and urine samples were obtained from 22 individuals (11 women and 11 men, aged 23–59 years, mean 39 years) and serum samples were obtained from 22 patients with renal insufficiency undergoing chronic

Table 2 ELISA recovery test

	Endogenous hepcidin (ng/ml)	Average recovery (%)	Range of recovery (%)
Serum			
1	273.8	93.1	91.8–94.3
2	116.8	94.7	89.2–98.7
3	82.3	97.1	94.5–105.7

Table 3 ELISA dilution test

	Endogenous hepcidin (ng/ml)	Average recovery (%)	Range of recovery (%)
Serum			
1	591.6	99.1	90.6–108.2
2	157.5	107.9	106.3–107.2
3	179.4	104.6	92.3–111.6

hemodialysis (11 women and 11 men, aged 25–77 years, mean 48 years). All patients with chronic renal insufficiency were treated two to three times a week with 3000 IU recombinant human erythropoietin (Hoffmann-La Roche, Grenzach-Whylen, Germany). During sample collection, healthy volunteers and patients had no infection and no bleeding. Ten milliliter blood samples were withdrawn into serum tubes and 10 ml urine samples were also collected in urine tubes and centrifuged at 2500 *g* for 10 min at 4 °C. Determinations were performed in duplicate using 96-well microtiter plates as described (Kulaksiz *et al.* 2004). In brief, microtiter plates were coated with 200 µl/well rabbit anti-hepcidin antibody EG(2)-HepN diluted 1:4000. Fifty microliter standards containing various amounts of synthetic peptides (0, 20, 100, 500 and 1000 ng/ml) or human serum and urine samples and 150 µl N-terminally biotinylated hepcidin-(28–47) (Peptide Speciality Laboratories GmbH, Heidelberg, Germany; 2 ng/well) were added to each well and incubated for 1 h at room temperature. After washing with TBS with 0.05% Tween 20, the biotinylated antigen-antibody complexes were detected by streptavidin-peroxidase enzyme (Dako, Hamburg, Germany) with the substrate tetramethylbenzidine (DRG Instruments GmbH, Marburg, Germany); the color reaction was

stopped with 1 M H₂SO₄ and the extinction of the solution was read at 450/630 nm wavelength.

Statistics

Possible differences between the groups were analyzed using the ANOVA method with pairwise *t*-tests after global evaluation. Differences were considered as significant at *P*<0.05.

Results

Expression of hepcidin in the mammalian kidney

RT-PCR analysis revealed clear expression of hepcidin not only in the liver (positive control, data not shown; see Kulaksiz *et al.* 2004), but also in human, rat and mouse kidney (Fig. 1). A 192 bp expected PCR product for human, a 193 bp product for mouse and a 201 bp product for rat were detected in the liver (data not shown) and kidney of these species. The sequence analysis revealed that the PCR-generated products had a complete homology with the cDNAs of the corresponding peptides.

At the translational level, the presence of hepcidin was confirmed by immunoblot studies with region-specific antibodies (Fig. 1). Antisera directed against the C and N terminus of the hepcidin precursor molecule coincidentally identified an immunoreactive band of ~9.5 kDa in extracts of human, rat and mouse kidney.

Cellular localization of hepcidin

Identification of kidney structures For a precise assignment of hepcidin synthesis to identified portions of the renal tubule, we used morphological criteria as well as the segmental coexpression of defined markers (Bachmann

Figure 1 (A) RT-PCR analyses of human (lane 2), mouse (lane 3) and rat (lane 4) kidney hepcidin expression. A bp DNA ladder is indicated (lanes 1 and 5). (B and C) Immunoblot analyses of hepcidin in extracts of human (lanes 1), rat (lanes 2) and mouse (lanes 3) kidney, as well as in human urine (lanes 4) with (B) N-terminal and (C) C-terminal antibodies. Note the immunoreactive bands at 9.5 kDa obtained with both antibodies recognizing different epitopes in the hepcidin precursor. (Molecular mass markers used: phosphorylase B, 105 kDa; glutamic dehydrogenase, 53 kDa; carbonic anhydrase, 34 kDa; myoglobin-blue, 23 kDa; myoglobin-red, 17 kDa; lysozyme, 13 kDa; aprotinin, 7 kDa; insulin, 3 kDa.)

et al. 1999, Campan *et al.* 2001). The proximal tubule was recognized by the presence of megalin and brush border; thin limbs and vasa recta by their location within the vascular bundles; medullary and cortical thick ascending limb (mTAL and cTAL respectively) by the presence, and macula densa by the absence, of Tamm-Horsfall protein (THP); distal convoluted tubule (DCT) by the presence of the $\text{Na}^+\text{--Cl}^-$ cotransporter (NCC); and parts of the DCT and the connecting tubule (CNT) by

calbindin. Collecting ducts were identified by the presence of aquaporin-2 as well by the presence of intercalated cells.

Localization The distribution of immunoreactive hepcidin was analyzed using region-specific antibodies against the C-terminal and N-terminal portions of hepcidin. Staining with these antibodies showed an apical immunoreactive signal in the mTAL and cTAL and in the medullary collecting duct (MCD) of rats and mice. A more cytosolic localization was detected in the CNT and cortical collecting duct (CCD). Along the nephron, the onset of the hepcidin localization began with the onset of mTAL at the boundary between the inner medulla and the inner stripe of the outer medulla (Fig. 2A) as identified by the co-localization with THP (Fig. 2B). Signal continued the mTAL along the cortical border. The signal was faint but apically enhanced within the mTAL cells (arrows in Fig. 2A). In the cortex, stronger hepcidin immunoreactivity was found along some of the cTAL segments (Fig. 2C), but some were negative. In the positive cTAL portions, the signal ended at the transition to the DCT (Fig. 2C and D). Macula densa cells of the juxtaglomerular apparatus were identified by their microanatomical location and by the absence of THP; hepcidin was principally absent from these cells (Fig. 2E and F). Immunostaining of the DCT, as identified by staining with antibody to NCC in consecutive sections, was negative for hepcidin (Fig. 2G–K). These results were found with N- and C-terminal primary anti-hepcidin antibodies.

Antibody to calbindin, showing the signal beginning with the terminal DCT (DCT2 in rats; for review see Bachmann *et al.* 1999; data not shown) and continuing to the transition of the CNT to the collecting duct (Fig. 3A–D), showed an overlap with anti-hepcidin as revealed by double-staining in the rat and the mouse using both N- and C-terminal antibodies. Both hepcidin and calbindin were located only in the principal cells, whereas the intercalated cells were negative (arrowheads in Fig. 3E and F). The localization of hepcidin continued within the

Figure 2 Distribution of immunoreactive hepcidin in rat kidney ((A and I) C-terminal hepcidin antibody and (C, E and G) N-terminal hepcidin antibody)). For segment identification, double staining with anti-THP (B, D and F) and staining in consecutive sections with anti-NCC (H and K) are shown. (A and B) Inner stripe of medulla showing faint hepcidin labeling at the luminal aspect (arrowheads) in TAL segments which are THP positive. A strong signal of MCD segments is shown in collecting ducts (see also Fig. 3). (C and D) The white lines indicate the junction between hepcidin- and THP-positive post-macula segment of TAL and hepcidin-negative, weakly THP-positive DCT (asterisks). (E and F) Hepcidin-positive, THP-positive TAL, opposite to macula densa (between the lines) showing the absence of hepcidin and THP. (G–K) Hepcidin-positive, NCC-negative staining of TAL between the lines and hepcidin-negative, NCC-positive staining of DCT (asterisks). Immunofluorescence microscopy using different fluorochromes. Magnification approximately $\times 400$.

CCD and outer medullary collecting duct (OMCD) principal cells as shown by serial sectioning using anti-aquaporin-2 (Fig. 3 G and H). In the papillary tip, anti-hepcidin labeling presented a weak apical staining in the inner medullary collecting duct (IMCD) cells as evaluated by double-labeling with aquaporin-2 (Fig. 3I and K).

In human tissue, the localization of hepcidin revealed the same distribution pattern as in rats and mice. Positive staining with hepcidin antibodies showed labeling in the cTAL portion located opposite to the macula densa, as pointed out by arrowheads in Fig. 4A, whereas the macula densa cells were unreactive. Hepcidin-positive segments were also found in the vicinity of the cortical interlobular vein, suggesting that they were CNT segments (Fig. 4B and C).

The distribution of immunoreactive hepcidin across the investigated species is summarized in Fig. 5.

Detection of hepcidin prohormone in serum and urine

A stable hepcidin ELISA assay with high reproducibility and sensitivity was developed with the specific N-terminal hepcidin antibody EG(2)-HepN (Kulaksiz *et al.* 2004). This antibody identifies pro-hepcidin; no ELISA assay exists at this time to detect the biologically active hepcidin-25. The ELISA employed revealed that pro-hepcidin is present in human serum. Pro-hepcidin was measured in the range from 68.5 to 139.2 ng/ml (means $\pm$ S.E.; 104.2 ± 19.5 ng/ml) in the serum of healthy subjects. The pro-hepcidin concentrations varied from 63.9 to 327.3 ng/ml (means $\pm$ S.E.; 156.8 ± 61.9 ng/ml) in the serum of patients suffering from chronic renal insufficiency and were significantly increased compared with that in the control group. Using the sensitive hepcidin ELISA, pro-hepcidin was detected in human urine from the control group in the range from 13.9 to 456.0 ng/ml (means $\pm$ S.E.; 180.1 ± 94.8 ng/ml). The presence of pro-hepcidin in human urine was also confirmed by immunoblot analysis. Hepcidin antisera identified in extracts of human urine a single immunoreactive band of ~ 9.5 kDa molecular mass that co-migrated exactly with the immunoreactive hepcidin in kidney tissues (Fig. 1).

Discussion

The recently discovered hormone hepcidin represents an antimicrobial peptide and a central regulator of iron

Figure 3 Distribution of immunoreactive hepcidin in rat kidney ((A and I) N-terminal hepcidin antibody and (C, E and G) C-terminal hepcidin antibody). For segment identification, double staining with anti-calbindin (B, D and F), and staining in consecutive sections with anti-aquaporin-2 (AQP2) (H and K) are shown. (A–D) The white lines indicate the junction between mildly to strongly hepcidin-positive, strongly calbindin-positive CNT and weakly hepcidin-positive, calbindin-negative CCD (asterisks). (E and F) Hepcidin-positive, calbindin-positive CNT profiles showing absence of staining in intercalated cells (arrowheads). (G and H) Hepcidin-positive, AQP2-positive OMCD profiles showing absence of staining in intercalated cells (arrowheads). (I and K) Hepcidin-positive, AQP2-positive IMCD profiles. Both signals are apically enhanced. Immunofluorescence microscopy using different fluorochromes. Magnification approximately $\times 400$.

Figure 4 (A and B) Distribution of immunoreactive hepcidin in human kidney using antibody to the N-terminal (A and B) and C-terminal (C) sequence. (A) Hepcidin-positive TAL (arrowheads) opposite to hepcidin-negative macula densa. (B) Hepcidin-positive CNT segments next to a cortical interlobular vein (asterisk). Staining is apically enhanced. (C) Hepcidin-positive distal tubules. Note the strong immunostaining at the apical pole of the epithelial cells (arrowheads). Immunoperoxidase staining. Magnification approximately $\times 450$.

homeostasis (Krause *et al.* 2000, Nicolas *et al.* 2001, 2002a, Park *et al.* 2001, Pigeon *et al.* 2001). In previous studies, it was shown that the liver is the main source of hepcidin (Park *et al.* 2001, Kulaksiz *et al.* 2004). Although hepcidin was originally isolated from human urine (Park *et al.* 2001) and hemofiltrate (Krause *et al.* 2000), no expression of this regulatory peptide has been described in the kidney (Pigeon *et al.* 2001).

Using the appropriate primer specifications and combinations successfully employed in the liver (Gehrke *et al.* 2003, Kulaksiz *et al.* 2004), the present RT-PCR analysis clearly revealed that hepcidin is not only expressed in the liver, but also in the kidney of three mammalian species – human, rat and mouse. To verify the presence of the translated hepcidin peptide in the kidney, we raised different region-specific antisera against hepcidin and used them in immunoblot analysis and immunohistochemistry. Immunoblot analysis confirmed expression of hepcidin in the kidney. Four different antisera recognizing different epitopes in the hepcidin precursor molecule concurrently identified an immunoreactive peptide of ~ 9.5 kDa in the kidney of three different species, which corresponds to the predicted molecular mass of hepcidin prohormone deduced from the respective cDNA sequence (Pigeon *et al.* 2001). The apparent molecular mass of this immunoreactive peptide is also in accordance with the molecular mass of hepcidin prohormone detected in the liver (Kulaksiz *et al.* 2004). Our findings unequivocally demonstrated that hepcidin is not liver specific, but is also expressed in the kidney.

Immunocytochemical investigations with N-terminal and C-terminal region-specific hepcidin antisera revealed that in human, mouse and rat kidney, the bioactive peptide is specifically localized to tubules of the renal

cortex, medulla and papilla. Hepcidin immunoreactivity was strong in cTAL and CNT, moderate expression was found in mTAL, IMCD and collecting ducts of the papilla, which were identified by specific markers. The coincident staining by different region-specific antibodies not only in the human, but also in mouse and rat kidney assumes that these tubules are the actual source of renal hepcidin. No immunoreactivity for hepcidin was detected in the glomerulus, proximal tubule and descending and ascending thin limb. Moreover, macula densa cells of the juxtaglomerular apparatus, identified by their microanatomical location and by their absence of THP, and intercalated cells of CNT and MCD were unreactive for hepcidin.

In all zones of the kidney, hepcidin immunoreactivity was confined to epithelial cells. Remarkably, in the cortex strong hepcidin immunoreactivity was found along some of the cTAL segments, but some were completely negative. Distinct intercellular differences exist between the epithelial cells even of the same tubule with respect to the density of hepcidin immunoreactivity that may reflect intercellular differences in expression or secretion of hepcidin.

Conspicuously, in some tubules immunoreactivity for hepcidin was localized in the whole cytoplasm of the epithelial cells, whereas in other tubules hepcidin immunoreactivity was concentrated at the apical pole of the epithelial cells. In CNT and CCD, hepcidin antibodies produced a cytoplasmatic granular immunoreactivity pattern, assuming localization of the bioactive peptide in small secretory vesicles of the respective cells that have already been identified in these cells by electron microscopy (Brenner 2000). In mTAL, cTAL and MCD, hepcidin antibodies showed an immunoreactive signal at

Figure 5 Schematic representation of the distribution of hepcidin immunoreactivity along the mammalian nephron and collecting duct system. Strong (red), intermediate (blue) and weak (broken blue line) staining intensities are indicated.

the apical cell pole. This peculiar distribution pattern of hepcidin at the cellular level may assume a lumenally directed release of hepcidin. We did not detect hepcidin immunoreactivity at the blood-side basal pole of renal tubule cells, suggesting that renal hepcidin is not released into the blood by these epithelial cells lining the tubules.

It is widely believed that control of iron homeostasis of the body is mainly dependent on tight regulation of iron uptake from the diet in the proximal small intestine. However, recent studies have shown that the kidney plays an important role in iron homeostasis (Gunshin *et al.* 1997, Ferguson *et al.* 2003, Wareing *et al.* 2003). Wareing *et al.* (2000) convincingly demonstrated that a metabolically significant amount of iron is filtered at the glomerulus and only 0.8–1.5% of the filtered iron is actually excreted in the urine. Thus, there must be a very effective pathway and a distinct regulation for reabsorption of iron along the renal tubules. Indeed, Ferguson *et al.* (2001) localized the divalent metal transporter 1 (DMT-1) in the tubular system of the kidney; this protein is proposed to be the major pathway for uptake of dietary iron by the gastrointestinal tract (Gunshin *et al.* 1997). Notably, DMT-1 expression has been shown to be highest at the apical pole of epithelial cells of distal tubules and collecting ducts, where we also found hepcidin. Furthermore, recent studies have demonstrated that altered dietary iron intake strongly modulates the renal DMT-1 expression (Wareing *et al.* 2003). Based on these findings, we believe that hepcidin is involved in a sophisticated regulation of renal iron transport.

The potential release of hepcidin into the urine was substantiated by immunoblot and ELISA studies. The region-specific hepcidin antisera coincidentally identified a strong immunoreactive band of the correct molecular mass (Kulaksiz *et al.* 2004) in urine that co-migrated with the immunoreactive hepcidin of kidney tissue extracts. These findings propose that hepcidin is synthesized by epithelial renal tubule and duct cells and suggest that it is released lumenally into the urine where it apparently escapes tubular proteolysis and recycling. The possibility that the hepcidin seen in the kidney could be a consequence of filtration of the small circulating peptide cannot be totally excluded, however, our RT-PCR experiments show that hepcidin is intrinsically produced in the kidney.

To measure the pro-hepcidin concentration in human urine, a sensitive ELISA with a detection sensitivity of ~4 ng/well was developed. ELISA analyses with the hepcidin antiserum EG(2)-HepN, which was already successfully used in previous ELISA studies (Kulaksiz *et al.* 2004), revealed a high concentration of pro-hepcidin in the range from 13.9 to 456.0 ng/ml (means $\pm$ S.E.; 180.1 ± 94.8 ng/ml) in the urine of healthy subjects. The mean concentration in urine is considerably higher than the pro-hepcidin mean concentration in the serum of these subjects (68.5 – 139.2 ng/ml; means $\pm$ S.E., 104.2 ± 19.5 ng/ml). Notably, no correlation was found between

the circulating pro-hepcidin and the serum iron or ferritin levels (Kulaksiz *et al.* 2004), nor between circulating pro-hepcidin and urinary pro-hepcidin. Likewise, no correlation was detected between urinary pro-hepcidin and serum iron or ferritin levels (data not shown), which are thought to regulate the expression of liver hepcidin (Pigeon *et al.* 2001, Nemeth *et al.* 2002, Ganz 2003). We therefore suggest that the regulation of renal/urinary pro-hepcidin is not directly influenced by serum iron or ferritin.

The evaluation of pro-hepcidin regulation in renal-insufficient patients undergoing chronic hemodialysis revealed that mean concentrations of pro-hepcidin in the serum of these patients were significantly increased from 104.2 ng/ml in healthy subjects to 156.8 ng/ml. Enhanced levels of pro-hepcidin in dialysis patients suggests that the kidneys are not only involved in the synthesis of hepcidin, but that they may also be involved in the elimination of the circulating peptide. Interestingly, in a previous study, the kidney hormone erythropoietin was shown to downregulate the liver hepcidin gene expression (Nicolas *et al.* 2002b). Thus, another explanation for the enhanced pro-hepcidin concentrations in dialysis patients could be the relative deficiency of erythropoietin which is encountered regularly in terminal renal insufficiency (Eckardt 2000, Santoro 2002). However, enhanced levels of pro-hepcidin were measured in our patients with chronic renal insufficiency, although they were treated with the hepcidin inhibitory hormone erythropoietin, supporting the concept of renal filtration of hepcidin. Based on our present findings, we assume that urinary hepcidin originates partly from the kidney and partly from the liver.

In summary, we have localized the peptide hormone hepcidin in the kidney of three mammalian species, indicating that hepcidin is not liver specific. Besides its elimination in the kidney, hepcidin is also produced as an intrinsic peptide in the epithelial tubule and duct cells of the kidney and may be released lumenally into the urine, implicating a regulatory role for hepcidin in the kidney and/or urinary tract. Further studies are needed to analyze the regulatory mechanism of hepcidin in the renal tubular system.

Acknowledgements

We thank Dr M Franke for providing blood from patients with chronic renal insufficiency. The technical assistance of K Bents (University Hospital Heidelberg) is greatly acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft (KU 1253/5-1) and by the Forschungsförderungs-Programm of the University Hospital Heidelberg (8/2001, 76/2002, 58 and 79/2003). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 27 October 2004

Accepted 27 October 2004