Megalin and Cubilin are Endocytic Receptors Involved in Renal Clearance of Hemoglobin

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Abstract. The kidney is the main site of hemoglobin clearance and degradation in conditions of severe hemolysis. Herein it is reported that megalin and cubilin, two epithelial endocytic receptors, mediate the uptake of hemoglobin in renal proximal tubules. Both receptors were purified by use of hemoglobin-Sepharose affinity chromatography of solubilized renal brush-border membranes. Apparent dissociation constants of 1.7 μM for megalin and 4.1 μM for cubilin were determined by surface plasmon resonance analysis. The binding was calcium dependent in both cases. Uptake of fluorescence-labeled hemoglobin by BN-16 cells was inhibited by anti-megalin and anti-cubilin antibodies as well as by receptor-associated protein, a chaperone for LDL-receptor family proteins. Partial inhibition by myoglobin was observed, whereas bovine serum albumin, intrinsic factor-cobalamin complexes, and β2-microglobulin did not affect the uptake. By use of immunohistochemistry, it was demonstrated that uptake of hemoglobin in proximal tubules of rat, mouse, and dog kidneys occurs under physiologic conditions. Studies on normal and megalin knockout mouse kidney sections showed that megalin is responsible for physiologic clearance of hemoglobin. Labeling intensities in kidneys from normal and cubilin-malexpressing dogs were similar, which suggests that, in the normal state, the role of cubilin in uptake of hemoglobin is rather limited. However, cubilin is likely to assist hemoglobin endocytosis in settings of hemoglobinuria. In conclusion, the study provides a molecular explanation for long-standing observations of hemoglobin uptake in renal proximal tubules that involve the endocytic receptors megalin and cubilin. The findings may prove to be essential for further research on the pathophysiology of hemoglobinuric acute renal failure and proteinuria-associated tubulointerstitial nephritis.

Many aspects of hemoglobin nephrotoxicity have been thoroughly investigated for the last several decades, because there is high prevalence of hemolytic disorders as well as a worldwide need for safe blood substitutes (1,2). As a result, a multifactorial model of renal pathology in settings of hemoglobinuria has emerged. Three molecular mechanisms are currently believed to exert the greatest affect on kidney impairment: direct toxicity of hemoglobin to proximal tubular epithelium, its vasoconstrictive action due to nitric oxide scavenging, and distal tubule obstruction by intraluminal hemoglobin precipitation (3,4). While the latter phenomena occur extracellularly, the toxic lesions in proximal tubules are related to the intracellular catabolism of reabsorbed hemoglobin. Although tubular uptake of hemoglobin has been well documented (5–8), the molecular mechanism(s) of hemoglobin entry into proximal epithelial cells has not been elucidated. However, several studies have clarified the molecular events responsible for tubular uptake of a subset of glomerular filtrate proteins by two endocytic receptors expressed by tubular epithelium: megalin, a 600-kD transmembrane glycoprotein that belongs to the LDL-receptor family, and cubilin, a 460-kD glycoprotein that lacks a classical transmembrane domain and is internalized via association with megalin. A variety of glomerular filtrate proteins have been demonstrated to be megalin ligands. The list is composed of vitamin-binding proteins such as retinol and vitamin D–binding proteins, enzymes (e.g., lipoprotein lipase), lipoproteins, the hormone carrier transthyretin, and a number of other low-molecular-mass proteins (9,10). Severe abnormalities, including proteinuria, in surviving megalin knockout mice underscores the physiologic relevance of this receptor (11).

The cubilin ligands identified to date include intrinsic factor-cobalamin complexes (IF-B12), albumin, Ig light chains, apolipoprotein A-I, HDL, and Clara cell secretory protein, but it is likely that the 27 CUB repeats that constitute the majority of cubilin structure can also bind other filtrate proteins (10,12).
The significance of cubilin as a protein scavenger in normal proximal tubules is supported by the finding of proteinuria in patients who have megaloblastic anemia type 1, a disorder caused by cubilin mutations in some families (13,14) and in dogs affected with an inherited abnormality of cubilin trafficking to the apical membrane (15,16).

We recently demonstrated the presence of specific binding sites for hemoglobin on renal brush-border membranes (17). Because their characteristics shared similarities with those of megalin and cubilin, we examined the possibility that renal uptake of hemoglobin is mediated by these receptors.

Materials and Methods

Proteins

Rat, mouse, and dog hemoglobins were prepared as described elsewhere (17). Human hemoglobin A0, human haptoglobin 1 to 1, bovine serum albumin (BSA), and ovalbumin were purchased from Sigma Chemical Co. (St. Louis, MO). IF-B12 purified from gastric mucosa was kindly provided by Dr. Ebba Nexø (Dept. of Clinical Chemistry, Aarhus University Hospital, Aarhus, Denmark). Recombinant receptor-associated protein (RAP) was prepared as described elsewhere (18).

Antibodies

Antisera to rat, mouse, and dog hemoglobins were raised in rabbits by repeated immunization with 0.2 mg of the antigen intravenously and subcutaneously. Sheep anti-rat megalin and rabbit anti-rat cubilin antisera were obtained as described elsewhere (19,20). Sera IgG fractions were prepared by Protein A-agarose affinity chromatography according to the manufacturer’s instructions (Pierce Chemical Co.). Specific anti-hemoglobin IgG was affinity-selected by hemoglobin-Sepharose chromatography as follows: IgG fraction (5 ml) was passed through 0.5 ml hemoglobin-Sepharose, and the bed was washed with 20 ml PBS (pH 7.4). Specific IgG was eluted with 3 ml of 5 (6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) to hemoglobin amino groups by use of the Fluorescin Labeling Kit, according to the manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, 4 mg of rat hemoglobin dissolved in phosphate-buffered saline (PBS; pH 7.4) was incubated with 0.4 mg FLUOS for 2 h at room temperature with gentle mixing. Unbound FLUOS was removed by Sephadex G-25 gel filtration. The approximate fluorescence to protein ratio of the preparation was 10. Aliquots of FLUOS-hemoglobin (1 mg/ml) were stored at −20°C until used. Protein concentrations were determined by use of Protein Assay Reagent (Pierce Chemical Co., Rockford, IL).

Purification of Hemoglobin Receptors by Affinity Chromatography

Rat renal brush-border membranes were prepared from 20 2-mo-old Wistar rats, each weighing 250 g. Before excision, kidneys were perfused via the abdominal aorta with saline that contained heparin (15 U/ml) at 120 mmHg for 5 min. Cortical tissue was excised and stored frozen at −20°C. Brush-border membranes were isolated from frozen cortices by a magnesium precipitation and differential centrifugation technique, as described by Biber et al. (21). All steps were performed at 4°C. The tissue was thawed in 5 volumes of 2 mM Tris-HCl (pH 7.0) that contained 10 mM mannitol and protease inhibitors (100 µg/ml Pefabloc, 5 µg/ml leupeptin, 1 µg/ml pepstatin, and 2 µg/ml E-64; Roche Diagnostics GmbH) and homogenized 3 × 30 s in a Waring blender. The homogenate was filtered through two layers of surgical gauze, and 0.01 volume of 1 M MgCl2 was added to the filtrate. The mixture was stirred for 15 min. Precipitated material was removed by centrifugation at 3000 × g for 12 min and discarded. The supernatant was centrifuged at 50,000 × g for 1 h. The pellet containing brush-border membranes was washed twice with 20 volumes of 50 mM phosphate buffer (pH 7.4) by dispersion and centrifugation at 50,000 × g for 1 h. The final preparation was diluted with the washing buffer to 10 mg/ml protein concentration and stored frozen at −20°C. Specific activity of alkaline phosphatase, a brush-border membrane marker, in the final membrane fraction was 7.2-fold to 8.5-fold greater than in the starting homogenate (22).

Membranes (30 ml) were solubilized by addition of Triton X-100 to the final concentration of 1% and mixing end-over-end for 4 h. Nondissolved material was removed by centrifugation at 50,000 × g for 1 h. The supernatant was recirculated at 0.2 ml/min flow through a 1.5 ml rat hemoglobin-Sepharose column equilibrated with PBS (pH 7.4), 0.6 mM CaCl2, and 0.5% CHAPS. The column was washed with 30 ml PBS (pH 7.4), 0.6 mM CaCl2, 0.5% CHAPS, 30 ml of the same buffer containing 0.5 M NaCl, and again with 10 ml of the first buffer. Bound proteins were eluted with PBS (pH 5.0), 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% CHAPS. Collected 1-ml fractions were concentrated 10 times by ultrafiltration by use of Centricons YM 10 (Millipore, Bedford, MD) and analyzed under nonreducing conditions by 4% to 16% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein bands were visualized by GELCODE blue stain reagent (Pierce Chemical Co.). For immunological analysis, proteins were blotted onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Nitrocellulose membranes were blocked by 5% skimmed milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, and 0.1% Tween 20 [pH 7.5]) for 1 h and incubated with primary antibody in PBS-T overnight at 4°C. After washing with PBS-T, the blots were incubated with horseradish peroxidase–conjugated secondary antibody diluted 1:3000 in PBS-T, ECL-PLUS reagent (Amer sham Pharmacia Biotech) and Fluor-s imaging system (Bio-Rad Laboratories Inc., Hercules, CA) was used for chemiluminescent visualization.

Kinetics of Hemoglobin Binding to Cubilin and Megalin

The binding of human hemoglobin to megalin and cubilin was studied by surface plasmon-resonance (SPR) analysis on a BiaCore 2000 instrument (BiaCore AB, Uppsala, Sweden). The procedure was essentially as described elsewhere (23). Briefly, BiaCore sensor chips type CM5 were activated with a 1:1 mixture of 0.2 M N-ethyl-N-(3-dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxysuccinimide in water, according to the manufacturer’s instructions. Megalin and
cubilin were purified by RAP or IF-B12 affinity chromatography, respectively. The preparations gave single bands in sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by coomassie brilliant blue staining. No cross-contamination of the two proteins could be detected. The proteins were immobilized at concentrations up to 50 μg/ml in 10 mM sodium acetate (pH 4.5), and the remaining binding sites were blocked with 1 M ethanolamine (pH 8.5). The resulting receptor densities were 23 fmol megalin/mm² and 38 fmol cubilin/mm², respectively. A control flow cell was made by performing the activation and blocking procedures only. In some experiments, immobilized receptor proteins were reduced by injection of 0.5% dithiothreitol in 6 M guanidine hydrochloride, 5 mM EDTA, and 50 mM Tris (pH 8.0) into the flow cell. Samples were dissolved in 10 mM HEPES, 150 mM NaCl, 2 mM CaCl₂, and 0.005% Tween-20 (pH 7.4) or in 10 mM HEPES, 150 mM NaCl, 20 mM ethyleneglycol-bis(β-aminooethyl ether)-N,N′-N′-tetraacetic acid, and 0.005% Tween-20 (pH 7.4). Sample and running buffer were identical. Regeneration of sensor chips after each analysis cycle was performed with 1.6 M glycine-HCl buffer (pH 3.0). The BioCore response is expressed in relative response units, i.e., the difference in response between protein and control flow channel. Kinetic parameters were determined by use of BIAevaluation 3.1 software.

**Immunohistochemistry**

Immunohistochemical studies were performed on kidneys excised from normal rats or rats injected with rat hemoglobin (200 mg/kg body wt in PBS (pH 7.4)) into the femoral vein at 5, 10, 20, and 30 min after injection, respectively. Kidneys from normal and megalin knockout mice (24) as well as normal and cubilin-malexpressing dogs (25) were also studied but without hemoglobin infusion. Cortex tissue specimens were prepared from kidneys after fixation with 1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) by retrograde perfusion through abdominal aorta or directly through the renal arteries in dogs. Blocks of tissue were further fixed by immersion in the same solution for 1 h and transferred to 2.3 M sucrose in PBS (pH 7.4) for 0.5 h before being frozen in liquid nitrogen. Semithin cryosections (0.8 μm) were cut by use of a Reichert Ultracut S microtome (Richert-Jung, Vienna, Austria) and placed onto glass slides. Endogenous peroxidase activity was quenched with PBS (pH 7.4), 10% methanol, and 3% H₂O₂, and nonspecific binding was blocked with PBS (pH 7.4), 1% BSA, and 0.05 M glycine. The sections were subsequently incubated with the appropriate anti-hemoglobin affinity-selected IgGs diluted 1:20 to 1:200 and peroxidase-conjugated goat IgG anti-rabbit immunoglobulins diluted 1:100. The reaction was visualized with diaminobenzidine. The sections were counterstained with Mayer’s hematoxilin.

**Uptake Studies on Cell Culture**

Rat yolk sac carcinoma cells BN-16 (20) were routinely grown in 25 cm² plastic culture flasks (Corning Costar, Badhoevedorp, Holland), in Eagle’s Minimal Essential Medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal calf serum (Biological Industries, Fredensborg, Denmark), 2 mM L-glutamine, 50 μM penicillin, and 50 μg/ml streptomycin (Bio-Whittaker) in humidified atmosphere that contained 5% CO₂ at 37°C. Cells were passaged every fourth day with a split ratio of 1:5 by trypsinization with 500 mg/L Trypsin and 200 mg/L EDTA (Bio-Whittaker).

For uptake experiments, cells were cultured in eight-chamber glass slides (Nalge Nunc International, Naperville, IL). One day before the cells reached confluence, the medium was replaced with medium supplemented with 0.5% ovoalbumin instead of 10% serum (serum-free medium). Cell monolayers were incubated with 10 μg/ml FLUOS-hemoglobin in serum-free medium for 10 min and fixed with 1% paraformaldehyde in PBS (pH 7.4) for 3 min. For inhibition studies, the following proteins were added to the incubation mixture: 20 μM unlabeled hemoglobin, 20 μM BSA, 20 μM IF-B12, 1 μM RAP, 200 to 400 mg/L sheep anti-rat megalin IgG serum fraction, sheep nonimmune IgG serum fraction, or rabbit anti-rat cubilin IgG serum fraction, rabbit nonimmune IgG serum fraction. The slides were mounted with 50% glycerol, 2% N-propylgallat, 2.4% Tris, and examined by use of a fluorescence microscope (Leica DMR) equipped with a color video camera (Sony 3CCD).

**Results**

Affinity chromatography of solubilized rat renal brush-border membranes on hemoglobin-Sepharose column yielded two main proteins, which in sodium dodecyl sulfate–polyacrylamide gel electrophoresis comigrated with cubilin and megalin, seen as the two predominant bands at the top area of the brush-border membrane fraction lane (Figure 1A). Western blot analysis of the peak fraction using specific antibodies confirmed the identity of the eluted proteins as cubilin and megalin (Figure 1B).

We examined the interactions of hemoglobin with cubilin and megalin by means of SPR (Figure 2). Again, we observed that hemoglobin bound to both receptors. The estimated dis...
Discussion

This study provides evidence that two previously characterized clearance receptors, cubilin and megalin, are involved in hemoglobin reabsorption in the renal proximal tubule. The receptors could be purified by use of affinity chromatography of solubilized renal brush-border membranes on hemoglobin-Sepharose in nearly equal amounts. Because the interaction between purified cubilin and megalin is quite strong (Kₐ ~7 nM) (27) in comparison to other ligands, one could speculate that the presence of both receptors in the eluate might be due to their association. However, in other studies, only trace amounts of megalin, as detected by immunoblotting, copurified with cubilin in affinity chromatography that used sole cubilin ligands such as IF-B12 or HDL (23,28). Apparently, in solubilized membranes the interaction between cubilin and megalin is largely abolished. Thus the results obtained by affinity chromatography, at least in the case of cubilin, are not consid-

Figure 2. Surface plasmon-resonance analysis of hemoglobin binding to cubilin and megalin. Megalin and cubilin were immobilized onto sensor chips, and samples of Hb (40 µl) were injected over the flow cells at 5 µl/min. The sensograms with hemoglobin concentrations in the range from 0.1 to 5 µM were recorded for evaluation of kinetic parameters (solid curves). The affinity of Hb for megalin was determined as Kₐ = 1.7 µM and the affinity of Hb for cubilin as Kₐ = 4.1 µM, under the assumption of one class of binding sites for each receptor. The binding of hemoglobin (2 µM) to the both receptors was abolished when 20 mM EDTA was included in the sample and running buffer, which shows calcium dependency of the interactions (dotted curves).

in a study elsewhere (26). When the cells were incubated with FLUOS-hemoglobin, an increasing green fluorescence appeared within endosomal-lysosomal structures. An excess of unlabeled hemoglobin (20 µM) virtually prevented fluorescence labeling (not shown), which suggests that labeling was due to binding-dependent uptake. After 10 min incubation, the signal was strong enough to perform the inhibition study. Uptake was inhibited by 1 µM RAP, a protein that affects the binding of all megalin and some cubilin ligands, as well as by antibodies raised against purified megalin or cubilin in concentrations of 200 and 400 µg/ml, respectively (Figure 3). Partial inhibition by myoglobin was observed, whereas BSA, IF-B12, and β₂-microglobulin did not affect the uptake (not shown).

Immunohistochemistry revealed evidence that megalin-mediated reabsorption of hemoglobin occurs under physiologic conditions. Using affinity-selected anti-rat hemoglobin IgG, we detected hemoglobin in normal rat kidney (Figure 4, A and B). In most proximal tubules, the apical plasma membrane and adjacent endosomes were stained, whereas in some tubules the reaction was also seen in more central aspects of the cells. Distal tubule cells had no hemoglobin immunoreactivity. Specificity of the anti-hemoglobin antibodies was examined in sections obtained from rats injected with hemoglobin (200 mg/kg). The immunoreaction progressively increased in intensity and distribution with time after intravenous hemoglobin administration. After 5 min, the apical-endosomal staining was more intense, and after 20 min many, vesicular structures located deeper in cells were also labeled (Figure 4, C and D).

Given that the uptake of hemoglobin occurs under physiologic conditions, we examined the kidneys from normal and megalin knockout mice (Figure 4, E and F). With normal mouse tissue, we obtained a pattern of labeling similar to that in rat cortex. In contrast, no vesicular hemoglobin immunostaining could be detected in renal cortex from the megalin knockout mice. In kidney sections from both normal and cubilin-malexpressing dogs, there was a vesicular labeling similar to that in normal rat tissue, but there was no difference in the reaction intensity between dogs (not shown).

Discussion

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erably influenced by a copurification phenomenon. We verified the binding of hemoglobin to both receptors using SPR. On the basis of dissociation constants, 1.7 μM for megalin and 4.1 μM for cubilin, the interactions can be classified as “low-affinity binding,” which applies to many ligands of these receptors. Both the affinity chromatography results and the SPR data indicate that hemoglobin binds to cubilin and megalin in a calcium-dependent manner, as do all other of their characterized ligands.

We further examined the significance of those interaction for

Figure 3. Characterization of hemoglobin uptake by BN-16 cells by use of fluorescence microscopy. Cells were grown to confluence on glass slides and incubated with 10 μg/ml FLUOS-Hb in Eagle’s Minimum Essential Medium that contained 0.5% ovoalbumin for 10 min alone (A) or in the presence of 1 μM recombinant receptor-associated protein (RAP) (B), 200 mg/L nonimmune sheep IgG (C), 200 mg/L sheep anti-megalin IgG (D), 400 mg/L nonimmune rabbit IgG (E), or 400 mg/L rabbit anti-cubilin IgG (F). The uptake of hemoglobin is markedly inhibited by RAP and anti-megalin or cubilin antibodies (compare A with B, C with D, and E with F). Magnification, ×700.
cellular uptake of hemoglobin using a cell-culture model. Because in cultured cells of proximal tubule origin, like LLC-PK1 or OK cells, the expression of megalin and cubilin is much lower in comparison to those in vivo, we chose BN-16 cells for these experiments. BN-16 cells, which originate from yolk sac epithelium, are structurally and functionally similar to renal

Figure 4. Immunohistochemical detection of hemoglobin in rat and mouse kidney cortex. Cryosections were incubated with affinity-selected anti-rat (A) or mouse hemoglobin IgG (B through F), and the reaction was visualized by immunoperoxidase method. Apical membrane and endosomal/lysosomal labeling can be detected in rat kidney proximal tubules with anti-rat (A) or mouse (B) hemoglobin antibodies. The increased reaction in cortex from kidneys excised from rat 5 min (C) or 20 min (D) after intravenous administration of rat hemoglobin (200 mg/kg body wt) confirms the specificity of the antibodies. A similar staining pattern can be seen in mouse kidney cortex (E), whereas no labeling can be detected in cortex from megalin knockout mouse (F). (▲) apical membrane staining; (→) endosomal/lysosomal labeling. Magnifications: ×800 in A, C, and D; ×1050 in B, E, and F.
proximal tubule cells but exhibit high rates of cubilin- and megalin-mediated endocytosis. These cells have been used elsewhere as an in vitro model of proximal tubular endocytosis (20,29). Indeed, we observed a rapid accumulation of fluorescence-labeled hemoglobin in the cells. Involvement of cubilin and megalin in the uptake was further demonstrated by the inhibitory action of antisera raised against purified receptors. Moreover, the uptake was virtually abolished by RAP, a chaperone that affects binding of most megalin and cubilin ligands (30). We also observed inhibition of hemoglobin uptake by myoglobin. These proteins share structural similarity, so it is plausible that they can occupy common binding sites on these receptors. Elucidation of myoglobin catabolic pathways in the kidney would be of particular clinical importance, because myoglobinuria underlies as many as 15% of all acute renal failure cases (3).

Renal uptake of hemoglobin has been demonstrated repeatedly in different experimental models of hemoglobinuria (5–8). Our immunohistochemical study documents that hemoglobin uptake also occurs under physiologic conditions. Comparison of kidneys of normal and megalin knockout mice confirmed that megalin is responsible for physiologic clearance of hemoglobin. The physiologic significance of hemoglobin uptake in the kidney is open to question and may be a mechanism of iron retrieval. One can calculate, taking free plasma hemoglobin concentration of 0.03 g/L (31) and the fractional filtration coefficient for hemoglobin of 0.03 (3), that lack of hemoglobin reabsorption in humans would result in a loss of 0.6 mg iron daily, which is rather negligible in comparison to 4 g overall iron body pool (32). However, extensive withdrawal of hemoglobin from the primary filtrate may protect the lower urinary tract from bacterial invasion. Endogenous host hemoproteins are commonly used by pathogenic bacteria as an iron source. Inaccessibility of this element, as well as heme in bacteria unable to synthesize protoporphyrin IX, is the factor most limiting for their growth and virulence (33,34). Recent findings that Neisseria gonorrhoeae and Gardnerella vaginalis, pathogenic bacteria that provoke urinary tract infections, can acquire iron from human hemoglobin would support this hypothesis (35,36).

We found no difference in renal hemoglobin uptake between normal dogs and those that do not express cubilin in the apical membrane of proximal tubule cells. It is conceivable that, under physiologic conditions, megalin itself is sufficient to scavenge the small amounts of filtered hemoglobin. Generally, hemoglobin released into blood circulation is irreversibly captured by the plasma protein haptoglobin and the complex, being too large to be filtered in the glomeruli, is catabolized by the mononuclear phagocyte system in liver and spleen (37). Recently, the receptor for the hemoglobin-haptoglobin complex has been identified as the acute phase–regulated and signal-inducing monocyte/macrophage protein CD163 (38). The role of cubilin may also be limited by its lower affinity for hemoglobin. However, cubilin may become an essential scavenger in hemolytic disorders when glomerular filtrate hemoglobin concentration is greatly elevated. The involvement of two receptors would serve as a high-capacity mechanism for hemoglobin uptake in such circumstances. The existence of an effective reabsorption mechanism for hemoglobin suggests that it may have a role in the development of hemoglobinuria/proteinuria-induced interstitial tubular nephritis. To date, investigations of other potentially nephrotoxic plasma proteins have focused mainly on albumin and transferrin (39).

In conclusion, this study reveals a molecular mechanism of hemoglobin uptake in the renal proximal tubule that involves the endocytic receptors megalin and cubilin. Identification of the receptors for tubular hemoglobin uptake may be essential for development of new therapeutic agents to block hemoglobin entry into tubular epithelial cells and prevent its cytotoxicity in hemoglobinuric states. To our knowledge, this is the first report demonstrating the uptake of endogenous hemoglobin under physiologic conditions, the pathophysiologic role of which remains to be elucidated.

Acknowledgments

This work was supported by the Danish Medical Research Council, the University of Aarhus Research Foundation, the NATO Science Fellowships Program (to J.G.), the NOVO-Nordisk Foundation, and the Biomembrane Research Center of Aarhus University. The skillful technical assistance of Hanne Sidelmann, Inger Kristoffersen, and Pia Kamuk Nielsen is greatly appreciated.

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