

Cardiovascular, Pulmonary and Renal Pathology

Plasma Protein Haptoglobin Modulates Renal Iron Loading

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Haptoglobin is the plasma protein with the highest binding affinity for hemoglobin. The strength of hemoglobin binding and the existence of a specific receptor for the haptoglobin-hemoglobin complex in the monocyte/macrophage system clearly suggest that haptoglobin may have a crucial role in heme-iron recovery. We used haptoglobin-null mice to evaluate the impact of haptoglobin gene inactivation on iron metabolism. Haptoglobin deficiency led to increased deposition of hemoglobin in proximal tubules of the kidney instead of the liver and the spleen as occurred in wild-type mice. This difference in organ distribution of hemoglobin in haptoglobin-deficient mice resulted in abnormal iron deposits in proximal tubules during aging. Moreover, iron also accumulated in proximal tubules after renal ischemia-reperfusion injury or after an acute plasma heme-protein overload caused by muscle injury, without affecting morphological and functional parameters of renal damage. These data demonstrate that haptoglobin crucially prevents glomerular filtration of hemoglobin and, consequently, renal iron loading during aging and following acute plasma heme-protein overload. (*Am J Pathol* 2005, 166:973–983)

Iron is a crucial agent in virtually all cell types for a vast number of cellular processes, including ATP generation, oxygen transport, and detoxification.¹ It has catalytic

function within heme or iron-sulfur clusters, or directly bound to proteins. Iron metabolism disorders are quite common in the human population and are related to both iron deficiency and overload.² Therefore, it is most valuable to understand iron metabolism not only at the molecular and cellular levels but also at the level of the whole organism.

Normally in humans, about 1 mg of iron is absorbed daily by the intestine, and, at the same time, an approximately equal amount is eliminated from the body. Remarkably, this dietary iron accounts for only 1 to 3% of the iron that is supplied daily to the blood. Most of the iron requirement is provided through reutilization from existing total body stores of 3 to 4 g, of which about 70% is maintained within hemoglobin.³ From these facts, it is clear that heme-iron metabolism constitutes a major component of iron homeostasis. Nevertheless, the mechanism and regulation of heme-iron reutilization are poorly understood. Among proteins potentially involved in heme-iron metabolism, haptoglobin (Hp) may have a crucial role.

Hp is the plasma protein with the highest binding affinity for hemoglobin ($K_d \approx 1$ pmol/L).⁴ Release of hemoglobin into plasma is a physiological phenomenon associated with intravascular hemolysis occurring during destruction of senescent erythrocytes and enucleation of erythroblasts. However, intravascular hemolysis becomes a severe pathological complication when it is accelerated in various autoimmune, infectious (such as malaria) and inherited (such as sickle cell disease) disorders. In plasma, stable Hp-hemoglobin complexes are formed and these are subsequently delivered to the reticuloendothelial system by CD163 receptor-mediated endocytosis.⁵ In this way, Hp is believed to reduce loss of hemoglobin through the glomeruli, hence protecting against peroxidative kidney injury, and allowing heme-

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iron recycling. The increased susceptibility to hemoglobin-driven lipid peroxidation demonstrated in conditions of hypo- or anaptoglobinemia in humans and in Hp-deficient mice supports this hypothesis.⁶⁻⁸

Hp is synthesized as a single chain polypeptide, which is cleaved into an amino-terminal α -chain and a carboxy-terminal β -chain. The basic mammalian isoform Hp(1-1) is a homodimer in which two Hp molecules are linked by a single disulfide bond through their respective α -chains. In humans, a variant with a longer α -chain, apparently originating from an early intragenic duplication, is also present. The short and long α -chains are designated as α^1 and α^2 , respectively. As the cysteine forming the intermolecular disulfide bond between α -chains is also duplicated, humans homozygous for the long variant allele show a multimeric Hp phenotype designated Hp(2-2). Hp(2-1) refers to the phenotype (both Hp dimers and multimers) seen in humans heterozygous for the two variant alleles. Complexes of hemoglobin and multimeric Hp (the 2-2 phenotype) exhibit higher functional affinity for CD163 than do complexes of hemoglobin and dimeric Hp (the 1-1 phenotype).⁹ These functional differences between the various Hp types have important biological and clinical consequences. In healthy men, the Hp(2-2) type is related to higher serum iron and ferritin levels than the Hp(2-1) and Hp(1-1) types. Moreover, in healthy men carrying the Hp(2-2) type, a fraction of Hp-hemoglobin complexes is shunted into monocyte-macrophages, resulting in partial iron retention.¹⁰ Finally, it has recently been proposed that Hp might be a genetic modifier of Hfe-associated hemochromatosis as Hp(2-2) type was over-represented in hemochromatotic patients and iron loading was more pronounced in patients carrying Hp(2-2).¹¹

These data suggest that Hp participates in iron homeostasis. However, to what extent Hp contributes to overall iron metabolism and how it exerts its action are hitherto open questions. To further investigate these issues, we used Hp-null mice to evaluate the impact of Hp gene inactivation on iron metabolism. Here, we show that, in Hp-null mice, free hemoglobin accumulates predominantly in the kidney instead of in the liver and spleen as is the case in wild-type mice. This difference in organ distribution of hemoglobin in Hp-deficient mice results in iron loading in proximal tubules during aging. Moreover, Hp-null mice also accumulate iron in the kidney after renal injury during which hemoglobin is released from erythrocytes. Finally, the kidney of wild-type mice show local expression of Hp and its receptor following injury. These data represent the first *in vivo* evidence that Hp prevents glomerular filtration of hemoglobin and, consequently, renal iron loading in both physiological and pathological conditions.

Materials and Methods

Animals

Hp-null mice were generated as previously described⁶ and kindly provided by F. Berger (University of South

Carolina, Columbia, SC) and H. Baumann (Roswell Park Cancer Institute, Buffalo, NY). Mice used in these experiments were littermates derived by breeding F₁ heterozygous Hp^{+/-} mice in the mixed genetic background C57BL/J6 × 129Sv.

Plasma Clearance and Organ Distribution of ¹²⁵I-Hemoglobin

For mouse hemoglobin preparation blood was collected from anesthetized mice by retro-orbital bleeding. Hemoglobin was purified as previously described and quantified using a kit from Sigma (S527-A; Sigma, St Louis, MO). Hemoglobin was radiolabeled with ¹²⁵I by Chloramin T method.¹² To ascertain that Chloramin T method did not alter the structure of the protein through oxidation, we confirmed the binding of iodinated preparation to Hp by Sephadex G-100 gel filtration and non-denaturing polyacrylamide gel electrophoresis. Age-matched adult males, weighing 25 g, were injected into the tail vein with 0.2 μ g of purified ¹²⁵I-hemoglobin (specific activity, 0.87 μ Ci/ μ g). For blood collection, the tip of the tail was cut and 20 μ l samples were taken into heparinized capillary tubes at various time points after ¹²⁵I-hemoglobin injection and radioactivity measured using a gamma counter (Cobra AutoGamma, Packard, Canberra, Canada). After last sampling, mice were anesthetized with Nembutal (100 mg/kg body weight) and the tissues were perfused via heart with saline. Organs were collected, blotted dry on filter paper, weighed and radioactivity counted on a gamma counter.

Autoradiography of Organs

For autoradiographic studies, mice were injected into the tail vein with 50 μ Ci of ¹²⁵I-hemoglobin (specific activity: 0.42 μ Ci/ μ g). After 20 minutes, organs were fixed by retrograde perfusion through abdominal aorta with 1% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4), postfixed in 1% osmium in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 30 minutes and in 0.5% uranyl acetate in maleate buffer (pH 5.2) for 1 hour, washed in the same buffer, dehydrated and embedded in Epon. One- μ m-thick tissue sections were prepared for light microscope autoradiography using Ilford K2 emulsion (Polysciences, Inc., Eppelheim, Germany). Sections were exposed for 2 to 4 weeks and examined on a Leica DMR microscope.

Renal Ischemia-Reperfusion Injury

For ischemia-reperfusion injury (IRI), age-matched adult males were anesthetized with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich, St. Louis, MO). After surgical skin preparation, the left kidney was exteriorized through a 2-cm subcostal incision. The left renal peduncle was ligated for 35 minutes and the mice kept warm at 37°C. Reperfusion after this period was checked by blood reflow to the ischemized kidney. The peritoneum and skin

were closed in separate layers using 4.0 absorbable suture. Mice were rested for 24 hours or 7 days. The non-ischemic contralateral right kidney was used as control.

Glycerol-Induced Acute Muscle Injury

To induce acute muscle injury, age-matched adult males were anesthetized with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich) and a solution of 50% glycerol in PBS was injected into the two posterior thigh muscles, at a concentration of 7.5 ml/kg body weight. Blood was taken at 6 hours, 1 day, and 3 days after treatment, and creatine kinase, urea, and creatinine were assessed as index of muscle injury and kidney functionality, respectively. Creatine kinase and urea were determined with the kits 47-10 and 535 from Sigma (Sigma-Aldrich), respectively, according to the manufacturer's instructions.

Histopathological and Immunohistochemical Analyses

At different times after IRI or glycerol-induced acute muscle injury, mice were perfused via aorta with PBS, tissues were dissected, fixed in 10% formalin for 48 hours and embedded in paraffin. Kidney sections were stained with periodic acid-Schiff base (PAS) or Perl's staining followed by DAB (methanol 3,3' diaminobenzidine; Boehringer Mannheim, Germany) development. For immunohistochemistry, kidney sections were analyzed with rat monoclonal anti-mouse CD18 or anti-mouse F4/80 antibodies (BMA Biomedicals AG, Switzerland) as described elsewhere.¹³ Slides were then stained with hematoxylin and mounted with DPX (BDH Laboratory Supplies, Leicester, UK).

The extent of epithelial cell necrosis and neutrophil infiltration was evaluated using a microscope coupled to an image analyzer (Image Pro Plus 4.0) and was graded according to the scheme of Kelly and colleagues.¹⁴ The percentage of tubules with epithelial cell necrosis and/or necrotic debris in the outer medulla and corticomedullary junction was estimated at 400 \times magnification and a score was assigned as follows: 0, none; 1+, <10%; 2+, 10 to 25%; 3+, 26 to 75%; and 4+, >75%. The extent of neutrophil infiltration was derived from the estimated mean number of neutrophils per high-power field (\times 400) in 5-10 consecutive fields from the outer medulla and corticomedullary junction starting at the most-involved area and proceeding in the direction of greatest involvement. Scores were assigned based on these counts as follows: 0, 0-1; 1+, 2-10; 2+, 11-20; 3+, 21-40; and 4+, >40 or too many to count. All sections were coded so that the observer was blinded to treatment group.

Determination of Tissue Iron Content

Mice were perfused via aorta with PBS, and tissue non-heme iron content determined with the colorimetric method using bathophenanthroline reagent as chromo-

gen.¹⁵ Results were expressed as μ g iron/g dry tissue weight.

Analysis of Oxidative Damage

Lipid peroxidation from tissue extracts was measured using the colorimetric assay kit Bioxytech LPO-586 from Oxis International (Portland, OR) according to the manufacturer's instructions.

Northern Blot

Total and poly(A)⁺ RNA were extracted with RNeasy Midi Kit and Oligotex mRNA Mini kit (Qiagen, Hilden, Germany), respectively, and analyzed by Northern blotting using standard techniques. Filters were sequentially hybridized with a ³²P-labeled DNA probe for Hp and β -actin.

Plasmid Construction and Antibody Preparation

cDNA sequence encoding amino acids 1069-1121, corresponding to the cytoplasmic tail of murine CD163, was amplified by PCR with the following primers: 5'-CGGAAT-TCAAGCGACGACGGATTGACGCG-3' and 5'-CGGGATC-CGTCATCCGTGCGTTGCCTGG-3'. The PCR product was sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and cloned in pMALc2 vector (New England Biolabs) and pGEX 4T2 vector (Pharmacia Biotech) in frame with maltose binding protein (MBP) and with glutathione S-transferase (GST) coding sequences, respectively.

MBP-CD163 fusion protein was produced by expressing the peptide sequence cloned in pMALc2 vector (New England Biolabs) in *Escherichia coli* BL21 bacterial strain. MBP-CD163 was purified on an amylose column according to the manufacturer's instructions (New England Biolabs). Rabbits were immunized by repeated intramuscular injections of the purified fusion protein (500 μ g) emulsified in complete Freund's adjuvant. The specificity of the antiserum was demonstrated in Western blots on protein extracts from *E. coli* expressing GST-CD163 fusion protein (35 kd), and GST alone (29 kd) as negative control. To affinity purify antibodies from rabbit serum MBP and MBP-CD163 fusion proteins were purified and coupled to Sepharose. Rabbit serum was loaded on the MBP column to eliminate antibodies to MBP and then reloaded on the MBP-CD163 column. Antibodies adsorbed on the MBP-CD163 Sepharose column were eluted with glycine-HCl buffer (pH 3).

Western Blot

One μ l of plasma, collected from the tail vein, was separated on 6% SDS-PAGE and analyzed by Western blotting using standard techniques with goat antiserum against human Hp (Sigma H 5015; Sigma) that also cross-reacts with mouse Hp. Plasma membrane proteins were extracted as previously described¹⁶ and protein

concentration was determined using Bio-Rad Assay (Bio-rad, München, Germany). 50 μg of protein extracts were separated on 6% SDS-PAGE and analyzed by Western blotting with polyclonal antibody to CD163 and a monoclonal antibody to vinculin.

Statistical Analysis

Results were expressed as mean \pm SD or mean \pm SEM. Statistical analyses were performed using the one-way analysis of variance. A *P* value of less than 0.05 was regarded as significant.

Results

Plasma Clearance and Organ Distribution of Hemoglobin

Hemoglobin clearance was assessed by injecting a small amount of ^{125}I -hemoglobin (0.2 μg) intravenously through the tail vein of mice and measuring the loss of radioactivity from plasma over time. Hemoglobin clearance was similar in wild-type and Hp-null mice, ie, plasma radioactivity was reduced to 50% within 5 minutes and by about 80% 15 minutes after injection (Figure 1A).

To determine whether the Hp-null mutation affected organ distribution of hemoglobin, mice were sacrificed 20 minutes after injection of ^{125}I -hemoglobin and total radioactivity in various organs was measured. In wild-type mice, ^{125}I -hemoglobin accumulated primarily in the liver and spleen, much less in the kidney and, at negligible levels in lung, heart, skeletal muscle and intestine. In Hp-null mice, ^{125}I -hemoglobin accumulated more in the kidney than in the liver and spleen, and, to a much lower extent, in the other organs (Figure 1B). These data clearly showed that, Hp null mutation affected organ distribution of hemoglobin, demonstrating an essential role of Hp as carrier of hemoglobin to the liver and the spleen.

Identification of Cell Types Taking Up Hemoglobin

To identify which cells in the liver, spleen and kidney take up hemoglobin, mice were injected with a higher dose of ^{125}I -hemoglobin (100 μg) and sacrificed 20 minutes later. Under these conditions, radioactivity found in the kidney of wild-type mice was comparable to that of Hp-null mice. On the other hand, a significantly greater amount of ^{125}I -hemoglobin deposited in the liver and spleen of wild-type mice than in those of Hp-null ones ($P < 0.05$; Figure 2A). Autoradiography showed that hemoglobin accumulated in hepatocytes and Kupffer cells in the liver, in macrophages in the spleen and in proximal tubular cells in the kidney both in wild-type (Figure 2B) and in Hp-null mice (data not shown).

Thus, after injection of a high dose of hemoglobin, Hp null mutation did not affect renal uptake of hemoglobin, but strongly decreased the amount of hemoglobin taken

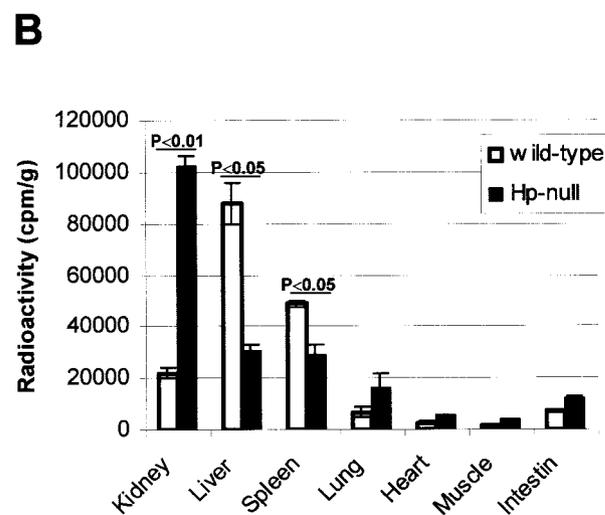
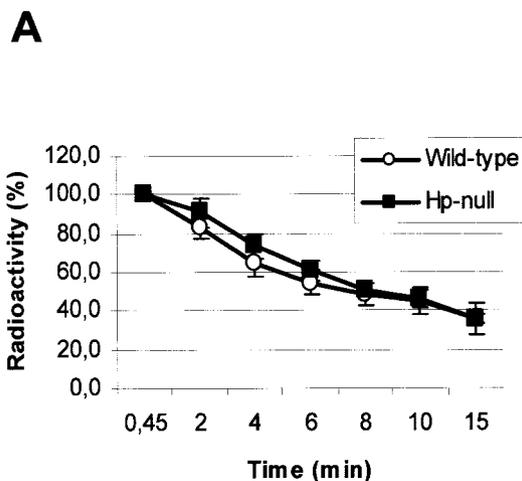


Figure 1. Plasma clearance and organ distribution of ^{125}I -hemoglobin. **A:** ^{125}I -hemoglobin was injected intravenously through the tail vein of mice as described in Materials and Methods. The radioactivity of blood samples obtained after 45 seconds was in the range of 88,300 to 122,300 cpm/ml. Three mice of each genotype were used and the average (\pm SD) at each data point is indicated. **B:** Mice were sacrificed 20 minutes after ^{125}I -hemoglobin injection and total radioactivity of various organs was counted as described in Materials and Methods. Three mice of each genotype were analyzed. Data represent mean \pm SD.

up by the liver and the spleen. However, the same cell types were able to take up hemoglobin in both wild-type and Hp-null mice.

Iron Loading during Aging

Our initial results showed that, after the injection of a low dose of hemoglobin, the Hp-null liver and kidney received less and more hemoglobin, respectively, than the corresponding organs of wild-type mice (Figure 1B). As hemoglobin is an important source of iron, we addressed the question of whether Hp-null mutation affects hepatic and renal iron deposits. Therefore, we measured liver and kidney iron content at 1, 2, and 3 months of age. In the liver, iron content was similar in wild-type and Hp-null mice at all time points (Figure 3A).

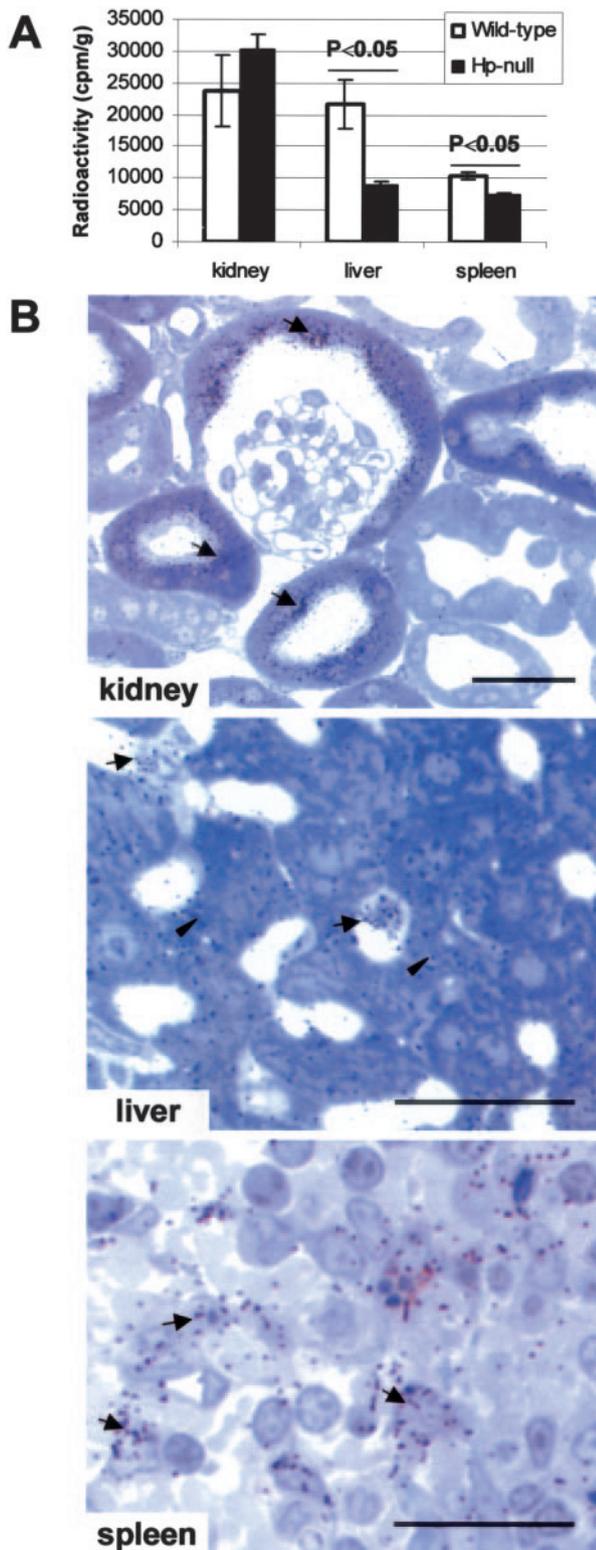


Figure 2. Identification of cell types taking up ^{125}I -hemoglobin. **A:** ^{125}I -hemoglobin was injected intravenously through the tail vein, mice were sacrificed 20 minutes later, and total radioactivity of the kidney, liver, and spleen was counted as described in Materials and Methods. Three mice of each genotype were analyzed. Data represent the mean \pm SD. **B:** Autoradiography of kidney, liver and spleen sections of a wild-type mouse. Sections were prepared as described in Materials and Methods. Note silver grain accumulation in proximal tubular cells (arrows) in the kidney, in hepatocytes (arrows) and Kupffer cells (arrowheads) in the liver and in macrophages (arrows) in the spleen. The same pattern was seen in Hp-null mice (data not shown). Scale bar = 25 μm .

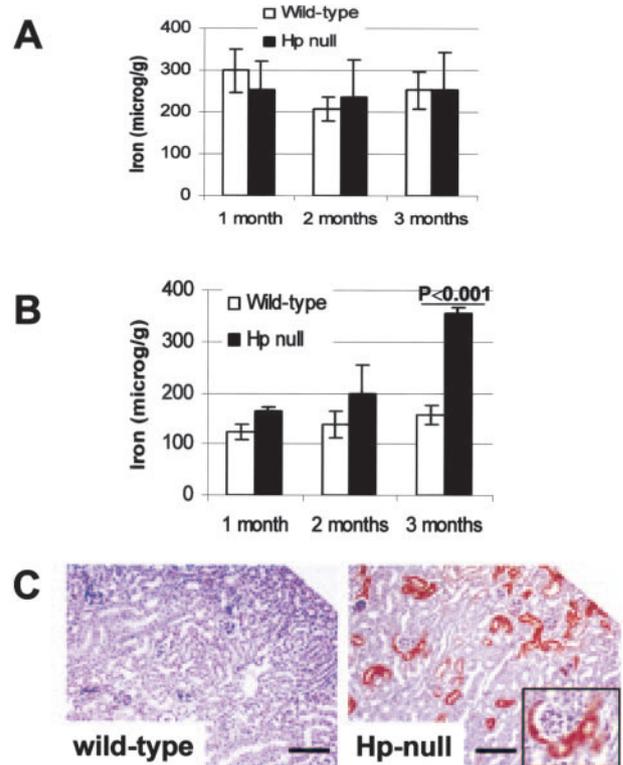


Figure 3. Iron loading during aging. **A:** Liver iron content was measured in wild-type and Hp-null mice at increasing ages. At least six mice of each genotype were analyzed. Data represent mean \pm SD. **B:** Kidney iron content was measured in wild-type and Hp-null mice at increasing ages. At least six mice of each genotype were analyzed. Data represent mean \pm SD. **C:** Kidney sections of a wild-type and a Hp-null mouse at 3 months of age stained with Perl's reaction. Note iron loading in tubular cells in Hp-deficient mouse (at high magnification in the inset). Scale bar = 100 μm .

In the kidney, iron content was slightly, but not significantly, higher in Hp-deficient mice than in wild-type controls at 1 and 2 months of age, but augmented significantly in 3 months-old Hp-null mice compared to age-matched wild-type controls ($P < 0.001$; Figure 3B). Perl's staining of kidney sections showed that, in Hp-deficient mice, iron accumulated in proximal tubular cells (Figure 3C). The pattern of Perl's staining was comparable to that of autoradiography at 20 minutes after injection of labeled hemoglobin (compare inset in Figure 3C to Figure 2B). Thus, the absence of Hp did not affect hepatic iron storage, but caused abnormal kidney iron loading with age.

Hp Expression after Renal Ischemia-Reperfusion Injury

Data reported in the previous section showed that Hp modulates renal hemoglobin handling. As heme is a prooxidant molecule involved in kidney damage after ischemia-reperfusion¹⁷ and hemoglobin released from damaged erythrocytes at sites of tissue injury is a source of heme,^{18,19} we investigated whether Hp-null mice were more susceptible to oxidative damage after ischemia reperfusion. Therefore, we analyzed iron loading and tissue damage in the kidney of wild-type and Hp-null

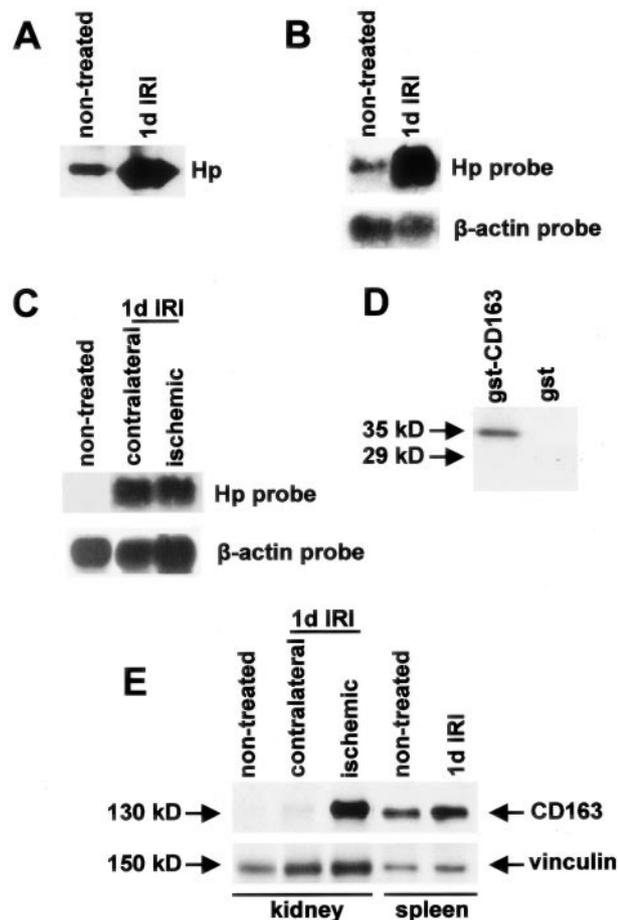


Figure 4. Hp expression after IRI. **A:** Western blotting of plasma proteins of a wild-type mouse before and one day after IRI assayed with an anti-Hp antibody. **B:** Northern blotting of total RNA extracted from the liver of a non-treated wild-type mouse and of a mouse subjected to IRI one day after surgery, sequentially analyzed with 32 P-dCTP-labeled probes to Hp and β -actin. **C:** Northern blotting of poly-A⁺ RNA extracted from the kidney of a non-treated wild-type mouse and from the contralateral and ischemic kidney of a wild-type mouse subjected to IRI one day after surgery, sequentially analyzed with 32 P-dCTP-labeled probes to Hp and β -actin. **D:** Western blot analysis to test anti-CD163 antibody specificity. Total protein extracts from *E. coli* expressing GST-CD163 fragment 1069–1121 and GST alone were subjected to SDS-PAGE, blotted on nitrocellulose membrane and probed with purified anti-CD163 polyclonal antibodies. Only recombinant CD163 (35 kd), but not GST (29 kd), was recognized by the purified anti-CD163 polyclonal antibody. **E:** Western blot on protein extracts from the kidney and the spleen of a non-treated wild-type mouse and of a mouse subjected to IRI one day after surgery, sequentially analyzed with the purified anti-CD163 and anti-vinculin antibodies. A CD163 band (130 kd) was detected in the ischemic kidney and in the spleen.

mice after IRI. We used a well-established murine model of IRI, in which the structural and functional consequences of brief periods of unilateral renal ischemia have been documented.^{14,20,21}

First, we demonstrated that IRI induced Hp expression in wild-type mice. As shown in Figure 4A, 24 hours after IRI, plasma level of Hp increased by more than five folds on the basal level. Increased protein expression in plasma was in agreement with a rise in Hp mRNA in the liver (Figure 4B). Induction of Hp expression was part of a general inflammatory reaction because other acute phase proteins such as serum amyloid A and P were also induced (not shown). Moreover, specific expression of Hp was detected in both contralateral and ischemic kid-

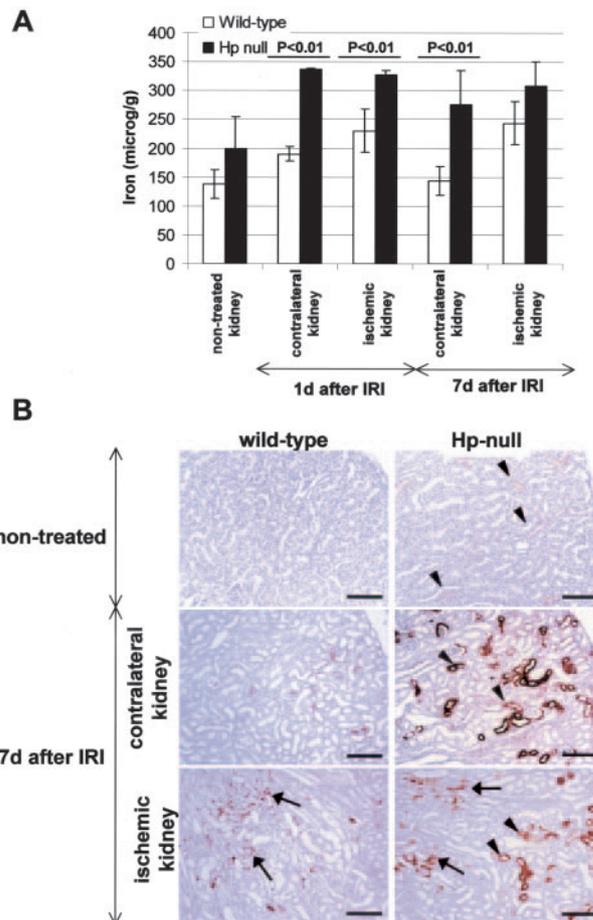


Figure 5. Iron loading after IRI. **A:** Kidney iron content was measured in non-treated mice and in mice subjected to IRI 1 and 7 days after surgery both in contralateral and ischemic organ. Six mice of each genotype were analyzed. Data represent mean \pm SD. **B:** Kidney sections from non-treated and ischemic (both ischemic and contralateral organ 7 days after injury) of wild-type and Hp-null mice stained with Perl's reaction. Note weak staining of some tubular cells in non-treated Hp-null mouse compared to more pronounced labeling of proximal tubules in both ischemic and contralateral kidney of Hp-null mouse subjected to IRI (arrowheads). Infiltrating macrophages in renal medulla of the ischemic kidney (arrows) were labeled in both wild-type and Hp-deficient mice. Scale bar = 100 μ m.

ney 24 hours after surgery (Figure 4C). Finally, consistent with IRI-induced inflammation, 24 hours after IRI, specific expression of the Hp-hemoglobin receptor, CD163, was detected in the ischemic kidney, but not in the contralateral one (Figure 4, D and E). Induction of Hp expression after IRI both in plasma and in the kidney indicated that this was an appropriate model for analyzing the impact of Hp gene inactivation on renal damage.

Iron Loading after Renal Ischemia-Reperfusion Injury

To investigate if Hp gene inactivation could modulate iron loading in the kidney after IRI, unilateral ischemia was performed and Hp-null and wild-type mice were sacrificed after 1 and 7 days of reperfusion. The experiments were performed on 2-month-old mice, an age at which only small differences in kidney iron content were detectable between wild-type and Hp-null mice (Figure 3).

One day after ischemic injury, iron content increased in the ischemic as well as in the contralateral non-ischemic kidney in both wild-type and Hp-null mice. The increase in renal iron was much more pronounced in Hp-null mice than in wild-type controls (Figure 5A).

Seven days after ischemic injury, iron content in the contralateral non-ischemic kidney corresponded to basal level in wild-type mice, but was significantly higher than basal level in Hp-null mice ($P < 0.05$; Figure 5A). Perl's staining on kidney sections showed that iron accumulated in proximal tubules only in Hp-null mice (Figure 5B). In ischemic kidney seven days after injury, iron content significantly increased with respect to the basal level in both wild-type ($P < 0.01$) and Hp-deficient mice ($P < 0.01$). Perl's staining on ischemic kidney sections showed that in Hp-null mice, iron accumulated in proximal tubules, while in wild-type mice only a weak staining was detectable in some tubular cells. In addition, infiltrating macrophages in the medulla and in the corticomedullary junction of the ischemic kidney were strongly positive to Perl's reaction in both genotypes (Figure 5, A and B). Thus, after IRI, renal accumulation of hemoglobin in Hp-null mice resulted in iron loading in proximal tubules, not only in ischemic kidney, but also in the contralateral one.

Renal Morphology and Function after Ischemia-Reperfusion Injury

Renal morphology and function were analyzed 1 and 7 days after IRI. Histopathological changes in the kidney one day after IRI included loss of brush-border membranes, tubular dilation, flattened tubular epithelium, luminal debris, and interstitial infiltrates.

The histological parameters of injury measured in this study were epithelial cell necrosis and extent of neutrophil infiltration evaluated on kidney sections stained with PAS reaction or processed by immunohistochemistry with the anti-CD18 antibody, respectively. Both measures were remarkably elevated in ischemic kidney 24 hours after surgery in both wild-type and Hp-null mice subjected to IRI, whereas non-ischemic kidneys appeared normal. There were no differences between wild-type and Hp-null mice regarding epithelial cell necrosis as well as extent of neutrophil infiltration (epithelial necrosis score: 3.00 ± 0.46 in wild-type vs. 3.14 ± 0.55 in Hp-null mice; neutrophil infiltration score: 3.38 ± 0.26 in wild-type vs. 3.50 ± 0.22 in Hp-null mice, $n = 8$).

Renal function was assessed by measuring plasma urea nitrogen and serum creatinine concentration. Twenty-four hours after IRI, blood urea nitrogen (BUN) level was slightly increased compared to basal level in both wild-type (0.28 ± 0.10 mg/ml vs. 0.15 ± 0.02 mg/ml, $n = 8$) and Hp-null mice (0.27 ± 0.06 mg/ml vs. 0.15 ± 0.02 mg/ml, $n = 7$). No significant differences between wild-type and Hp-null mice were detected. In agreement with this, 24 hours after IRI serum creatinine also was unchanged compared to the basal level in both wild-type (0.34 ± 0.03 mg/dl vs. 0.40 ± 0.05 mg/dl, $n = 4$) and Hp-null mice (0.38 ± 0.10 mg/dl vs. 0.40 ± 0.05 mg/dl, $n = 4$).

Seven days after IRI, renal morphology was similar in wild-type and Hp-null mice, with signs of damage as flattened epithelium and brush border loss (not shown). Lipid peroxidation was measured on kidney extracts 7 days after IRI to assess the extent of oxidative injury. Lipid peroxidation was higher in ischemic kidney than in control ones in both wild-type and in Hp-null mice with no significant differences between genotypes (245 ± 61 pmol MDA/mg protein vs. 125 ± 30 pmol MDA/mg protein in wild-type mice and 260 ± 85 pmol MDA/mg protein vs. 130 ± 10 pmol MDA/mg protein in Hp-null mice, $n = 8$). These data demonstrated that Hp null mutation neither worsened renal susceptibility to IRI nor affected renal recovery after damage.

Iron Loading after Glycerol-Induced Acute Muscle Injury

The IRI model showed that, after a renal insult, Hp was able to prevent glomerular filtration of hemoglobin and, consequently, its reabsorption by the renal tubules. To further confirm this observation, we used another model of heme-protein plasma overload, the glycerol model of acute muscle injury. Indeed, the intramuscular injection of hypertonic glycerol induced myolysis and hemolysis thereby releasing into plasma large amounts of myoglobin and hemoglobin.^{22,23}

We used experimental conditions that, in wild-type mice, were able to cause muscle damage without compromising renal function. Two month-old wild-type and Hp-deficient mice were used. Plasma level of creatine kinase was measured as a marker of muscle injury. Six hours after injection of hypertonic glycerol, plasma creatine kinase concentration was increased dramatically in both wild-type and Hp-deficient mice (651 ± 97 Sigma Units/L vs. 13 ± 1 U/L in wild-type mice and 496 ± 93 U/L vs. 21 ± 7 U/L in Hp-deficient mice, $n = 8$), whereas it fell to basal level 24 hours after injection (6 ± 1 U/L in wild-type mice and 13 ± 1 U/L in Hp-null mice, $n = 8$).

To investigate whether Hp null mutation modulates renal iron deposits under these conditions, mice were injected with glycerol and sacrificed 1 and 7 days later. One day after glycerol injection kidney iron increased slightly in wild-type mice, but significantly more in Hp-deficient mice ($P < 0.05$; Figure 6A). Seven days after glycerol injection, kidney iron content had returned to basal level in wild-type controls, but remained significantly higher than basal level in Hp-null mice ($P < 0.001$; Figure 6A). Perl's staining demonstrated that iron accumulated in proximal tubular cells of the kidney (Figure 6B). Again, Hp was crucial in preventing tubular accumulation of iron.

Renal Morphology and Function after Glycerol-Induced Acute Muscle Injury

The kidney is the most exposed tissue to heme protein-mediated damage. As mentioned above, we used experimental conditions that, in wild-type mice, were able to

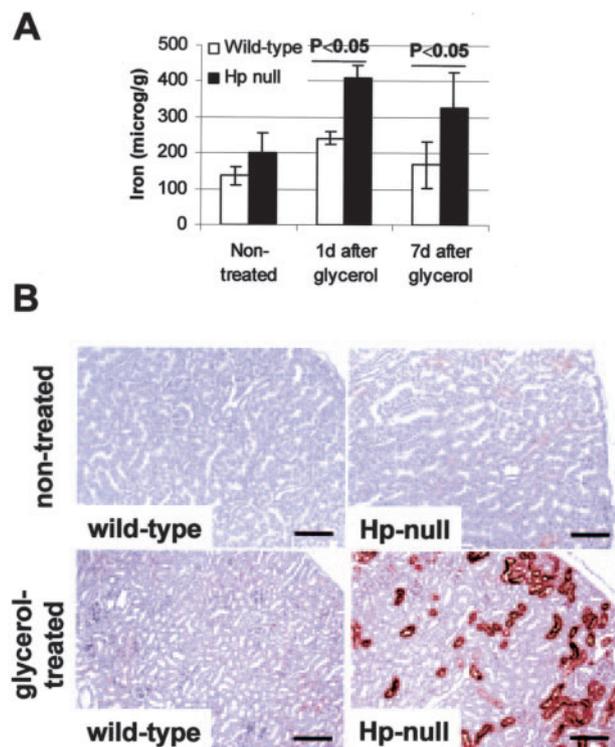


Figure 6. Iron loading after glycerol-induced acute muscle injury. **A:** Kidney iron content was measured in non-treated mice and in glycerol-treated mice 1 and 7 days after injection. Six mice of each genotype were analyzed. Data represent the mean \pm SD. **B:** Kidney sections from non-treated and glycerol-treated (7 days after injection) wild-type and Hp-null mice stained with Perl's reaction. Note weak staining of some tubular cells in non-treated Hp-null mouse compared to strong labeling of proximal tubules in Hp-null mouse 7 days after glycerol injection. In wild-type mouse only a weak stain of some proximal tubules was detected after glycerol treatment. Scale bar = 100 μ m.

induce muscle damage without compromising renal function. To assess the susceptibility of Hp-null mice to muscle injury, we analyzed renal morphology and function after glycerol injection in Hp-deficient mice and compared them to wild-type controls.

Kidney histology, one day after glycerol injection, showed loss of brush-border membranes, tubular dilation, flattened tubular epithelium, luminal debris in both wild-type and Hp-null mice with no between-genotype differences. Seven days after glycerol injection, only isolated tubules with evident signs of tubular necrosis were observed. Again, no differences were detected between wild-type and Hp-null mice (not shown).

Blood urea nitrogen (BUN) and serum creatinine were determined as markers of renal functionality. Twenty-four hours after glycerol injection, BUN levels were similar to basal level in both wild-type (0.17 ± 0.05 mg/ml vs. 0.15 ± 0.02 mg/ml, $n = 8$) and Hp-deficient mice (0.25 ± 0.05 mg/ml vs. 0.15 ± 0.02 mg/ml, $n = 8$). Similarly, serum creatinine was unchanged compared to the basal level in both genotypes (0.34 ± 0.13 mg/dl vs. 0.40 ± 0.05 mg/dl in wild-type mice and 0.44 ± 0.18 mg/dl vs. 0.40 ± 0.05 mg/dl in Hp-null mice, $n = 4$). Finally, lipid peroxidation in kidney extracts 7 days after glycerol injection was slightly increased with regard to basal level in both wild-type and Hp-null mice with no differences between genotypes (178 ± 37 pmol MDA/mg protein vs.

140 ± 56 pmol MDA/mg protein in wild-type mice and 180 ± 66 pmol MDA/mg protein vs. 157 ± 48 pmol MDA/mg protein in Hp-null mice, $n = 8$). Therefore, Hp did not affect renal susceptibility to glycerol-induced acute muscle injury.

Discussion

The present study establishes that Hp prevents renal iron loading by virtue of its ability to direct free hemoglobin to the liver and spleen and to prevent its renal filtration.

After injection of a low dose of labeled hemoglobin, kidneys of Hp-null mice accumulated five times more radioactivity than those of wild-type mice. On the other hand, livers and spleens of Hp-null mice accumulated only 30% and 50%, respectively, of the radioactivity measured in the corresponding organs of wild-type mice. This is the first *in vivo* evidence that Hp directs hemoglobin principally to the liver and spleen and prevents its glomerular filtration.

Uptake of hemoglobin from glomerular filtrate in Hp-null mice is accounted for by megalin- and cubilin-mediated endocytosis. Megalin and cubilin are multiligand endocytic receptors expressed at the apical membrane of proximal tubules. Their primary function is to reabsorb small molecules that pass the glomerular filtration barrier. Previously reported data have shown that hemoglobin is one of their ligands.^{24,25}

The differences in liver and spleen radioactivity between wild-type and Hp-null mice demonstrated that Hp mediated hepatic and splenic recovery of a significant amount of free hemoglobin. Nevertheless, the liver and spleen of Hp-null mice were able to take up some hemoglobin, suggesting the existence of another system involved in the recovery of free hemoglobin. These data are in agreement with previously reported results showing that, after injection of ¹²⁵I-hemoglobin-Hp complexes in rats, the complexes accumulated in liver parenchymal cells.^{26,27} Moreover, Oshiro and collaborators²⁸ showed that, after internalization into liver parenchymal cells, organelles containing the hemoglobin-Hp complex distribute in the microsome fraction where the complex dissociates into two subunits that are subsequently degraded.

After injection of a high dose of ¹²⁵I-hemoglobin, a greater amount of hemoglobin was deposited in the kidney of wild-type mice than after the injection of a low dose of hemoglobin. This indicates that the high dose of hemoglobin saturated the binding capacity of circulating Hp in wild-type mice and, consequently, not bound hemoglobin was filtered by the glomeruli and reabsorbed by proximal tubules. This is accounted for by the fact that, after internalization of the hemoglobin-Hp complex, Hp is not recycled.³

Autoradiography showed that hemoglobin accumulated in the same cell types in wild-type and Hp-deficient mice, ie, proximal tubular cells, hepatocytes, Kupffer cells and splenic macrophages. Labeling of Kupffer cells and macrophages in wild-type mice was expected because CD163, the specific receptor of Hp-hemoglobin complex is exclusively expressed by the monocyte/mac-

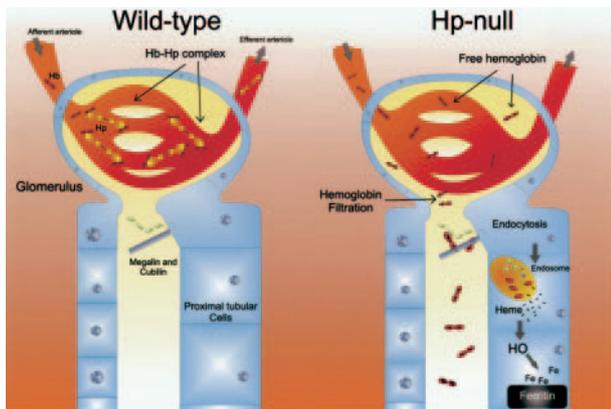


Figure 7. Model to explain renal iron loading in Hp-null mice. In wild-type mice free plasma hemoglobin is bound by Hp and transported to the monocyte/macrophage system. In Hp-null mice, free hemoglobin is filtered through the glomerular barrier and reabsorbed by proximal tubular cells through the endocytic receptors megalin and cubilin. In tubular cells, hemoglobin is degraded in the endosomal compartment and heme is metabolized by heme oxygenase. Iron derived from heme catabolism is stored associated to ferritin. Hb, hemoglobin; Hp, haptoglobin; HO, heme oxygenase.

rophage cell line.^{5,29} Label of hepatocytes in wild-type mice suggests the existence of another receptor for the Hp-hemoglobin complex as reported by *in vivo* and *in vitro* experiments on hepatocytes and hepatoma cell lines.^{26–28,30,31} However, detection of labeled hemoglobin in the same cell types of Hp-null mice suggests the existence of yet another system able to take up hemoglobin. An Hp-independent system may be responsible for hemoglobin recovery in wild-type mice, under conditions of hemoglobin overload when the buffering capacity of circulating Hp is saturated. This system would be activated by default in Hp-deficient mice.

Lack of Hp resulted in iron loading of the kidney during aging. We postulate that, in tubular cells, hemoglobin is degraded in the endosomal/lysosomal compartments and heme is catabolized by heme oxygenase. Iron derived from heme catabolism is stored associated to ferritin, resulting in iron loading (Figure 7).

Iron loading in the kidney was slow in the first two months of life and strongly accelerated at 3 months of age. This could be due to higher iron demand in younger mice resulting in higher rate of iron export from storage tissues, including kidney. According to this hypothesis, the kidney would be able to respond to soluble signals that control iron recycling. One of these could be the plasma peptide hepcidin, which is filtered by glomerulus and is a central modulator of iron homeostasis.³²

Despite iron loading in the kidney of Hp-null mice, no differences in iron deposits were detectable in the liver. This was probably due to the activation of compensatory systems to maintain iron homeostasis, including increased iron absorption and/or accelerated iron turnover in reticuloendothelial cells. However, it is possible to speculate that, under conditions of iron deficiency as in the anemia of inflammation, observed in patients with chronic infections, malignancies, trauma, and inflammatory disorders,³³ the “Hp status” could indeed modulate hepatic iron stores.

Lack of Hp resulted in iron loading of the kidney also after a renal insult in which release of hemoglobin into plasma occurs and after an acute plasma heme-protein overload caused by muscle injury. After intramuscular injection of glycerol as well as on the contralateral kidney of ischemized mice, 7 days after treatment, iron content increased by about 40% and 60%, respectively, compared to baseline in Hp-deficient mice, while it remained at basal levels in wild-type controls. Renal iron deposits were localized exclusively in proximal tubules. In ischemic kidney, iron content increased in both Hp-null and wild-type mice. However, kidney histology showed that while iron accumulated in infiltrating macrophages in both wild-type and Hp-null mice, it only accumulated in proximal tubular cells in the latter. The fact that the bathophenanthroline assay failed to register the difference in iron content observed on kidney sections can be explained by the fact that ischemic injury caused the formation of coagulates which were difficult to remove by perfusion. This might invalidate tissue iron content determination by the spectrophotometric assay.

Models of IRI and muscle injury indicate that acute release of hemoglobin in the bloodstream causes saturation of the iron turnover capacity of tubular cells, thus resulting in iron loading. However, despite the difference in iron content, no difference in lipid peroxidation after IRI and glycerol-induced acute muscle injury was measured between Hp-null and wild-type mice. This fact is not surprising as the amount of iron stored in the kidney of Hp-null mice is far below the amount responsible for peroxidative tissue damage in mouse models of iron loading such as the Hfe knock-out model of hereditary hemochromatosis.³⁴

Nevertheless, previously reported data have shown that, after intravascular hemolysis caused by phenylhydrazine injection, the kidney of Hp-deficient mice suffered from higher oxidative damage than that of wild-type mice.^{6,7} Since the extent of hemoglobin overload following phenylhydrazine injection caused depletion of circulating Hp in wild-type mice (E. Tolosano, unpublished observations), possibly masking some of the effects of Hp gene knockout, we chose models of kidney injury in which hemoglobin overload is low. The accumulation of iron in the kidney of Hp-null mice under these conditions demonstrates that Hp has a role in heme-iron recovery also in the case of hemoglobin overload not exceeding the binding capacity of circulating Hp. Moreover, lack of renal oxidative damage in Hp-null mice, under these conditions as well as under physiological situations, supports the role of the kidney as an iron storage tissue. The capability of the kidney to accumulate iron could become important under particular conditions as in cases of congenital or acquired anaptoglobinemia³⁵ or when other components of the heme-iron recovery pathways in the liver and spleen are impaired. Further studies are needed to elucidate whether the kidney is able to mobilize iron deposits. The expression of the basolateral iron transporter ferroportin in proximal tubules is concordant with this hypothesis.³⁶

The lack of difference in lipid peroxidation between wild-type and Hp-null mice is in agreement with results

showing renal histological and functional parameters that were comparable in wild-type and Hp-deficient mice either after IRI or rhabdomyolysis. Although previous reports have shown that extrarenal heme proteins, such as hemoglobin, might contribute to renal damage after ischemia-reperfusion and glycerol injection,^{18,19,37} our data indicate that free hemoglobin contribution to kidney oxidative injury in our experimental conditions was negligible. Heme released from intracellular hemoproteins as cytochrome P450 and catalase, in the case of IRI and, from myoglobin, in the case of glycerol injection is probably the main reason for kidney damage. However, it is possible that other plasma heme-binding proteins as albumin, hemopexin³⁸ and α 1-microglobulin³⁹ may compensate for the lack of Hp.

Plasma hemoglobin overload induced circulating Hp expression following increased Hp mRNA expression in the liver. Also, a specific expression of Hp was detected in the kidney. This is in agreement with previous reports showing renal expression of Hp after LPS administration.⁴⁰ The ability of the kidney to express Hp further supports the hypothesis that this organ is actively involved in hemoglobin handling. On the other hand, local expression of Hp has recently been reported in arteries after flow-induced arterial remodeling and in arthritic and oncological tissues.⁴¹ Therefore, local Hp expression at sites of hemoglobin release could be an important strategy to remove heme-iron, thus assisting circulating Hp.

Finally, we showed renal expression of CD163 following renal injury. CD163^{5,29} was probably expressed by infiltrating macrophages in the ischemic kidney and would therefore be involved in the resolution of inflammation after injury, as well as in elimination of Hp-hemoglobin complexes.

In conclusion, in this study we demonstrate that Hp is an important modulator of iron homeostasis. In humans, a correlation between Hp haplotype and amount of transferrin-bound iron as well as macrophage iron retention has been reported.^{9,10} Our data provide a rationale for these observations. In particular, the extent of renal iron accumulation in Hp-null mice indicates that Hp mediates the recovery of a significant amount of iron, suggesting that different binding capacities of human Hp variants to their ligand could indeed modulate heme-iron recovery. Moreover, our data highlight the role of the kidney in hemoglobin handling in addition to its well-known role in non-heme iron recovery.

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