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Poor lysosomal membrane integrity in proximal tubule cells of haptoglobin 2-2 genotype mice with diabetes mellitus

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Abstract

The haptoglobin (Hp) genotype is a major determinant of progression of nephropathy in individuals with diabetes mellitus (DM). The major function of the Hp protein is to bind and modulate the fate of extracorporeal hemoglobin and its iron cargo. We have previously demonstrated an interaction between the Hp genotype and the DM on the accumulation of iron in renal proximal tubule cells. The primary objective of this study was to determine the intracellular localization of this iron in the proximal tubule cell and to assess its potential toxicity. Transmission electron microscopy demonstrated a marked accumulation of electron-dense deposits in the lysosomes of proximal tubules cells in Hp 2-2 DM mice. Energy-dispersive X-ray spectroscopy and electron energy loss spectroscopy were used to perform elemental analysis of these deposits and demonstrated that these deposits were iron rich. These deposits were associated with lysosomal membrane lipid peroxidation and loss of lysosomal membrane integrity. Vitamin E administration to Hp 2-2 DM mice resulted in a significant decrease in both intralysosomal iron-induced oxidation and lysosomal destabilization. Iron-induced renal tubular injury may play a major role in the development of diabetic nephropathy and may be a target for slowing the progression of renal disease.

Keywords

Diabetes; Nephropathy; Lysosome; Iron-induced injury; Haptoglobin genotype; Oxidative stress

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) and accounts for approximately 40% of all patients who require renal replacement therapy [1]. Traditional risk factors and glycemic control are important but inadequate for predicting the incidence and severity of DN. The interindividual variability in the risk for developing DN and its clustering within families suggest a substantial genetic predisposition [2,3]. As reactive oxygen species, particularly those derived from iron, have been implicated in the progression of DN and other vascular complications of Diabetes, polymorphic genetic loci encoding variants in enzymes protecting against iron-induced oxidative stress serve as potential susceptibility determinants for the development of DN [4–7].

Haptoglobin (Hp) is an acute phase protein whose primary function is to neutralize the prooxidative activity and accelerate the clearance of extracorporeal hemoglobin (Hb) [8]. In humans there exists a common functional allelic polymorphism at the Hp locus with two classes of alleles denoted 1 and 2. Seven independent longitudinal studies have demonstrated a direct relationship between the Hp genotype and the incident cardiovascular disease in DM with the Hp 2-2 genotype being associated with a 2- to 5-fold increased risk [9]. In the only published longitudinal study examining the relationship between incident DN and the Hp genotype Costacou and colleagues demonstrated in the EDC cohort that the Hp 2-2 genotype was an independent determinant of early renal functional decline and progression to ESRD in individuals with Type I DM [10]. We have recently confirmed in a second longitudinal cohort this association between incident early renal functional decline and progression to ESRD in Type I DM and the Hp genotype in the DCCT/EDIC cohort (A. Levy, unpublished observations).

We previously demonstrated in C57Bl/6 mice that in the setting of DM, replacement by homologous recombination of the wild-type Hp 1 allele with the Hp 2 allele converted this mouse strain from a nephropathy resistant to a nephropathy prone state. Hp 2-2 DM mice were shown to develop histological and functional (changes in creatinine clearance) changes representative of the early changes found in humans with DN [11]. Moreover, we demonstrated that these nephropathic changes occurring in Hp 2-2 DM mice could be prevented by vitamin E, suggesting that these changes were due to oxidative stress [11]. A striking histological feature in Hp 2-2 DM mice was a marked accumulation of iron (documented with Perl's stain) *exclusively* in renal proximal tubule cells and this iron accumulation was associated with marked tubular hypertrophy [11]. A growing body of evidence suggests that the deterioration of renal function in DN correlates best with proximal tubular injury with tubular hypertrophy preceding and inducing hypertrophy and sclerosis of the glomeruli [12]. The primary objective of the present study was to determine the intracellular localization of iron in the renal proximal tubule cell of DM mice and to assess how it may result in proximal tubular cell damage.

Research design and methods

Animal studies

All procedures were approved by the Animal Care Committee of the Technion (protocol number IL-112-11-11). All mice were of a C57Bl/6 genetic background. The Hp 2 allele is present only in humans. All other species have only an Hp 1 allele, which is highly homologous with the human Hp 1 allele. Thus, wild-type mice carry the Hp 1 allele (referred herein as Hp 1-1 mice). The construction of the murine Hp 2 allele and the targeting of its insertion by homologous recombination to the murine Hp genetic locus have been previously described [13]. Mice were fed normal chow and in mice in which we sought to induce DM, intraperitoneal streptozotocin was administered (50 mg/kg for 5 subsequent days) at 10 weeks of age. Mice were sacrificed after a DM duration of 3 months (non-DM mice and DM mice were sacrificed at the same age). There were no differences in spot

glucose levels between mice with the different Hp genotypes. For vitamin E studies, DM mice were treated with placebo or vitamin E (40 mg/kg/day administered in the drinking water [11])) beginning 1 month after the onset of DM; mice were sacrificed after 2 months of vitamin E or placebo treatment. After mice were sacrificed the kidneys were removed and washed in saline and either fixed in formalin for morphometric and immunohistological analysis [13], in glutaraldehyde for electron microscopy analysis, or placed in liquid nitrogen for biochemical and cell fractionation studies.

Transmission electron microscopy (TEM) [14,15]

Kidney tissue samples from Hp 1-1 and Hp 2-2 mice were immersed immediately on isolation into 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h and then postfixed in osmium tetroxide for 1 h, dehydrated through a series of ethanol solutions, and embedded in epoxy. Semithin sections were used for orientation and selection. Ultrathin sections (60 nm) were cut with a diamond knife, mounted on 300-mesh copper grids, and either left unstained or stained with lead citrate for 2 min. Sections of four different blocks were viewed and photographed with a Jeol 100SX electron microscope operated at 80 kV. Electron micrographs at a magnification of 2000–80,000 were captured with an 11.1 megapixel CCD (SIA, Duluth, GA).

Energy dispersive X-ray spectroscopy (EDX) and electron energy loss spectroscopy (EELS)

EDX and EELS were performed with a Zeiss Libra 120 transmission electron microscope that was equipped with a Pheonix X-ray detector (EDAX) and an in-column Omega energy filter. EELS spectra were recorded with a digital camera at 120 kV and an energy resolution of 1.5 eV using the EFTEM software of Olympus soft imaging solutions. Sections were used without any on-section staining and were about 50 nm (EELS) or 80–120 nm (EDX) in thickness.

Lysosome purification

Lysosomes were purified from murine kidneys as previously described with some modifications [16–18]. One kidney was removed to a chilled petri dish containing homogenization medium (HM: 0.25 M sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.0) and finely minced. The tissue was suspended in 3 ml HM and transferred to a Potter-Elvehjem homogenizer and homogenized with 7 strokes. The homogenate was centrifuged twice for 10 min at 750 g at 4 °C, and the postnuclear supernatant (PNS) removed. The PNS was then incubated with 1.25 mM calcium chloride (resulting in swelling of the mitochondria, making them less dense and easier to separate from the lysosomal fraction during Percoll density centrifugation). The PNS was then centrifuged at 20,000g for 10 min at 4 °C. The resulting pellet was resuspended in 5.5 ml HM and mixed with 4.5 ml 90% Percoll in HM. This solution was then centrifuged for 30 min at 47,000g at 4 °C in Beckman ultracentrifuge tubes (No. 344059) after which the top 7 ml was removed with a pipette and the bottom 1.5 ml removed with a syringe connected to a needle. The bottom 1.5 ml was spun at 100,000g for 1 h in Beckman microultracentrifuge tubes (No. 343778) to pellet the Percoll. The turbid layer above the pellet was collected, suspended in HM, and kept at 4 °C until analysis. Protein concentrations were determined using the Bradford reagent. We monitored the purification of the lysosomes by following the specific activity of the lysosomal-specific markers acid phosphatase and cathepsin D (Sigma). We observed an approximately 50-fold enrichment during this purification procedure, similar to that previously described [16,18], in the specific activity of these lysosomal-specific markers compared to the whole cell homogenate. In order to assess contamination of the lysosomal preparation from other cell organelles we also assessed the activity of cytochrome *c* oxidase (mitochondria), cytochrome *c* reductase (endoplasmic reticulum), and catalase (peroxisome) (all assessed

using marker-specific enzymatic assays from Sigma). We did not find detectable activity of these other organelle-specific markers, as previously described [16,18], in our lysosomal preparations used in these studies.

Assessment of lysosomal membrane integrity

Lysosomal membrane integrity was determined by measuring the activity of the lysosomal enzyme β -hexosaminidase using the fluorimetric substrate 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside [17]. Since intact lysosomal membranes prevent substrate access to the intralysosomal enzyme, no substrate turnover in a preparation of lysosomes indicates integrity of the lysosomal membrane.

Briefly, 50 μ l of the lysosome preparation was incubated for 1 min at 37 °C with 250 μ l of 100 mM citrate buffer, pH 4.5, containing 0.25 M sucrose, 20 mM sodium phosphate, pH 4.5, and 1.2 mM 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside with and without 0.2% Triton X-100. The reaction was terminated after 1 min by the addition of 1 ml of 0.5 M glycine/sodium carbonate buffer, pH 10. The liberated 4-methylumbelliferone was determined by measuring the fluorescence in a BMG GalaxyFlouroStar microplate reader with a 365/450 nm excitation/emission filter pair. The degree of lysosome injury was expressed as percentage fragility ([activity in the absence of detergent/activity in the presence of detergent] $\times 100$).

Assessment of lysosomal membrane lipid peroxides

Lysosomal lipid peroxides were measured by adding to 100 μ l of the lysosomal preparation, 1 ml of lipid peroxide reagent (0.2 M potassium hydrophosphate, 0.12 M potassium iodide, 0.15 M sodium azide, 2 g/L igepal, 0.1 g/L alkylbenzyltrimethylammonium chloride, 10 μ M ammonium molybdate). The mixture was vortexed and incubated for 30 min in the dark, and then the OD of the sample was measured at 365 nm. The concentrations of lipid peroxides were calculated as previously described [19].

Measurement of redox-active chelatable iron in kidney lysosomes

Assessment of redox-active chelatable iron has been described previously [20]. Briefly, dihydrorhodamine (DHR) was used as a sensitive fluorescent indicator of oxidative activity. To assess the amount of redox-active chelatable iron in lysosomal preparations, quadruplicates of 20 μ l of purified lysosomes were transferred to clear-bottom 96-well plates and incubated with DHR (50 μ M) in 180 μ l iron-free Hepes-buffered saline containing 40 μ M ascorbate in the presence and absence of 50 μ M iron chelator (deferiprone). The kinetics of fluorescence increase were followed at 37 °C in a BMG GalaxyFlouroStar microplate reader with a 485/538 nm excitation/emission filter pair for 40 min. The slopes of the DHR fluorescence intensity over time were then determined. The difference in the rate of oxidation of DHR in the presence and absence of the chelator represents the specific component of iron that is catalytically redox active. The free redox-active iron concentrations (in μ M) were then determined from calibration curves using Hepes-buffered saline with increasing concentrations of Fe:nitrilotriacetic acid (NTA).

Measurement of vitamin E

α - and γ -tocopherol concentrations, as well as total cholesterol concentrations (see below) were determined [21]. Tocopherols were analyzed by high performance liquid chromatography (HPLC) with electrochemical detection, using a modification of Podda et al. [21]. Briefly, samples were mixed with 2 ml 1% ascorbic acid in ethanol and 1 ml Milli-Q water (Millipore, Billerica, MA). Following addition of 300 μ l saturated KOH, samples were saponified at 70 °C for 30 min. After cooling on ice and addition of 25 μ l BHT, the

samples were extracted with hexane, taken to dryness under nitrogen, resuspended in 70 μ l 50:50 (v/v) ethanol–methanol, and then transferred to HPLC injection vials. The HPLC system consisted of a SCL-10 A VP Shimadzu (Kyoto, Japan) system controller, two LC-10AD VP Shimadzu pumps, a SIL-10AD VP Shimadzu autoinjector with sample cooler, a Brownlee Spheri-5 RP-18 precolumn (5 μ m 30 $\times$ 4.6 mm), a Symmetry Shield RP18 (3.5 μ m $\times$ 4.6 mm $\times$ 100 mm) column (Waters, Milford, MA), and a LC-4C amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN). The mobile phase consisted of 0.1% (w/v) lithium perchlorate and 98:2 (v/v) methanol–water and was run isocratically at 0.6 ml/min/pump for 8 min. Cholesterol concentrations of the hexane extracts used for tocopherol analysis were determined by taking a known aliquot of the hexane to dryness under nitrogen. Cholesterol was then assayed using the Amplex Red Cholesterol Assay Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Specifically, cholesterol concentrations were determined fluorometrically using a SpectraMax Gemini XS microplate spectrofluorometer (Molecular Devices, Silicon Valley, CA) with an excitation wavelength of 545 nm and an emission wavelength of 590 nm. α -Tocopherol was normalized to cholesterol and expressed as nanomole α -tocopherol per milligram cholesterol.

Statistical analysis

All results are reported as means $\pm$ SEM. Comparison between groups was performed using ANOVA and the Tukey-Kramer honestly significant difference method for pairwise comparisons, with a *P* value of <0.05 considered significant. For determination of the percentage of tubule cell lysosomes which contained electron-dense deposits per mouse, all lysosomes from 30 fields per mouse (TEM, magnification 10,000 $\times$) were used. For determination of the average size of electron-dense deposits in the lysosome per mouse, in those lysosomes which had visible deposits, the average percentage of lysosomal area of 10 lysosomes per mouse was determined (also determined at 10,000 $\times$ magnification). In order to ascertain if there was a significant difference between Hp 1-1 and Hp 2-2 DM mice in the percentage of lysosomes which had electron-dense deposits or in the average size of these deposits, *P* values were determined comparing the average values calculated for each mouse.

Results

Increased iron-rich deposits in lysosomes of proximal tubule cells in Hp 2-2 DM mice

We sought to assess the intracellular localization of iron within the proximal tubules using TEM. TEM demonstrated a marked accumulation of electron-dense deposits in the lysosomes of proximal tubule cells (Fig. 1A). EDX and EELS were used to perform elemental analysis of these deposits and demonstrated that these deposits were iron rich (Fig. 1B). Lysosomes containing these iron deposits were more frequent in the proximal tubule of Hp 2-2 (65 $\pm$ 4% of all lysosomes) compared with Hp 1-1 DM mice (41 $\pm$ 4% of all lysosomes, *P* <0.05) (Fig. 1A). The deposit sizes were approximately double within Hp 2-2 DM lysosomes as compared to those within lysosomes from Hp 1-1 DM mice (*P* <0.05) (Fig. 1A). Fig. 1C demonstrates that the electron-dense deposits were surrounded by a single membrane (not visible at lower magnifications as in Fig. 1A) and hence are located completely within the lysosome.

Intralysosomal redox-active iron concentrations are markedly increased in Hp 2-2 DM mouse kidneys

In order to further characterize the biochemical properties and functional significance of these iron-rich lysosomal deposits, we isolated lysosomes from Hp 1-1 and Hp 2-2 DM mice monitoring the specific activity of lysosomal and other organelle-specific enzymes to assess the purity of the preparation. In order to determine if the increased iron-rich deposits in Hp

2-2 DM mice had the potential to produce reactive oxygen species, we assessed redox-active chelatable iron in lysosomes of Hp 1-1 and 2-2 DM mice. We found a 2-fold increase in the amount of redox-active iron in the lysosomes of Hp 2-2 DM mice ($0.56 \pm 0.07 \mu\text{M}$) as compared with those from Hp 1-1 DM mice ($0.23 \pm 0.14 \mu\text{M}$, $n=6$ in each group, $P=0.036$).

Lysosomal membrane lipid peroxides are increased in Hp 2-2 DM proximal tubule cell lysosomes

Having demonstrated that lysosomes from Hp 2-2 DM mice contain increased redox-active iron, we next sought to determine if this iron was associated with an increase in lysosomal membrane lipid peroxidation. Lysosomal lipid peroxides were significantly increased in Hp 2-2 DM mice ($n=6$ in each group, $P<0.0001$ for one-way ANOVA and $P<0.001$ for all pairwise comparisons between the Hp 2-2 DM and the other three groups) (Fig. 2).

Lysosomal membrane integrity is compromised in Hp 2-2 DM proximal tubule cell lysosomes

In order to determine if the increased lysosomal membrane peroxidation was associated with the loss of lysosomal membrane integrity, we measured the specific activity of an intralysosomal enzyme, β -hexosaminidase, in lysosomal preparations from Hp 1-1 and Hp 2-2 mice with and without DM. Membrane integrity was significantly decreased in the lysosomes purified from the kidneys of Hp 2-2 DM mice as compared to all other groups ($n=6$ in each group, $P<0.003$ by ANOVA comparing all 4 groups, and $P<0.01$ for pairwise comparisons between Hp 2-2 DM and the other three groups) (Fig. 3).

Proof of concept that the loss of lysosomal membrane integrity in Hp 2-2 DM proximal tubule cells is due to oxidative damage

We sought to evaluate the role of lipid peroxidation in the maintenance of lysosomal membrane integrity by showing that chronic administration of the lipid-soluble antioxidant, vitamin E, could decrease lysosomal membrane oxidation and maintain lysosomal membrane integrity. Vitamin E supplementation resulted in a significant 45% reduction in lysosomal redox-active iron in Hp 2-2 DM mice ($n=6$, $P<0.04$) with no significant effect on lysosomal redox-active iron in Hp 1-1 DM mice. Moreover, we found that vitamin E supplementation significantly decreased lysosomal lipid peroxides in Hp 2-2 DM kidneys as compared with lysosomal preparations of Hp 2-2 DM mice treated with placebo (75.7 ± 8.7 nmol lipid peroxides/mg protein for Hp 2-2 DM with vitamin E vs 109.2 ± 8.8 nmol lipid peroxides/mg protein for Hp 2-2 DM without vitamin E, $n=6$ in each group, $P=0.03$). There was no significant reduction in lysosomal lipid peroxides in Hp 1-1 DM mice treated with vitamin E (Fig. 4A). Moreover, there was a significant correlation between lysosome membrane α -tocopherol concentrations and the degree of lysosomal membrane oxidation in Hp 2-2 DM mice but not in Hp 1-1 DM mice (Fig. 5A; $n=12$ for both groups, correlation coefficient=0.79, $P<0.003$ for correlation of vitamin E and lysosomal lipid peroxides in Hp 2-2 DM and correlation coefficient=0.54, $P=0.07$ for correlation of vitamin E and lysosomal lipid peroxides in Hp 1-1 DM mice). Finally, we found a significant reduction in the loss of lysosomal membrane integrity in lysosomes purified from kidneys of Hp 2-2 DM mice treated with vitamin E as compared with those treated with placebo ($24.1\% \pm 2.3$ for vitamin E group vs $30.7\% \pm 1.7$ for placebo group, $n=6$ per group, $P=0.03$). No significant differences in lysosomal membrane integrity were found after vitamin E administration to Hp 1-1 DM mice as compared to those treated with placebo ($19.9\% \pm 2.7$ for vitamin E group vs $22.1\% \pm 2.3$ for placebo group, $n=6$, $P=0.24$) (Fig. 4B). There was a significant correlation in Hp 2-2 DM mice, but not in Hp 1-1 DM mice, between the concentration of vitamin E in the lysosomal membrane and the lysosomal membrane integrity (Fig. 5B; $n=12$ for both groups, correlation coefficient 0.72, $P<0.008$ for correlation of vitamin E and lysosomal integrity in Hp 2-2 DM and correlation coefficient=0.53, $P=0.08$ in Hp 1-1 DM mice).

Discussion

In this study we have demonstrated that increased lysosomal redox-active iron results in lysosomal membrane injury in renal cells of Hp 2-2 DM mice. These data therefore provide a novel pathophysiological mechanism explaining why the progression to end-stage renal disease is increased in DM individuals with the Hp 2-2 DM genotype. Moreover, the interaction between the vitamin E and the Hp genotype on lysosomal injury suggests that a pharmacogenomic paradigm of selective administration of vitamin E to Hp 2-2 DM individuals may offer considerable renal protection similar to that recently demonstrated for cardiovascular disease [22].

There exists prior evidence that proximal tubule cell lysosomal iron overload is a common pathogenic feature of early diabetic nephropathy [23]. Nankivell and colleagues demonstrated a dramatic increase in lysosomal iron deposition in biopsies from individuals with DN as compared to normal controls [23]. Moreover, the amount of proximal tubule cell iron deposition was highly correlated with the degree of loss of renal function in these individuals. While the Hp genotype of these individuals in this prior study was not reported this prior study demonstrates the relevance of the findings of the current study done in mice to DN in man.

Proximal tubule cell lysosomal iron accumulation has also been demonstrated in human biopsy specimens from individuals with early diabetic nephropathy, although the Hp-type relationship in these specimens was not investigated. As lysosomes were derived from whole kidneys, theoretically the compromise in lysosome membrane integrity demonstrated in this study may have occurred in any renal cell type. However, we assert that the changes described here in lysosomal structure and function are specific for proximal tubule cells. We have shown that the lysosomal injury is due to iron accumulating in the lysosome. The focus on proximal tubule cells is based on prior data [11,20] showing that Perl's iron stain is present only in proximal tubule cells and not in the glomerulus and its constituent cells nor in other tubular or interstitial cells. It is axiomatic that in the absence of a Perl's stain there is no possibility that cells other than the proximal tubular cells are iron overloaded in the Hp 2-2 DM mice. Furthermore, these other renal cell types do not have iron deposits by EM as we have shown here in proximal tubule cells.

The source of excess proximal tubule iron in Hp 2-2 DM is Hb [20]. In Hp 2-2 DM the normal clearance mechanism via the CD163 receptor [24] for the Hp-Hb complex is severely impaired with an approximate 5-fold increase in the half-life of the complex [22]. Consequently the steady-state levels of the complex are higher in Hp 2-2 DM [22]. Moreover, in DM the glomerular filtration barrier is perturbed, thereby allowing complexes such as Hp-Hb to cross the barrier. Proximal tubule cubulin and megalin have both been demonstrated to bind to Hb in the glomerular filtrate, mediating Hb uptake into proximal tubule cells, and may serve as an entry point into these cells for the Hp-Hb complex [25]; however other siderophores such as neutrophil gelatinase-associated lipocalin may also mediate this process [26]. The endocytosed Hp-Hb complex is then targeted to the lysosome where it is degraded [25]. As the half-life of Hp 2-2-Hb and Hp 1-1-Hb in the lysosome does not appear to be different [27], the differences observed in the steady-state amount of proximal tubule iron in mice with the Hp 1-1 or Hp 2-2 genotypes results from the greater flux of iron through the renal proximal tubule cell in Hp 2-2.

The form in which excess iron is found in lysosomes of Hp 2-2 DM mice appears to be as cross-linked iron-rich protein complexes such as ferritin, hemosiderin, and lipofuscin based on the characteristic features of these complexes on TEM [15]. This excess iron in Hp 2-2 DM renal proximal tubule lysosomes is redox active and may therefore result in cellular

toxicity. The lysosome membrane appears to be better geared for oxidative insults than other cellular membranes, as it is enriched with antioxidants (vitamin E); the vitamin E content of the lysosome membrane is 40 times greater than that of the cytoplasmic membrane [28]. While the lysosome may be geared to deal with oxidants such as iron, the unique properties of the Hp 2-2-Hb complex in the setting of DM may overwhelm its antioxidative defenses [29,30]. The Hp 2-2-Hb complex may act as a Fenton reagent, and in the setting of DM, peroxide production may be sufficiently high to promote a large increase in hydroxyl radical formation via Fenton chemistry [31].

Lysosomal injury has recently been suggested as a new pathophysiological paradigm playing a major role in a wide range of chronic diseases ranging from atherosclerosis to neurodegenerative disease [32]. A loss of lysosomal membrane integrity would be expected to lead to the ability of degradative enzymes present in the lysosome to enter the cytosol and wreak havoc, leading to cell injury and death. We propose that any cell that endocytoses the Hp-Hb complex may develop lysosomal injury but with the exception of macrophages, no other cell type other than the proximal tubule cell has a mechanism to endocytose this complex. Consistent with this hypothesis, we have recently demonstrated that macrophages release cathepsin D (an intralysosomal enzyme) into the cytoplasm after taking up Hp 2-2-Hb complexes (unpublished observations) and this may explain the association of the Hp type with atherosclerotic plaque rupture in DM [33]. The loss of lysosomal integrity would also be expected to interfere with the maintenance of the acidic pH gradient present in the lysosome and therefore interfere with lysosome degradative functions conceivably leading to a defect in autophagy [32].

In conclusion, we have provided evidence for a novel mechanism whereby the Hp genotype may predispose to renal injury in the setting of DM. Elimination of excess proximal tubule iron, or its dangerous oxidative potential, possibly by a combination of iron chelating agents [34] and/or antioxidants [35] may thus prove helpful in the prevention of DN in DM individuals with the Hp 2-2 genotype.

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Abbreviations

DN	Diabetic nephropathy
ESRD	End-stage renal disease
Hp	Haptoglobin
DM	Diabetes mellitus
EDC	Epidemiology of diabetic complications
DCCT/EDIC	Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications

TEM	Transmission electron microscopy
EDX	Energy dispersive X-ray spectroscopy
EELS	Electron energy loss spectroscopy
DHR	Dihydrorhodamine

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Fig. 1.

Increased iron-rich deposits in lysosomes of proximal tubule cells in Hp 2-2 DM mice. TEM and EDX were performed as described in methods. (A) Increased presence and size of electron-dense deposits in lysosomes of Hp 2-2 DM mice. TEM of proximal tubule of DM mice with the Hp 1-1 (left panel) or Hp 2-2 genotype (right panel); original magnification 10,000 $\times$. Note, in Hp 1-1 DM mice, relatively few lysosomes are seen with discrete electron-dense deposits. In Hp 2-2 DM mice, most lysosomes contain electron-dense deposits. In Hp 2-2 DM mice 65 $\pm$ 4% of lysosomes contained deposits as compared to 41 $\pm$ 4% in Hp 1-1 DM mice (30 fields were screened for each mouse, $n=5$, $P<0.05$). Lysosomal electron-dense deposits were larger in Hp 2-2 DM mice as compared to Hp 1-1 DM mice (0.09 $\pm$ 0.01% vs 0.045 $\pm$ 0.01%, $P<0.05$). (Results expressed as percentage of lysosomal area of 10 lysosomes per mouse. All lysosomes used for this analysis had visible deposits). (B) Electron-dense deposits in lysosomes of proximal tubule cells of Hp 2-2 DM mice contain iron. The left image is an electron micrograph highlighting two regions by a circle. Circle 00 labels a cytoplasmic area containing a mitochondrion in a tubular cell with no electron-dense deposit. Circle 01 labels a single membrane-bound organelle showing electron-dense particles. The large electron-dense body at the bottom of the image is most likely a fat droplet. Regions 00 and 01 underwent analysis by EDX which allows elemental analysis in the selected region of the section by choosing a particular condenser aperture. The EDX spectra (on the right) show no iron peak for region 00 (arrow in bottom graph) but a high copper peak generated by the copper grid on which the tissue is mounted. In contrast, for region 01, a significant Fe peak (arrow in top graph) can be seen in addition to the peaks detected also in region 00. EELS was performed in a similar way and also revealed Fe in the respective spectrum and region (not shown). (C) High resolution examination of lysosomal electron-dense deposits in proximal tubule cells of Hp 2-2 DM mice. TEM (40,000 $\times$ magnification) of a proximal tubule lysosome containing an iron-rich electron-dense deposit (arrows) in a Hp 1-1 DM mouse (left panel) and a Hp 2-2 DM mouse (right panel). At this

higher magnification it is demonstrated that the electron-dense deposits are completely encircled by a single membrane (not visible at lower magnification as in panel A and hence are completely within the lysosome).

Fig. 2.

Lysosomal lipid peroxides are significantly increased in the lysosomes of Hp 2-2 DM kidneys. The amount of lipid peroxides is presented as the mean $\pm$ SEM for each group. Lysosomal lipid peroxides were significantly increased (*) in Hp 2-2 DM mice ($n=6$ in each group, $P<0.0001$ by ANOVA comparing all 4 groups; $P<0.001$ for all pairwise comparisons between Hp 2-2 DM and the other three groups).

Fig. 3.

Lysosomal membrane integrity is reduced in proximal tubule lysosomes in Hp 2-2 DM mice. Lysosomal membrane integrity was assessed in lysosomal preparations purified from kidneys of Hp 1-1 and Hp 2-2 DM and non-DM mice using the β -hexosaminidase assay as described in the methods section. There was a significant (*) reduction in lysosomal membrane integrity in Hp 2-2 DM mice ($n=6$ in each group, $P<0.003$ by ANOVA comparing all 4 groups; $P<0.01$ for all pairwise comparisons between Hp 2-2 DM and the other three groups).

Fig. 4.

Effect of vitamin E supplementation on lysosomal membrane lipid peroxides and membrane integrity. (A) Lysosomal membrane lipid peroxides were significantly reduced in Hp 2-2 DM mice which received vitamin E supplementation as compared to Hp 2-2 DM mice receiving placebo (* $P=0.03$). (B) Lysosomal membrane activity was reduced in Hp 2-2 DM mice which received vitamin E supplementation as compared to Hp 2-2 DM mice receiving placebo (* $P=0.03$).

Fig. 5.

Lysosomal vitamin E concentration is correlated with lysosomal membrane lipid peroxidation and lysosomal membrane integrity. Vitamin E was measured in lysosomal preparations as described in the methods section and normalized to cholesterol. Filled symbols are data points for mice receiving vitamin E supplementation while empty symbols are data points for mice who received placebo. (A) Lysosomal vitamin E and lysosomal lipid peroxidation. There was a highly significant correlation between the amount of lysosomal vitamin E and the lysosomal membrane lipid peroxidation in Hp 2-2 DM mice ($n=12$, half of which received vitamin E supplementation, correlation coefficient=0.79, $P<0.003$). There was no significant correlation between vitamin E and lysosomal lipid peroxidation in Hp 1-1 DM mice ($n=12$, half of which received vitamin E supplementation, $P=0.10$) (data not shown). (B) Lysosomal vitamin E and lysosomal membrane integrity. There was a highly significant correlation between lysosomal vitamin E and lysosomal membrane integrity in Hp 2-2 DM mice ($n=12$, half of which received vitamin E supplementation; correlation

coefficient=0.72, $P=0.008$). There was no significant correlation between vitamin E and lysosomal membrane integrity in Hp 1-1 DM mice (data not shown).