# 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 extracorpuscular 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(DN) and may be a target for slowing the progression of renal disease.

## Introduction

Diabetic nephropathy is the leading cause of end-stagerenal disease (ESRD) and accounts for approximately 40% of all patients who require renal replacementtherapy. Traditional risk factors and glycemic control are important but inadequate for predicting the incidence and severity of DN

Haptoglobin (Hp) is an acute phase protein whose primary function is to neutralize the prooxidative activity and accelerate the clearance of extracorpuscular hemoglobin (Hb) . In humans there exists a common functional allelic polymorphism at the Hp locus with two classes of alleles denoted 1 and 2.

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 GFR) changes representative of the early changes found in humans with DN.

## Research design and methods

All procedures were approved by the Animal Care Committee of the Technion. All mice were of a C57B1/6 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. 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. Mice were fed normal chow and in mice in which we sought to induce DM, Streptozotocin was administered IP (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). For vitamin E studies, DM mice were treated with placebo or vitamin E (40 mg/ kg/day administered in the drinking water 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 , in glutaraldehyde for electron microscopy analysis, or placed in liquid nitrogen for biochemical and cell fractionation studies.

## Results

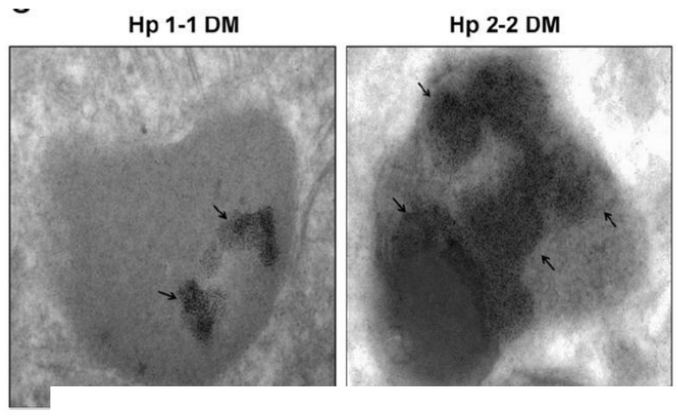


Fig. 1. Increased iron-rich deposits in lysosomes of proximal tubule cells in Hp 2-2 DM mice. High resolution examination of lysosomal electron-dense deposits in proximal tubule cells of Hp 2-2 D mice.

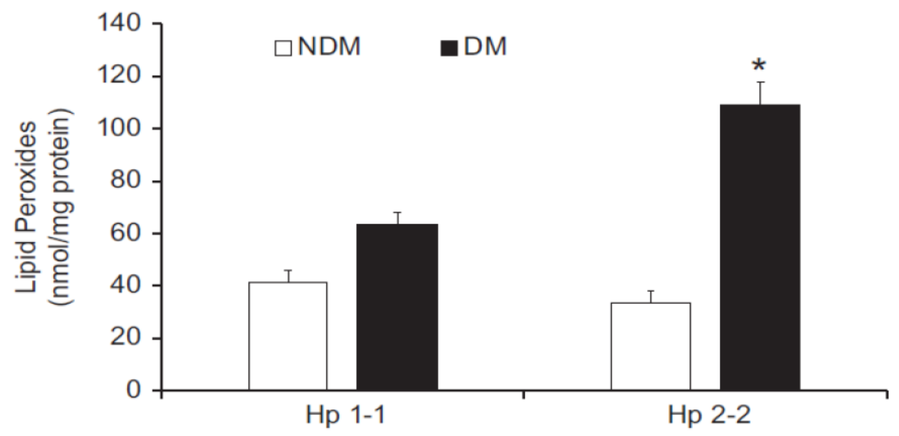


**Fig. 2.** Lysosomal lipid peroxides are significantly increased in the lysosomes of Hp 2-2 DM kidneys The amount of lipid peroxides is presented as the mean ± SEM for each group. Lysosomal lipid peroxides were significantly increased (*) in Hp 2-2 DM mice ($n=6$ in each group, $P<0.0001$ by ANOVA comparing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

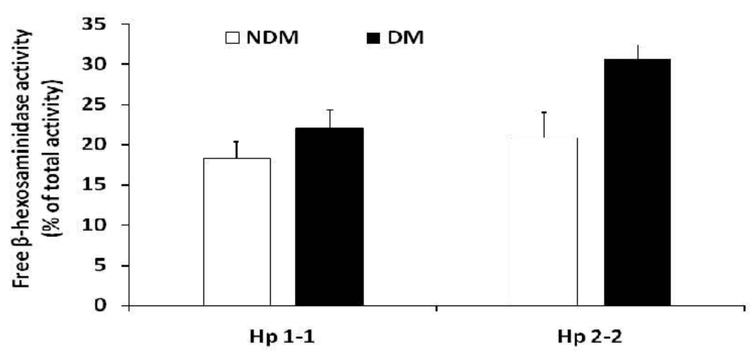


**Fig. 3**. Lysosomal membrane integrity is reduced in proximal tubule lysosomes in Hp 2-2DM mice. Lysosomal membrane integrity was assessed in lysosomal preparations purified from kidneys of Hp1-1 and Hp2-2DM and non-DM mice using the b-hexosaminidase assayas. There was a significant(*) reduction inlysosomal membrane integrityinHp2-2DM mice (n¼6 in each group, Po0.003 by ANOVA comparing all 4 groups; Po0.01 for all pair wise comparisons between Hp2-2DM and the Other three groups..

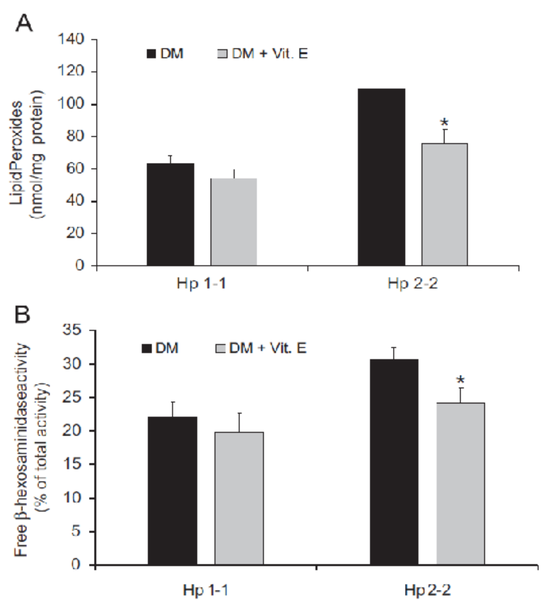


Fig. 4. Effect of vitamin E supplementation on lysosomal membrane lipid peroxides and membrane integrity. (A) Lysosomal membrane lipid peroxides were significantly reduced in Hp 2-2 DM mice which received vitamin E supplementation as compared to Hp 2-2 DM mice receiving placebo (* $P=0.03$). (B) Lysosomal 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 (* $P=0.03$).

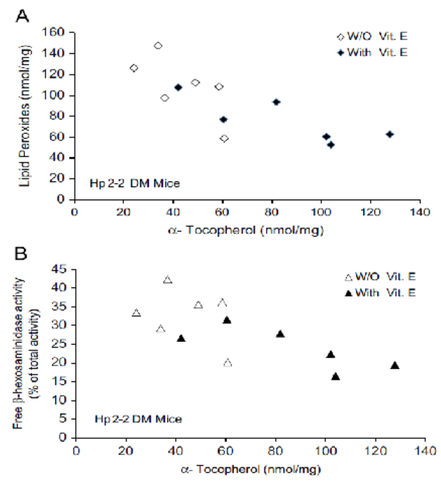


**Fig.5: Lysosomal vitamin E concentration is correlated with lysosomal membrane lipid peroxidation and lysosomal membrane integrity. Vitamin E was measured in lysosomal preparations as described in the methods section and normalized to cholesterol. Filled squares are data points for mice receiving vitamin E supple- mentation while empty squares are data points for mice who received placebo**

## Conclusions:

**1.** 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.

**2**. 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.

**3.** we have provided evidence for a novel mechanism whereby the Hp genotype may predispose to renal injury in the setting of DM.


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