Free Radical Biology and Medicine 53 (2012) 779–786

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

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

Rabea Asleh [a,1], Farid M. Nakhoul [a,b,*,1], Rachel Miller-Lotan [a], Hoda Awad [a], Dan Farbstein [a], Nina S. Levy [a], Nakhoul Nakhoul [b], Theodore C. Iancu [c], Irena Manov [c], Michael Laue [d], Maret G. Traber [e], Katie M. Lebold [e], Andrew P. Levy [a]

[a] Department of Anatomy and Cell Biology, Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, 1 Efron St. Bat Galim, P.O.B. 9649, Haifa, Israel
[b] Nephrology Department, Baruch-Padeh Poryia Medical Center, Bar Ilan University Faculty of Medicine, Lower Galilee, 15208, Poryia, Israel
[c] Pediatric Research and Electron Microscopy Unit, Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, 1 Efron St. Bat Galim, P.O.B. 9649, Haifa, Israel
[d] Robert Koch Institute, Nordufer 20, 13353, Berlin, Germany
[e] Linus Pauling Institute, Oregon State University, 307 Linus Pauling Science Center, Corvalis, OR, USA

ARTICLE INFO

Article history:
Received 22 March 2012
Received in revised form
5 June 2012
Accepted 11 June 2012
Available online 27 June 2012

Keywords:
Diabetes
Nephropathy
Lysosome
Iron-induced injury
Haptoglobin genotype
Oxidative stress

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 and may be a target for slowing the progression of renal disease.

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 extracorpuscular 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

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

* Corresponding author at: Poryia Medical Center, Bar Ilan University Faculty of Medicine, Poryia, Israel. fax: 972 4 6652587.
E-mail addresses: asl@yahoo.com (R. Asleh), fnakhoul@poria.health.gov.il (F.M. Nakhoul), lotanr@tx.technion.ac.il (R. Miller-Lotan), aw@yahoo.com (H. Awad), dan.farbstein@gmail.com (D. Farbstein), ninal@tx.technion.ac.il (N.S. Levy), n@yahoo.com (N. Nakhoul), tiancu@tx.technion.ac.il (T.C. Iancu), irenamanov@gmail.com (I. Manov), laueM@rki.de (M. Laue), maret.traber@oregonstate.edu (M.G. Traber), leboldk@gmail.com (K.M. Lebold), alevy@tx.technion.ac.il (A.P. Levy).
[1] Both authors contributed equally to this manuscript.

0891-5849/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2012.06.015

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 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. 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 20,000*g* 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 47,000*g* 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 100,000*g* 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