Pharmacogenomic effect of vitamin E on kidney structure and function in transgenic mice with the haptoglobin 2-2 genotype and diabetes mellitus

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Nakhoul FM, Miller-Lotan R, Awad H, Asleh R, Jad K, Nakhoul N, Asaf R, Abu-Saleh N, Levy AP. Pharmacogenomic effect of vitamin E on kidney structure and function in transgenic mice with the haptoglobin 2-2 genotype and diabetes mellitus. Am J Physiol Renal Physiol 296: F830–F838, 2009. First published January 28, 2009; doi:10.1152/ajprenal.90655.2008.—Polymorphic loci regulating oxidative stress are potential susceptibility genes for diabetic nephropathy (DN). Haptoglobin (Hp) is an antioxidant protein which serves to protect against oxidative stress induced by extracorpuscular hemoglobin. There are two alleles at the Hp locus, 1 and 2. The Hp 1 protein is a superior antioxidant to the Hp 2 protein. The Hp 2 allele has been associated with increased prevalence of DN and appears to be associated with a more rapid progression to end-stage renal disease. We sought to recapitulate this association between Hp genotype and DN in mice genetically modified at the Hp locus. We assessed morphometric, histologic, and functional parameters involved in the development and progression of DN in mice with diabetes mellitus (DM) with either the Hp 2-2 or Hp 1-1 genotype. Morphometric analysis demonstrated that glomerular and proximal tubular hypertrophy were significantly increased in Hp 2-2 DM mice. Histological analysis demonstrated that Hp 2-2 DM mice had significantly more collagen type IV, smooth muscle actin, and increased renal iron deposition. Studies of renal function demonstrated creatinine clearance time and albuminuria were increased in Hp 2-2 DM mice. Vitamin E provided significant protection against the development of functional and histological features characteristic of DN to Hp 2-2 DM but not to Hp 1-1 DM mice. These studies serve to strengthen the association between the Hp 2-2 genotype and diabetic renal disease and suggest a pharmacogenomic interaction may exist between the Hp genotype and vitamin E.

diabetic nephropathy; hyperglycemia

APPROXIMATELY ONE-THIRD of all patients with diabetes mellitus (DM) develop end-stage renal disease (ESRD) necessitating renal replacement therapy within 25 years of DM onset (20, 37, 38, 40). Long-term prospective and interventional studies have clearly demonstrated that the risk of developing diabetic nephropathy (DN) is directly related to exposure to hyperglycemia (7). However, family studies have clearly demonstrated that hyperglycemia is a necessary but not sufficient condition for the development of DN. Several lines of evidence have supported the concept that there exist polymorphic genetic loci which determine susceptibility to DN (10, 11, 19, 28, 39, 41, 45). First, DN has been shown to cluster within families. Second, there exist marked ethnic differences in the incidence of DN which cannot be explained by differences in conventional risk factors. Third, susceptibility to DN has been linked to chromosomal markers using specific population groups such as the Pima Indians. The importance of identifying such a predictive marker is that it would permit early identification of individuals at high risk of developing DN in whom more aggressive treatment might be initiated earlier and may provide additional insight into the pathophysiology of DN and the development of new preventative therapies.

Increased oxidative stress has been proposed to play a fundamental role in the development of DN (18, 36, 43, 44). Therefore, genetic loci encoding proteins regulating the level oxidative stress are prime potential candidate susceptibility genes for DN. Over the past 10–15 years a number of such candidate genes have been identified in genetic association studies with DN (23). Haptoglobin (Hp) is a serum antioxidant protein which serves to protect against oxidative stress induced by extracorpuscular hemoglobin (8). There are two common alleles at the Hp locus, denoted 1 and 2. The structure and function of the two Hp allele protein products are distinct. We and others showed in vitro and in vivo that the Hp 1 protein is a superior antioxidant to the Hp 2 protein (1, 2, 29).

In most Western societies, the two Hp alleles are in a balanced polymorphism with ~40% of the alleles being Hp 1 and 60% Hp 2 with the prevalence of the three Hp genotypes being Hp 1-1 (16%), Hp 2-1 (48%), and Hp 2-2 (36%) (8). The frequency of these two alleles is not different in individuals with and without DM (3). We examined the association of DN and the Hp polymorphism and found that the Hp 1-1 genotype was associated with a significantly lower prevalence of DN (22, 34) and an apparent slower rate of progression of DN to ESRD (9, 13) compared with individuals with Hp 2-1 and Hp 2-2. These findings have recently been confirmed by three independent groups in independent cohorts (4, 5, 12).

Genetically modified mice offer the most direct means to demonstrate a gene-disease association. Such mice are inbred allowing one to study the effect of a change in one single gene. The Hp 2 allele is found only in humans (8). All other animals including higher primates have only the Hp 1 allele and therefore the Hp 1-1 genotype. One approach to model the Hp polymorphism in mice is to introduce the human Hp 2 allele as a transgene. Human Hp 2 transgenic mice in an Hp knockout background have been used to study mice only expressing the Hp 2 allelic protein product (30, 31). However, these human Hp 2 allele transgenic mice have two serious shortcomings.

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First, insertion of the human Hp 2 allele is random in the genome, i.e., not in the normal location of the murine Hp gene on chromosome 8 and therefore the regulation of the Hp 2 allele is different from the murine Hp 1 allele. Second, the circulating levels of the protein product from the human Hp 2 allele are different from the levels of the wild-type murine Hp 1 allele. This directly affects the polymeric distribution of the circulating Hp polymers found in the serum of these mice. We recently overcame all of these problems by producing a transgenic mouse with a genetically engineered murine (as opposed to human) Hp 2 allele and targeted insertion of this murine Hp 2 gene to the murine Hp locus by homologous recombination (26). In this study, we report on the use of these mice to study the development and progression of DN.

MATERIALS AND METHODS

Animals

All protocols were approved by the Animal Care and Use Committee of the Technion Faculty of Medicine. The wild-type murine C57Bl/6 Hp gene is a type 1 Hp allele with over 90% homology to the human Hp 1 allele. The construction of C57Bl/6 mice with targeted insertion of a murine Hp 2 allele to generate mice with the Hp 2-2 genotype has been created by genetically engineering a Hp 2 allele by duplication of exons 3 and 4 in the genomic sequence of the murine Hp 2 allele and targeting this to the genomic sequence of the murine Hp locus by homologous recombination (26). In this study, we report on the use of these mice to study the development and progression of DN.
Hp 1 allele. We then inserted this murine Hp 2 allele at the endogenous Hp locus using a targeting strategy that is specifically selected for a homologous recombination event between the murine Hp 2 allele and the endogenous murine Hp 1 allele (31). Mice were fed a standard mice chow with free access to water. There was no significant difference in blood glucose between Hp 1 and Hp 2 DM mice with and without DM. The total number of mice at the beginning and end of the study appears in Table 1.

**Streptozotocin-Induced Diabetes**

Streptozotocin was administered at 6 wk of age in a low-dose 5-day protocol as recently described by the National Institutes of Health-sponsored Diabetes Consortium (50 mg/kg for 5 days). For all studies, a group of littermates who were not injected with streptozotocin was followed in parallel so that the only difference between the groups was the presence or absence of DM. Therefore, the parameters described below were measured for four groups of animals: Hp 1-1 DM and Hp 2-2 DM. There was no difference in spot glucose or HbA1c between Hp 1-1 and Hp 2-2 DM mice. For all analysis measurements were made when mice were 4 mo of age.

**Preparation of Renal Tissues for Histochemical Analysis**

Mice were killed at 4 mo of age. Kidneys were excised and weighed and the half middle portion was fixed in 4% buffered formaldehyde and embedded in paraffin and stained with PAS.

**Morphometric Analysis Glomeruli and Tubules**

Morphometric analysis of glomeruli. Glomerular area in PAS-stained, paraffin-embedded sections was measured using Image Pro software analysis in a cohort of animals 4–5 mo old with and without diabetes and is reported in $\mu m^2 \times 10^{-3}$. All values are expressed as means ± SE of a minimum of 6 animals from each group and each animal value was expressed as the mean of 30 glomeruli measured for each animal. One reader scored all glomeruli in the study and was blinded to the genotype of the mice.

Morphometric analysis of tubules. Proximal tubules in PAS-stained, paraffin-embedded sections were analyzed using Image Pro software analysis. One reader scored all proximal tubules in the study and was blinded to the genotype of the mice. Proximal tubule area was measured using Image Pro software analysis in a cohort of animals 4–5 mo old with and without diabetes and is reported in $\mu m^2 \times 10^{-3}$. All values are expressed as means ± SE of a minimum of 5 animals from each group and each animal value was expressed as the mean of 40 proximal tubules measured for each animal. P values are for the direct comparison between Hp 1-1 mice and Hp 2-2 mice with or without diabetes.

**Quantitation of Iron Deposition**

Perl’s stain was used for the detection of iron as previously described (21). Quantitation was performed with Image Pro Plus without knowledge of the Hp genotype of the sample. For each
kidney, the results are expressed as the average total area stained for iron in the 10 most heavily stained fields (hot spots) examined at ×40 magnification.

**Immunohistochemical Analysis for Collagen and Smooth Muscle Actin**

Collagen immunohistochemistry was performed using polyclonal goat anti-collagen IV Ab (Southern Bio). Vectastain (Vector Labs) ABC kit was used for the secondary antibody and for detection with orange-brown staining indicating collagen. Quantitation of the immunostaining area was done using Image Pro analysis and is reported in percent area of the glomeruli. Immunohistochemical identification of smooth muscle actin (orange-red) was performed using a monoclonal antibody to mouse smooth muscle actin and the ABC kit described above for collagen. Quantitation of actin staining was performed similar to collagen (as % of glomerular area).

**Assessment of Glomerular Filtration Rate**

The creatinine clearance time (CCT) was measured in mice 1 day before death (4 mo of age, 2.5 mo of DM) using the relationship CCT (ml/min) = [urinary creatinine in mg/dl] × [urinary flow rate in ml/min]/[plasma creatinine in mg/dl].

**Assessment of Albuminuria**

Albuminuria (mg) was assessed in a 24-h urine collection by Elisa (Exocell) 1 day before killing the mice (4 mo of age, 2.5 mo of DM).

**Assessment of Lipid Peroxides in Renal Tissue**

Kidneys were snap-frozen in liquid nitrogen and homogenized in 1 ml of homogenization buffer containing 250 mM sucrose, 10 mM HEPES, 5 mM EDTA, and 0.1 mM PMSF. Homogenates were centrifuged for 5 min at 14,000 rpm at 4°C. Supernatants were used for determination of lipid peroxidation. One milliliter of lipid peroxide reagent was then added (0.2 M potassium hydrophosphate, 0.12 M potassium iodide, 0.15 M sodium azide, 2 g/l igepl, 0.1 g/l alkylbenzylidimethyl ammonium chloride, 10 μM ammonium molybdate). After a 30-min incubation in the dark, the optical density (OD) of the sample was measured at 365 nM. The amount of nanomoles of lipid peroxides in a given sample under the reaction conditions defined above was derived using the known extinction coefficient for lipid peroxides (2.46 × 10^4 M^-1) using the relationship nanomoles lipid peroxides = measured(OD365) × (100)/2.46 (18).

**Vitamin E Supplementation**

Vitamin E was administered in the drinking water, for 6 wk, beginning 1 mo after onset of DM until the mice were killed at 4 mo of age. We used vitamin E from Merck which is water miscible as documented by the manufacturer (Merck cat. no. 500862). This is DL alpha tocopherol acetate and enters easily into water. We made up a stock solution of vitamin E 1 ml in 50 ml of water and then used 5 ml of this stock solution in a 250-ml bottle of water for the mice. As noted in Table 1, the volume of urine was ~30 ml a day for the DM mice.

**Fig. 3.** Increased mesangial collagen IV in Hp 2-2 DM mice. Collagen immunohistochemistry using polyclonal goat anti-collagen IV Ab (Southern Bio). Vectastain (Vector Labs) ABC kit was used for the secondary antibody and for detection with orange-brown staining indicating collagen. Quantitation of the immunostaining area was done using Image Pro analysis and is reported in % area of the glomeruli. All values are expressed as means ± SE (P < 0.001) with a minimum of 6 animals used from each group (30 glomeruli for each animal). There was a significant increase in collagen IV immunostaining (%) in Hp 2-2 DM mice vs. Hp 2-2 non-DM mice (P < 0.001). There was a significant decrease in collagen IV immunostaining area in Hp 2-2 DM mice with Vit-E (P < 0.05). *Hp2,2 D vs. Hp2,2 ND. #Hp2,2 D + Vit-E vs. Hp2,2 D.
mice and the DM mice were ~20 g so each DM mouse received ~600 mg·kg⁻¹·day⁻¹ during the course of treatment.

**Statistical Analysis**

All results are reported as means ± SE. 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.

**RESULTS**

**Morphometry: Increased Renal Hypertrophy in Hp 2-2 DM Mice**

Gross kidney size expressed as the kidney index (kidney mass/body mass) was significantly elevated in Hp 2-2 DM mice compared with their non-DM littermates and with Hp 1-1 DM mice (15.5 ± 0.97 g/kg for Hp 2-2 DM vs. 11.9 ± 1.1 g/kg for Hp 1-1 DM and 10.1 ± 0.4 g/kg for Hp 2-2 non-DM; P < 0.05 comparing Hp 2-2 DM mice with Hp 1-1 DM or Hp 2-2 non-DM mice). There was a significant increase in both total glomerular area and proximal tubule area in Hp 2-2 DM mice compared with Hp 1-1 DM mice [glomerular area: 4,852.9 ± 308.7 for Hp 2-2 DM vs. 3,176.8 ± 99.3 for Hp 1-1 DM, P < 0.001 (Fig. 1); proximal tubular area: 1,152.6 ± 42.4 for Hp 2-2 DM vs. 818 ± 17.15 for Hp 1-1 DM, P < 0.05 (Fig. 2)]. We observed no significant difference in the cellularity of Hp 1-1 vs. Hp 2-2 glomeruli or tubules suggesting that the glomerular expansion seen in Hp 2-2 DM mice was more likely to be due to hypertrophy than hyperplasia.

**Histology: Increased Collagen, Smooth Muscle Actin, and Iron in Hp 2-2 DM Mice**

Collagen type IV (Fig. 3) and smooth muscle cell actin (Fig. 4), proteins known to be increased in human DN glomeruli, were significantly increased in Hp 2-2 DM mice (0.10 ± 0.07 for Hp 2-2 DM and 0.03 ± 0.003 for Hp 1-1 DM, P < 0.001 (Fig. 3), and 0.14 ± 0.01 for Hp 2-2 DM and 0.04 ± 0.01 for 1-1 DM, P < 0.001 (Fig. 4), respectively). Significantly greater amounts of iron were found in the renal tissue (localized to the proximal tubular cells) of Hp 2-2 DM mice.
mice compared with Hp 1-1 DM mice \[1.34 \pm 0.19\] for Hp 2-2 DM and \[0.56 \pm 0.12\] for Hp 1-1 DM, \(P < 0.01\) (Fig. 5), respectively.

**Biochemistry: Increased Oxidative Stress in the Kidneys of Hp 2-2 DM Mice**

We assessed global oxidative stress in renal tissue of Hp 1 and Hp 2 DM mice by measuring total lipid peroxides in these tissues as described in MATERIALS AND METHODS. We found that lipid peroxidation in homogenized renal tissue was increased in Hp 2-2 DM vs. Hp 1-1 DM mice \[36.75 \pm 6.0\] for Hp 2-2 DM vs. \[27.18 \pm 7.7\] for Hp 1-1 DM, \(n = 5\), \(P < 0.05\).

**Function: Increased Hyperfiltration and Albuminuria in Hp 2-2 DM Mice**

Hyperfiltration, evident as an increase in the CCT, is a characteristic of early DN. We found a significant increase in the CCT in Hp 2-2 DM mice compared with Hp 1-1 DM mice \[0.20 \pm 0.03\] for Hp 2-2 DM and \[0.08 \pm 0.02\] for Hp 1-1 DM, \(P = 0.037\); Fig. 6). Albuminuria was assessed in a 24-h urine collection by Elisa. We found a marked increase in albuminuria in both Hp 1-1 DM and Hp 2-2 DM mice compared with their non-DM littermates and a nonsignificant two- to threefold increase in albuminuria in Hp 2-2 DM mice compared with Hp 1-1 DM mice \[95.3 \pm 38.0\] vs. \[37.9 \pm 11.9\], \(P = 0.16\).

**Prevention of DN in Hp 2-2 DM Mice with Vitamin E Supplementation**

As compared with Hp 2-2 DM mice which did not receive vitamin E, we found a significant reduction in total glomerular area \((P < 0.05;\) Fig. 1), proximal tubule area \((P < 0.05;\) Fig. 2), glomerular collagen content \((P < 0.001;\) Fig. 3), glomerular actin content \((P < 0.001;\) Fig. 4), and CCT \((P < 0.04;\) Fig. 6). We also found a nonsignificant reduction in albuminuria in Hp 2-2 DM mice receiving vitamin E \([18.5 \pm 7.2\) vs. \(95.3 \pm 38.0\), \(P = 0.16\)]. Vitamin E supplementation to Hp 2-2 DM mice also appeared to result in a significant 50% reduction in global oxidative stress in renal tissue slices assessed as lipid peroxidation \((n = 5\), \(P < 0.01\). In contrast, in Hp 1-1 DM mice, vitamin E did not affect any morphometric or functional parameter as demonstrated in Figs. 1–6 nor did it significantly decrease total lipid peroxides in renal tissue of Hp 1-1 DM mice.

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**Fig. 5.** Increased renal iron deposition in the proximal tubule of Hp 2-2 DM mice. Perl’s iron stain was used to localize iron in paraffin-embedded kidney sections in Hp 1-1 and Hp 2-2 mice with and without DM. Arrow indicates iron-induced stain in blue (×400 magnification) located within proximal tubular cells. There was a significant increase in iron staining in the renal tissue of Hp 2-2 DM (D) vs. Hp 1-1 DM (D) and Hp 2-2 non-DM mice \((P < 0.001;\) \(n = 6\) animals for each group), *Hp2,2 (D) vs. Hp2,2. #Hp2,2 (D) + Vit-E vs. Hp2,2 (D).
clearance of free hemoglobin via uptake by the CD163 macroprotein in binding to this free hemoglobin and neutralizing its half time (46). The Hp 1-1 protein is superior to the Hp 2-2 increased red cell fragility resulting in a shorter red blood cell Extracorpuscular hemoglobin is increased in diabetes due to the difference in the manner in which the two mice have more renal damage compared with Hp 1-1 DM mice between the Hp 2 allele and susceptibility to DN. Therefore, these studies serve to strengthen the association (hyperfiltration, glomerular hypertrophy, and albuminuria).

The fundamental mechanism explaining why Hp 2-2 DM mice have more renal damage compared with Hp 1-1 DM mice can be explained by differences in the manner in which the two Hp types regulate the disposition of extracorpuscular hemoglobin and more specifically hemoglobin-derived iron (1, 2, 29). Extracorpuscular hemoglobin is increased in diabetes due to increased red cell fragility resulting in a shorter red blood cell half time (46). The Hp 1-1 protein is superior to the Hp 2-2 protein in binding to this free hemoglobin and neutralizing its oxidative potential (29). Furthermore, the rate of clearance of hemoglobin is Hp type dependent. Hp 1-1 directs a more rapid clearance of free hemoglobin via uptake by the CD163 macrophage scavenger receptor (1). In the diabetic state, this is particularly important as the ability of Hp to block the oxidative activity of hemoglobin is impaired when hemoglobin becomes glycated (2). A further mechanism contributing to Hp type-dependent differences in protection against hemoglobin-driven oxidative stress is that the expression of the CD163 receptor for clearing hemoglobin is decreased in the setting of Hp 2 (27). Megalin and cubulin expressed on proximal tubular cells appear to be default receptors for the Hb and the Hp-Hb complex (16). We previously demonstrated and reported that Hp concentrations are identical in Hp 1 and Hp 2 streptozotocin mice after similar duration of DM. Furthermore, in human studies there is no evidence that Hp concentration has any effect on renal function in any of the epidemiological studies examining Hp and renal function, only the Hp type.

The increased iron present in the proximal tubular cells of Hp 2-2 DM mice likely reflects the impaired CD163 clearance mechanism operative in these animals in which the default mechanism for clearance of the Hp-Hb complex has kicked in.

Although the glomerulus has been the primary focus of where diabetic renal disease begins, recent studies in experimental animals have shown that the growth of proximal tubules accounts for most of the increase in renal mass in early DM and that the release of growth factors by the tubulointerstitium may be responsible for many of the structural changes in the glomerulus that are seen in DM (48). We propose that the increased deposition of iron in the renal proximal tubular cells of Hp 2-2 DM mice accentuates differences in tubular disease between Hp 1-1 and Hp 2-2 mice. Importantly, increased proximal tubular iron has been observed in patients with DN (35). Nankivell et al. (35) suggested that this iron causes the tubular damage of DN by the generation of reactive oxygen species.

The promise of pharmacogenomics is that not all individuals with a given disease may benefit from the same drug treatment. We demonstrated that vitamin E appears to provide renal protection to Hp 2-2 DM mice but does not appear to have any effect on Hp 1-1 DM mice. The pharmacogenomic implications of these findings are significant. Large-scale clinical trials of vitamin E to prevent macrovascular complications of diabetes have failed to show that vitamin E provided any clinical benefit (47). Studies assessing the effect of vitamin E on the progression of DN in humans with DM have yielded inconsistent findings (15, 17). Moreover, recent meta-analysis suggested that there is an increased risk of all cause mortality with high-dose vitamin E supplementation (6, 32). One explanation for the failure of vitamin E to provide benefit in human studies may be due to the inadequate nature of patient selection in these studies (42). We recently provided concrete evidence in humans for a pharmacogenomic interaction between the Hp genotype and vitamin E supplementation on the development of atherosclerotic cardiovascular disease. We found by analyzing stored blood samples from the HOPE study that individuals’ DM and the Hp 2-2 genotype appeared to receive significant clinical benefit from vitamin E (24). Moreover, we recently demonstrated in a prospective double blind clinical trial that vitamin E dramatically reduces cardiovascular disease in Hp 2-2 DM individuals (33). The ability of vitamin E to reduce features of renal disease characteristic of early human DN in Hp 2-2 DM mice but not in Hp 1-1 DM mice suggests that
there may also be an interaction between Hp genotype and vitamin E therapy on diabetic renal disease.

This study has several limitations which must be clearly acknowledged. First, we examined only one time point (2.5 mo) after the onset of DM. The changes we reported may not accurately reflect the long-term impact of the difference between Hp 1 and Hp 2 mice. We were unable to examine longer periods of diabetic exposure in the Hp 2 DM mice due to their extremely high mortality rate. Second, we cannot rule out that a more prolonged follow-up may be necessary to exclude a beneficial effect of vitamin E in DM mice with the Hp 1-1 genotype. Third, our measurements of renal function were based on 24-h urine collections without any documentation as to whether there existed irregularities in bladder emptying between the experimental groups which may have confounded our measurements.

In conclusion, these studies provide support for the hypothesis that the Hp genotype is a major determinant of the development and progression of diabetic renal disease. Moreover, they suggest that a pharmacogenomic interaction may exist between the Hp genotype and vitamin E in the ability to modulate the development and slow the progression of diabetic renal disease (25).

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