Correction of HDL Dysfunction in Individuals With Diabetes and the Haptoglobin 2-2 Genotype

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OBJECTIVE—Pharmacogenomics is a key component of personalized medicine. The Israel Cardiovascular Events Reduction with Vitamin E Study, a prospective placebo-controlled study, recently demonstrated that vitamin E could dramatically reduce CVD in individuals with diabetes and the haptoglobin (Hp) 2-2 genotype (40% of diabetic individuals). However, because of the large number of clinical trials that failed to demonstrate benefit from vitamin E coupled with the lack of a mechanistic explanation for why vitamin E should be beneficial only in diabetic individuals with the Hp 2-2 genotype, enthusiasm for this pharmacogenomic paradigm has been limited. In this study, we sought to provide such a mechanistic explanation based on the hypothesis that the Hp 2-2 genotype and diabetes interact to promote HDL oxidative modification and dysfunction.

RESEARCH DESIGN AND METHODS—Hp and lipid peroxides were assessed in HDL isolated from diabetic individuals or mice with the Hp 1-1 or Hp 2-2 genotypes. HDL function was assessed based on its ability to promote cholesterol efflux from macrophages. A crossover placebo-controlled study in Hp 2-2 diabetic humans and in Hp 1-1 and Hp 2-2 diabetic mice assessed the ability of vitamin E to favorably modify these structural and functional parameters.

RESULTS—Hp and lipid peroxides associated with HDL were increased and HDL function was impaired in Hp 2-2 diabetic individuals and mice. Vitamin E decreased oxidative modification of HDL and improved HDL function in Hp 2-2 diabetes but had no effect in Hp 1-1 diabetes.

CONCLUSIONS—Vitamin E significantly improves the quality of HDL in Hp 2-2 diabetic individuals. Diabetes 57:2794–2800, 2008

Pharmacogenomics is a key component of personalized medicine (1). Therapy targeted to a specific patient based on his or her genetically determined pathophysiology responsible for the disease offers the possibility of significantly improving patient care and reducing costs. However, despite the clear public health and economic benefits that would be attained by such an approach, this paradigm has not been successfully applied to a common disease.

Cardiovascular disease (CVD) is responsible for 75% of deaths among individuals with diabetes, and yearly expenditures for CVD in diabetes exceed $200 billion (2). Neither conventional risk factors nor the degree of glycemic control adequately predict which individuals with diabetes develop CVD, suggesting the existence of genetic susceptibility factors.

A polymorphism in the haptoglobin (Hp) gene may define which individuals with diabetes are at greatest risk of CVD. There exist two classes of alleles at the Hp locus denoted 1 and 2 with three possible Hp genotypes 1-1, 2-1, and 2-2 (3). In five independent longitudinal studies performed in ethnically and geographically diverse groups, individuals with the Hp 2-2 genotype and diabetes were found to have a two- to fivefold increased risk of CVD compared with diabetic individuals without the Hp 2-2 genotype (4–8). The prevalence of the Hp 2-2 genotype in the diabetic population in most Western countries is ~40%, making this a common polymorphism.

The Hp polymorphism differs from nearly all polymorphisms being assessed in genome-wide association studies because it is a functional polymorphism (3). Understanding functional differences between the Hp 1 and Hp 2 allelic protein products, particularly in diabetes, may provide insight into why Hp 2-2 diabetic individuals have more CVD and how this increased burden of disease might be reduced. The most well-understood function of Hp is to bind Hb released from erythrocytes (3). Each day, >6 g Hb is released into the bloodstream due to turnover of erythrocytes, and heme iron in this Hb is a powerful oxidizing agent (9,10). Hp, which is present in a 400-fold molar excess to free Hb under normal conditions, binds Hb, reducing its ability to mediate oxidative modifications and directing its removal from the blood via the monocyte/macrophage CD163 Hp-Hb scavenger receptor (11).

More than 5 years ago, motivated by in vitro studies demonstrating that the Hp 2-2 protein provides inferior protection against Hb-mediated oxidative stress (9,10), coupled with the suggested importance of oxidative stress in diabetic atherosclerosis (12), we sought to determine whether antioxidant therapy might be particularly beneficial to the Hp 2-2 diabetic cohort. We first tested this hypothesis by examining stored samples from the Heart

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Outcomes Prevention Evaluation (HOPE) study, which had failed to demonstrate benefit from vitamin E (13). We found that myocardial infarction and CVD death were reduced by >40 and 50%, respectively, in Hp 2-2 diabetic HOPE participants who received vitamin E (14). To prospectively test the hypothesis, we initiated a double-blind randomized placebo-controlled study of vitamin E in 1,434 Hp 2-2 diabetic individuals (Israel Cardiovascular Events Reduction with Vitamin E [ICARE] Study). We found that vitamin E supplementation was associated with a >50% reduction in the combined primary outcome of stroke, myocardial infarction, and cardiovascular death in Hp 2-2 diabetes (7).

Enthusiasm for these findings, despite the considerable public health and economic benefits that they suggest, has been muted. Our study comes in the wake of numerous large clinical trials that failed to demonstrate that vitamin E provides any protection against CVD and may be harmful (13, 15–20). Further hampering acceptance of this paradigm is the lack of a rational pathophysiological and pharmacogenetic mechanism to explain why Hp 2-2 diabetic individuals have an increased risk of CVD and how vitamin E mitigates this risk. In this study, we sought to provide a rationale for the pharmacogenomic application of the Hp genotype to prevent CVD in diabetes by elucidating the unique structural modifications and dysfunctional nature of HDL in Hp 2-2 diabetic individuals and how these structural and functional changes in HDL are rapidly reversed with vitamin E.

**RESEARCH DESIGN AND METHODS**

**Ethical approval.** These studies were approved by the institutional review boards of the Rambam Medical Center and the Technion. All participants provided informed consent.

**Human subjects.** All studies except where indicated were performed with type 2 diabetic individuals recruited from ICARE (7). The Hp type of participants was determined by gel electrophoresis, which has a 100% correspondence with the Hp genotype determined by PCR (21).

**Animal studies.** The Hp 2 allele is present only in humans. All other species have only the Hp 1 allele, which is highly homologous with the human Hp 1 allele. We have previously described the construction of a murine Hp 2 allele and the targeting of its insertion by homologous recombination to the murine Hp genetic locus (22). Mice were fed normal chow. Diabetes was produced with streptozotocin at 2 months of age and studied after a diabetes duration of 1 month.

**Measurement of the clearance rate of Hp-Hb in vivo.** Hp and Hb were labeled with 125I using chloramine T (23). 125I-labeled Hp-Hb was injected in murine serum using a rabbit anti-ApoA1 antibody and protein A/G Sepharose. Ultracentrifugation-purified HDL was prepared as previously described (24). Immunopurified HDL was prepared from human or murine serum using a rabbit anti-apoA1 antibody and protein A/G Sepharose. HDL-associated lipid peroxides and HDL-associated redox active iron. Total lipid peroxides (nanomoles) associated with HDL were assessed in 1 μg immunopurified HDL (25). For the assessment of redox active iron associated with HDL, the time-dependent oxidation of dihydrodihydroxyamine by immunopurified HDL was assessed in the presence and absence of desferroxamine (25).

**Assessment of the association of native Hp and Hp with Hp 2-2 HDL.** Hp and Hb were assessed in immunopurified HDL by Western blot with either rabbit anti-Hp or anti-Hb antiserum and alkaline phosphatase–coupled goat anti-rabbit antiserum for detection.

**Cholesterol efflux.** Serum from mice or humans treated with placebo or vitamin E was assessed for its ability to promote the efflux of \[^{3}H\]cholesterol from macrophages (26).

**Study drugs.** For murine studies, vitamin E was administered in the drinking water at 40 mg · kg⁻¹ · day⁻¹ for 30 days beginning 1 month after onset of diabetes. For human studies, placebo or vitamin E (400 IU natural source d-α-tocopheryl/day) capsules were provided in a double-blinded format.

**Human crossover study design.** The study (clinical trial reg. no. NCT00314379) was performed in 18 Hp 2-2 diabetic individuals who were not on antioxidant therapy at baseline (baseline characteristics provided in supplementary Table 1 available in an online appendix at http://dx.doi.org/10.2337/db08-0450). Blood was taken at baseline (test 1). Participants were randomly allocated to initially receive vitamin E or placebo for 2 months, after which another blood sample was taken (test 2), and this initial treatment was stopped. Two weeks later, the participants were crossed over to the other treatment, and a blood sample was taken after 2 months of treatment (test 3).

**Statistical analysis.** All results are reported as means ± SE. Comparison between groups was performed using Student’s t test or ANOVA and the
prepared from serum by either ultracentrifugation or immunoabsorption (Fig. 1A). We found that Hp is present in the HDL when the HDL is prepared by immunoabsorption but not if the HDL is prepared by ultracentrifugation. Although we found that Hp is present in the HDL of all individuals, because the Hp 2-2 protein is made up of 3–10 disulfide-linked Hp monomers compared with the Hp 1-1 protein, which is made up of only 2 disulfide-linked Hp monomers (3), significantly more Hp was detected in the HDL of Hp 2-2 individuals (Fig. 1B). The amount of Hp associated with HDL is increased in Hp 2-2 diabetic individuals. The binding of Hp to HDL and the high affinity of Hp for Hb suggested that Hp may tether Hb to HDL. Furthermore, the impaired clearance of Hp 2-2–Hb in diabetes would suggest that there might be more of the complex associated with HDL in Hp 2-2 diabetic mice or humans. We first investigated this possibility by assessing $^{125}$I-Hp-Hb in the HDL immunoprecipitate and found a dramatic increase, representing $>$25% of all injected cpm, in the amount of Hp 2-2–Hb associated with HDL in Hp 2-2 diabetic mice (Fig. 2A). However, in mice genetically deficient for Hp (Hp knockout), no $^{125}$I-Hb was found associated with HDL (zero cpm in HDL immunoprecipitate), demonstrating that Hp is critical for binding of Hb to HDL.

Parallel studies were performed in humans. First, we incubated serum from Hp 1-1, Hp 2-2, or Hp 0 (individuals in whom Hp was not detectable by gel electrophoresis) with $^{125}$I-Hb and assessed the amount of radioactive label in the HDL immunoprecipitate. We found significantly greater Hb associated with HDL in Hp 2-2 serum (Fig. 2B).

We then assessed the amount of endogenous Hb associated with HDL in Hp 1-1 and Hp 2-2 mice and humans with and without diabetes by Western blot. We detected substantial amounts of Hb associated with HDL in $>$90% of Hp 2-2 diabetic individuals but failed to find Hb associated with HDL in any Hp 1-1 diabetic individuals or in any individuals (Hp 1-1 or Hp 2-2) without diabetes (Fig. 3A). Similarly, we found a marked increase in the amount of endogenous Hb associated with HDL in Hp 2-2 diabetic mice (Fig. 3B).

**RESULTS**

The half-life of the Hp 2-2–Hb complex is markedly increased in diabetes. We sought to test the hypothesis that clearance of Hp-Hb from the plasmatic compartment is both Hp genotype and diabetes dependent. We tested this hypothesis by injecting $^{125}$I-Hp-Hb into Hp 1-1 or Hp 2-2 mice with or without diabetes. The half-life of Hp 1-1–Hb was $\sim$20 min with or without diabetes. The half-life of Hp 2-2–Hb was $\sim$50 min in mice without diabetes and $\sim$100 min in mice with diabetes (Table 1).

Hp is an HDL-associated protein in humans. Hp has been shown by some but not all investigators to be an HDL-associated protein (24,27–29). Critical analysis of these prior studies suggested that the key difference in these studies was in the manner in which the HDL was prepared. We assessed the presence of Hp in human HDL

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**FIG. 2.** The association of $^{125}$I-Hp-Hb and $^{125}$I-Hb with HDL is Hp genotype- and diabetes-dependent. A: Increased association of injected Hp-Hb with HDL in Hp 2-2 diabetic mice. $^{125}$I-Hp-Hb complex (one million cpm) was injected in the tail vein. The percentage of the injected cpm that coimmunoprecipitated with HDL at all time points after the injection (1–180 min) is shown ($n = 5$ for Hp 1-1 and Hp 2-2 nondiabetes and $n = 6$ for Hp 1-1 and Hp 2-2 diabetes). There was a significant increase in cpm in the HDL immunoprecipitate of Hp 2-2 diabetes ($P < 0.0001$ compared with Hp 2-2 nondiabetes). There was no significant difference in cpm in the HDL immunoprecipitate of Hp 1-1 diabetes compared with Hp 1-1 nondiabetes ($P = 0.24$). B: The ability of $^{125}$I-Hb to bind to human HDL in vitro is increased in Hp 2-2 and decreased in Hp 0. $^{125}$I-Hb was incubated with serum from individuals with Hp 1-1, Hp 2-2, or Hp 0. $^{125}$I-Hb associating with HDL was assessed by immunoprecipitation, and the mean ± SE for 10 individuals from each of the three groups is shown. There was significantly more $^{125}$I-Hb associated with HDL using serum from Hp 2-2 individuals compared with Hp 1-1 individuals ($P < 0.0001$). The amount of $^{125}$I-Hb associating with HDL using Hp 0 serum was significantly less than that observed in Hp 1-1 serum ($P < 0.002$). Note that Hp 0 does not indicate that these individuals lack Hp, but rather that the level of Hp is below the level of detection by gel electrophoresis.

Tukey-Kramer honestly significant difference method for comparisons of means test as appropriate, with a $P$ value of $\leq 0.05$ considered significant.

**FIG. 3.** Hb is an HDL-associated protein in Hp 2-2 diabetic humans and mice. A: The amount of Hb associated with HDL is increased in Hp 2-2 diabetic individuals. Western blot for Hb of HDL immunoprecipitate of serum of Hp 1-1 or Hp 2-2 diabetic individuals. Hb was identifiable in 14 of 15 diabetic individuals with the Hp 2-2 genotype and in 0 of 15 of the diabetic individuals with the Hp 1-1 genotype. Hb was not found associated with HDL from nondiabetic Hp 1-1 or Hp 2-2 individuals (not shown). Hb indicates purified Hb used as positive control. B: The amount of Hb associated with HDL is increased in Hp 2-2 diabetic mice. Western blot for Hb of HDL immunoprecipitate of serum of Hp 1-1 or Hp 2-2 mice with or without diabetes (D). Hb indicates purified Hb used as positive control.

The amount of Hb associated with HDL is increased in Hp 2-2 diabetic individuals. The binding of Hp to HDL and the high affinity of Hp for Hb suggested that Hp may tether Hb to HDL. Furthermore, the impaired clearance of Hp 2-2–Hb in diabetes would suggest that there might be more of the complex associated with HDL in Hp 2-2 diabetic mice or humans. We first investigated this possibility by assessing $^{125}$I-Hp-Hb in the HDL immunoprecipitate and found a dramatic increase, representing $>$25% of all injected cpm, in the amount of Hp 2-2–Hb associated with HDL in Hp 2-2 diabetic mice (Fig. 2A). However, in mice genetically deficient for Hp (Hp knockout), no $^{125}$I-Hb was found associated with HDL (zero cpm in HDL immunoprecipitate), demonstrating that Hp is critical for binding of Hb to HDL.

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The HDL of Hp 2-2 diabetic humans contains redox active iron and has increased lipid peroxides. The increased association of the pro-oxidant Hb with HDL in Hp 2-2 diabetic individuals may result in the increased oxidative modification of HDL-associated lipid and proteins and may paradoxically make the HDL a pro-oxidant (30). We assessed oxidation of HDL-associated lipids in the HDL of Hp 1-1 and Hp 2-2 diabetic individuals and found a marked increase in the amount of lipid peroxides in the HDL of Hp 2-2 diabetes (1.8 ± 0.2 nmol/µg HDL vs. 1.2 ± 0.2 nmol/µg HDL, n = 20, P = 0.04). HDL from Hp 2-2 diabetic individuals was also associated with an increased amount of iron capable of mediating oxidation (4.4 ± 0.8 pmol redox active iron/µg HDL vs. 1.8 ± 0.5 pmol redox active iron/µg HDL, n = 20, P = 0.02).

The HDL in Hp 2-2 diabetes is dysfunctional. We assessed the ability of serum from Hp 1-1 or Hp 2-2 diabetic mice or humans with type 1 or type 2 diabetes to promote cholesterol efflux from macrophages in vitro. We found a significant 30–40% decrease in HDL function in Hp 2-2 diabetes compared with Hp 1-1 diabetes (Fig. 4). No differences were found between Hp 1-1 and Hp 2-2 in the absence of diabetes or between Hp 1-1 with and without diabetes (data not shown) (26).

HDL oxidative modification and dysfunction can be corrected in Hp 2-2 diabetes with vitamin E. We assessed the ability of vitamin E to reduce HDL oxidative modification (HDL-associated lipid peroxides) and to improve HDL function in Hp 1-1 or Hp 2-2 diabetic mice. We found that vitamin E had no effect on HDL lipid peroxides but improved HDL function in Hp 2-2 diabetic mice (Fig. 5). There was a significant difference in cholesterol efflux elicited by serum from Hp 1-1 and Hp 2-2 diabetic mice (P = 0.002 comparing placebo groups). Vitamin E significantly improved cholesterol efflux in Hp 2-2 diabetic mice (P = 0.0006 comparing Hp 2-2 placebo vs. Hp 2-2 vitamin E). Eflux elicited by the serum of Hp 2-2 diabetic mice treated with vitamin E was not significantly different from that elicited by Hp 1-1 diabetic mice. Vitamin E had no effect on efflux in Hp 1-1 diabetic mice (P = 0.29). Vitamin E reduces HDL-associated lipid peroxides in Hp 2-2 diabetic mice but not in Hp 1-1 diabetic mice. There was a significant difference in HDL-associated lipid peroxides between Hp 1-1 and Hp 2-2 diabetic mice (P = 0.0001). Vitamin E significantly reduced lipid peroxides in Hp 2-2 diabetic mice (P = 0.001 comparing Hp 2-2 placebo vs. Hp 2-2 vitamin E) but had no effect on efflux in Hp 1-1 diabetic mice (P = 0.74 comparing Hp 1-1 placebo vs. Hp 1-1 vitamin E).
or function in Hp 1-1 diabetic mice. However, vitamin E significantly improved HDL function and reduced HDL lipid peroxides in Hp 2-2 diabetic mice, restoring function and reducing lipid peroxides to levels similar to those found in Hp 1-1 diabetes (Fig. 5).

In humans, we assessed the ability of vitamin E to improve HDL function and reduce HDL-associated lipid peroxides in Hp 2-2 diabetes in a crossover study. We found that vitamin E significantly improved HDL function by 30–40% and reduced HDL lipid peroxides by 20–30%. Notably, in this crossover design we found that after vitamin E had restored HDL function and reduced lipid peroxides and the vitamin E was then withdrawn, HDL function deteriorated, and HDL-associated lipid peroxides increased to levels seen at baseline within 2 months after cessation of vitamin E supplementation (Fig. 6).

DISCUSSION
In this translational study, we have provided a pathophysiological and pharmacogenomic rationale as to why vitamin E may provide cardiovascular benefit to individuals with the Hp 2-2 genotype and diabetes (Fig. 7). The main reason why Hp 2-2 diabetic individuals appear to uniquely derive benefit from vitamin E is that there is substantially more Hb associated with the HDL of Hp 2-2 diabetic individuals. This key structural difference between HDL in Hp 1-1 and Hp 2-2 diabetic individuals is the result of an impairment in the CD163-mediated clearance of Hp-Hb in Hp 2-2 diabetes (23,31).

The association of Hb with HDL results in the oxidative modification of HDL-associated lipids and proteins. The loss of function of HDL may be the direct result of its oxidative modification. Hb can oxidize ApoA1 (22), and oxidation of ApoA1 interferes with its ability to promote cholesterol efflux from macrophages (33). Oxidative modification of HDL-associated lipids can result in the inactivation of HDL-associated antioxidant enzymes such as glutathione peroxidase and paraoxonase (30).

A binding site for Hp on ApoA1 (amino acid residues 141–164) has been identified (27). Interestingly, lecithin acyl transferase (LCAT), whose activity is dependent on its binding to ApoA1, binds to ApoA1 residues 159–170 (34). Hazen and colleagues (34) have shown that nitration or oxidation of Tyr166 in ApoA1 results in an inhibition of the binding of LCAT to ApoA1. We have previously demonstrated a marked decrease in LCAT activity in Hp 2-2 diabetic individuals (25). We propose that binding of Hp-Hb to a site adjacent to the LCAT binding site may
result in the nitration or oxidation (35) of Tyr166, resulting in an impairment in LCAT activity. An impairment in LCAT activity would be expected to impair the maturation of HDL and its ability to promote cholesterol efflux (36). We have found a very tight correlation between LCAT activity and cholesterol efflux in diabetic individuals ($r = 0.81, P = 0.0002$) (26).

The ability of Hb associated with HDL in Hp 2-2 diabetic individuals to sequester nitric oxide (NO) (37) may have a clinical significance that is of greater importance (38) than the effect of Hb on the function of HDL in reverse cholesterol transport. HDL in Hp 2-2 diabetes may actually be proatherogenic and prothrombotic by limiting NO bioavailability.

These mechanisms are also relevant to the atherosclerotic plaque. Plaque hemorrhage is recognized as an important determinant of plaque stability (39). The Hp genotype may determine the response to plaque hemorrhage (40). Impaired clearance of Hb in Hp 2-2 diabetic plaques may lead to oxidative modification of HDL within the plaque and an impairment of its ability to promote reverse cholesterol transport.

The current focus of the medical community toward HDL has been to increase its concentration. The hypothesis presented here may help to explain the dramatically increased CVD risk in patients with type 1 diabetes, despite a usually normal HDL and lipoprotein profile. Moreover, increasing the amount of HDL in individuals in whom the HDL is dysfunctional and potentially proatherogenic may actually be harmful (30). We believe that this is the first demonstration in humans that HDL function can be improved in a specific population with vitamin E. However, not all HDL dysfunction can be attributed to Hb-mediated oxidation, and consequently, not all individuals would be expected to improve the quality of their HDL with vitamin E, as we have demonstrated here with Hp 1-1 diabetes.

In conclusion, we believe that we have provided a pathophysiological and pharmacogenomic rationale as to why vitamin E may provide benefit to the Hp 2-2 diabetic cohort. The potential public health and economic benefits from application of this paradigm are enormous. We hope that these findings will encourage testing of this hypothesis in a large-scale clinical trial that could result in the establishment of treatment guidelines for individuals with diabetes.

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