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SHORT COMMUNICATION

Haptoglobin genotype and endothelial function in diabetes mellitus: a pilot study

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Abstract Endothelial function (EnF) is impaired in patients with diabetes mellitus (DM) due in large part to an increase in oxidative stress. Haptoglobin (Hp) is a potent antioxidant protein which is encoded by two different alleles (1 and 2) with the Hp 1 protein being a superior antioxidant to the Hp 2 protein. We hypothesized that DM individuals with the Hp 2-2 genotype would have greater endothelial dysfunction as compared to DM individuals with the Hp 1-1 genotype. We studied EnF in 16 Hp 2-2, 14 Hp 1-1 DM individuals and 14 healthy subjects. DM patients' groups were matched in terms of age, cardiovascular risk factors and metabolic characteristics. EnF was assessed using post-ischemic reactive hyperemia and strain gauge plethysmography and expressed either as the maximal flow after the ischemic period or as the area under the flow–time curve (AUC). We showed that EnF indices, AUC and maximal flow, were also higher in the healthy and Hp 1-1 groups compared with Hp 2-2 genotype group (615 ± 60 and 600 ± 40 vs. 450 ± 50 ml dl^{-1}, 29 ± 2.6 and 25 ± 3 vs. 14 ± 1.8 ml min^{-1} dl^{-1}, $P < 0.003$ and $P < 0.05$, for AUC and maximal flow, one-way ANOVA, respectively). We concluded that Hp 2-2 diabetic patients had a worse EnF than controls and Hp 1-1 diabetic subjects.

Keywords Endothelial function · Haptoglobin · Reactive hyperemia

Abbreviations

EnF	Endothelial function
Hp	Haptoglobin phenotype
TOD	Target organ damage
AUC	Area under the flow–time curve

Introduction

Diabetes mellitus (DM) is one of the most common debilitating systemic diseases affecting approximately 5% of the general population in industrialized societies (Peters and Schriger 1998). The occurrence of target organ damage such as retinopathy, nephropathy and cardiovascular diseases accounts for a large percentage of hospital admissions (Howard and Magee 2000). In the past decade, investigators have focused on the quality of glycemic control rather than on the individual intrinsic predisposing factors for the development of diabetic vasculopathy (The Diabetes Control and Complications Trial Research Group 1993, 2000).

Vascular endothelial functional impairment is considered one of the consequences of DM predisposing factors (e.g. offspring of parents with diabetes, obese individual, individuals with insulin resistance, etc.) and of DM by itself (Levy et al. 2000; Nakhoul et al. 2000). Elevated oxidative

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stress appears to play an important contributory role in this phenomenon (Langlois and Delanghe 1996). Accordingly, genetically determined differences in antioxidant protection may determine the susceptibility to endothelial dysfunction in DM individuals. One susceptibility factor for diabetic complications appears to be a polymorphism in the haptoglobin (Hp) gene. Two classes of alleles exist at the Hp locus with correspondingly three possible Hp genotypes (1-1, 2-1 and 2-2). In three independent prospective and cross-sectional studies, the Hp 2-2 genotype has consistently been shown to be associated with a significant increase in diabetic eye, kidney and cardiovascular disease as compared to the Hp 1-1 genotype (Suleiman et al. 2005; Levy et al. 2002; Levy 2004).

We and others have demonstrated that the Hp 1 and Hp 2 proteins differ markedly in terms of their antioxidant function, with Hp 2-2 individuals having the least antioxidant protection (Bamm et al. 2004). Therefore, we proposed that the functional status of endothelium in patients with DM would be correlated with the Hp genotype. In order to test this hypothesis, we assessed endothelium-dependent vasodilatation in the forearms of Hp 1-1 and Hp 2-2 DM individuals, and compared the results with those obtained from healthy subjects that served as a control group.

Methods

Subjects

All subjects signed an informed consent form approved by the local institutional review board for human subjects' research. Subjects were recruited from the diabetic units of Rambam Medical Center and surrounding clinics from a cohort of 1,511 individuals that agreed to undergo Hp phenotyping. Diabetic patients were screened for Hp genotype for different ongoing studies, including ours. In this cohort, the frequency of the three Hp genotypes is 10% Hp 1-1, 50% Hp 2-1 and 40% Hp 2-2 (distribution not similar but rather close to that known to exist in the population; Hochberg et al. 2002). We set out to have a comparable number of Hp 1-1 and Hp 2-2 subjects for this study in order to permit the assessment of differences between the two groups. Subjects were recruited for the present study if they met the following criteria: capable to read and sign consent form, any type of DM for a known duration of at least 5 years (patients were not selected according to their DM type), plasma creatinine below 1.3 mg dl^{-1}, non-smokers, no clinical evidence of congestive heart failure or peripheral vascular disease.

The power calculation for the current study (80%) was estimated based on our previous experimental data (Lavi et al. 2006). We estimated that the number of subjects that will be required to prove our hypothesis is between 14 and 18 in each group (Levy et al. 2002). We used the software GraphPad StatMate (version 2.00, 2004, CA, USA) for this calculation.

Diabetic groups were also compared to 14 normotensive healthy subjects (age matched, non-smokers and with no known medical conditions) that may affect their blood flow or EnF. This group was recruited by advertising in a local magazine and screened for Hp genotype after the completion of the study.

Haptoglobin typing

Hp typing was performed from 10 ml of plasma by polyacrylamide gel electrophoresis according to established methods (Hochberg et al. 2002). A signature banding pattern was obtained from individuals who were homozygous for the Hp 1 allele (Hp 1-1), homozygous for the Hp 2 allele (Hp 2-2), or who were heterozygous at the Hp locus (Hp 2-1). We have established 100% concordance between the Hp phenotype as determined from plasma or serum and the Hp genotype as determined from genomic DNA by the polymerase chain reaction (Koch et al. 2002). An unambiguous Hp type was obtained from all subjects.

Endothelial function

All studies were performed in a quiet darkened room with an ambient temperature of approximately 24°C at the Recanati Autonomic Dysfunction Center, Rambam Medical Center. Subjects abstained from alcoholic and other beverages containing monoamine for 24 h prior to the study.

Throughout the entire experiment, a three-lead electrocardiogram and oscillometric blood pressure cuff (Datex-Engstrom, Helsinki, Finland) were recorded continuously. After instrumentation and being at rest for 20 min, a baseline forearm flow was measured and subsequently forearm EnF was assessed as previously described (Kuvin and Karas 2003; Joannides et al. 2006; Higashi and Yoshizumi 2003; Casino et al. 1993). Briefly, a sphygmomanometer cuff applied to the right arm was inflated to 50 mmHg for 7 s to prevent venous egress. During this period, forearm volume changes per time unit (correlates with blood flow changes) were measured by strain gauge plethysmography (ECR5, D.E. Hokanson Inc., Bellevue, WA). A 7-s interval was allowed between each baseline flow. It is established that this interval is sufficient to allow for system adaptation (Corretti et al. 2002). The cutaneous flow of the hand was excluded by inflating a wrist cuff to a level greater than systolic blood pressure. Baseline forearm blood flow was the average of at least four stable repeated flow measurements. Reactive hyperemia was induced by a pneumatic cuff (S300 Aneroid sphygmomanometer) placed above the

sphygmomanometric cuff, which was inflated to greater than systolic pressure for 5 min. A rapid deflation was then allowed and a series of post-ischemic forearm blood flow measurements as previously described were performed (Kuvin and Karas 2003; Joannides et al. 2006; Higashi and Yoshizumi 2003; Casino et al. 1993). Flow curves were analyzed by an independent interpreter that was unaware of the subjects' Hp genotype in order to avoid bias. These sequences of flow measurements are correlated with EnF as described (Kuvin and Karas 2003; Joannides et al. 2006; Higashi and Yoshizumi 2003; Casino et al. 1993).

The method in which we used to assess EnF is post-ischemic reactive hyperemia. The acceptable approach for evaluating changes in flow after ischemic cessation is to consider the parameters of area under the time–flow curve (AUC) and maximal flow increments compared with basal flow. The post-ischemic highest blood flow (maximal flow) and the AUC of the reactive hyperemia response are calculated and considered indices of forearm EnF. According to Joannides et al. (2006) review paper, this method is suitable for measurement of resistance arteries EnF when only the late phase is taken into account. Higashi and Yoshizumi (2003), however, demonstrated that the peak forearm blood flow in response to reactive hyperemia is almost identical to the forearm blood flow in response to acetylcholine, which is endothelial-dependent vasodilator. In the current research, we took both peak flow after ischemia cessation and the AUC as parameters of reactive hyperemia to cover both the aspects of the debate. Studying the reactive hyperemic response allowed us to assess EnF in resistance arteries.

Calculations and statistical analysis

Data were presented as mean ± SEM. One-way ANOVA and two-sided Student's unpaired *t* test were used to compare between the groups (the first to compare between the three groups including the control, and the second when only comparison between the two DM patients' groups was required). The Chi-square test was used to perform sub-analysis by type of diabetes of each Hp genotype groups (results not enclosed). Statistical significance was set at $P < 0.05$. Data were analyzed with Excel (Microsoft 2000, Redmond, WA) and GraphPad Prism (version 4.03, GraphPad Software Inc., San Diego, CA). Power calculation was determined using the software GraphPad StatMate (version 2.00, 2004, CA, USA).

Results

Patients' characteristics are depicted in Table 1. In the DM patient group, patients with Hp 1-1 genotype tended to be older, with longer duration of DM and a higher prevalence of hypertension than those with the Hp 2-2 genotype. Relevant metabolic characteristics (HbA1c, lipid profile and creatinine) were comparable between groups. No significant statistical difference was found between the groups in terms of medications intake, including ACE inhibitors, calcium channel blockers, statins and aspirin; none of our patients were on beta blockers and alpha-adrenoreceptor blocker. All patients with DM type 1 and four with MD type 2 were on insulin (see Table 1).

Healthy normotensive group was assembled of 14 subjects (9 males and 5 females, mean age 59 ± 2 years). Their Hp genotype composes three subjects with 1-1, seven 2-2 and four 2-1. Their hemodynamic (HR, 78 ± 4 beats per minute; SBP, 125 ± 3 mmHg; DBP, 78 ± 6 mmHg) and biochemical (lipid profile 3.3 ± 0.4, 1.2 ± 0.1 and 2.7 ± 0.1 system international units (SI) for TG, HDL and LDL cholesterol, respectively) characteristics were all within the normal range. None of them took any medication chronically.

Baseline flow in the healthy control groups was significantly higher than in the Hp 2-2 DM group, but comparable with the Hp 1-1 DM group (Fig. 1). Indices of EnF, i.e. reactive hyperemic response, expressed either as the maximal flow after the ischemic period or as the AUC, were also significantly higher in the Hp 1-1 DM and healthy groups as compared to the Hp 2-2 DM group (Fig. 2).

As shown in Table 1, the prevalence of Type 1 DM was significantly higher in the Hp 2-2 genotype group ($P = 0.03$). Considering the small number, a sub-analysis showed that DM type did not influence EnF indices ($P = 0.88$ and 0.35 for AUC and maximal flow, respectively).

Discussion

In this study we have demonstrated that there is a greater impairment in EnF in Hp 2-2 as compared to Hp 1-1 DM individuals. This is consistent with epidemiological studies showing a higher incidence of diabetic complications in the Hp 2-2 group.

Endothelial dysfunction in DM is mediated primarily by a decrease in the bioavailability of nitric oxide (NO). One probable mechanism by which NO is reduced in DM is via degradation by oxygen-derived free radicals such as the superoxide and hydroxyl radicals (Cosentino et al. 1997; Gryglewski et al. 1986).

We and others have previously shown that the Hp 1 protein is superior to the Hp 2 protein in preventing the formation of these radicals produced from extracorpuscular hemoglobin via Fenton chemistry (Suleiman et al. 2005; Miller et al. 1997; Melamed-Frank et al. 2001). An additional mechanism whereby NO can be consumed is via the

Table 1 Patients' general characteristics, hemodynamics and metabolic profile

	Haptoglobin 1-1 ($N = 14$)	Haptoglobin 2-2 ($N = 16$)	P
Age (years)	56.2 ± 3.3	49.1 ± 5.1	0.28
Females	6	9	0.72
T1DM	2	9	0.03
Known DM duration (years)	17.1 ± 1.9	13.2 ± 2.1	0.17
Hypertension	9	4	0.065
Known IHD	3	2	0.65
Systolic BP (mmHg)	145 ± 8	142 ± 6	0.74
Mean BP (mmHg)	103 ± 3.8	94 ± 3	0.07
Heart rate (bpm)	76 ± 4	73 ± 3	0.58
HbA1c	7.9 ± 0.3	7.3 ± 0.5	0.37
Creatinine (mg dl^{-1})	1.0 ± 0.05	0.83 ± 0.06	0.06
Total cholesterol (mg dl^{-1})	4.1 ± 0.4	4.7 ± 0.3	0.25
LDL (SI)	2.7 ± 0.4	2.6 ± 0.2	0.78
HDL (SI)	1 ± 0.2	1.2 ± 1	0.11
TG (SI)	4.2 ± 0.8	4.2 ± 1.1	0.99
Hypoglycemic medications (Ins/Ins + or/or)	3/1/10	9/1/6	
Other relevant medications (ASA, BB, CCB, ACE-I, statins)	5/3/0/2/2	4/2/1/2/3	

Ins insulin, *or* oral hypoglycemic, *SI* system international units, *ASA* aspirin, *BB* beta blockers, *CCB* calcium channel blockers, *ACE-I* angiotensin converting enzyme inhibitor

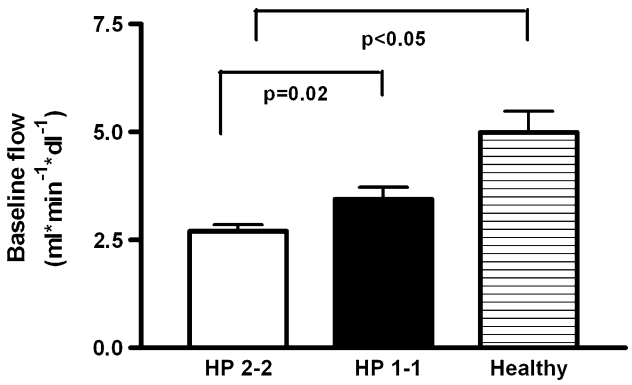

Fig. 1 Baseline blood flow measurements in healthy and DM patients' groups

deoxygenation reaction (Rother et al. 2005). The primary mediator of this reaction in vivo is the hemoglobin–haptoglobin complex (Rother et al. 2005). The Hp 1 protein is more rapid and more efficient than the Hp 2 protein in mediating the clearance of the haptoglobin–hemoglobin complex from the plasmatic compartment via the CD163 scavenger receptor (Asleh et al. 2005).

Study limitations

1. The distribution of DM types between the Hp genotype groups was not equal. We chose patients according to their haptoglobin genotype rather than the DM. According to our knowledge, it is the glycemic control that affects EnF, rather than DM type per se. The study groups were matched for conditions that tend to accompany type 2 DM (e.g. hyperglycemia) and have a role in endothelial dysfunction formation.
2. The control group was not similar, in terms of Hp genotype, to the diabetic patients' group. This could have influence the results.
3. Several parameters that are considered as effectors of EnF were not evaluated in the current research (e.g. insulin sensitivity, body mass index, waist circumference).
4. Three of the patients in the Hp 1-1 group had ischemic heart disease (IHD), which did not affect the entire group's EnF to differ from that of control's. One might consider it impossible since it is expected that improved EnF protects against the development of vascular pathology that leads to, among the rest, IHD.

To conclude, the current study, even though it is an anecdotal observation, provides support for the notion that the population of individuals with diabetes is not homogenous, and that optimizing risk stratification algorithms and treatment could require a pharmacogenomic approach. Indeed, given the prominent role of oxidative stress in Hp 2-2-mediated pathophysiology, it will be of considerable interest to assess the ability of antioxidant therapy to reverse endothelial cell dysfunction and provide cardiovascular benefit specifically in all Hp genotype subgroups, especially the Hp

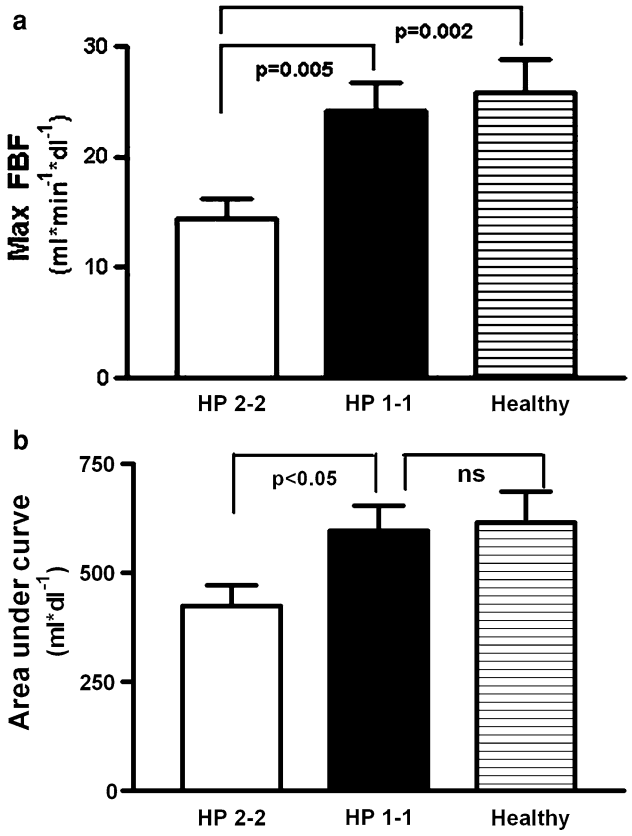

Fig. 2 Endothelial function indices, maximal forearm blood flow after ischemia (*upper image*) and the area after the flow–time curve after ischemia (*lower image*) in healthy and DM patients' groups

2-2 group which is the focus of a soon to be completed clinical trial.

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