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Inverse correlation of serum paraoxonase and homocysteine thiolactonase activities and antioxidant capacity of high-density lipoprotein with the severity of cardiovascular disease in persons with type 2 diabetes mellitus

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Abstract

Atherosclerotic risk is increased in diabetes partly because of increased plasma levels of the oxidized low-density lipoprotein and homocysteine, 2 independent and important cardiovascular disease (CVD) risk factors. Paraoxonase (PON) is a multifunctional antioxidant enzyme component of high-density lipoprotein (HDL), which can protect against low-density lipoprotein (LDL) oxidation. It also exhibits homocysteine thiolactonase (HCTL) activity that detoxifies homocysteine thiolactone, which can damage proteins by homocysteinylation of the lysine residues, thus leading to atherosclerosis. We conducted a cross-sectional study to correlate PON-1, HCTL activities, and the lag time of LDL oxidation in 15 healthy control subjects and in 55 subjects with type 2 diabetes mellitus with different degrees of CVD. Compared with healthy controls and diabetic subjects without evidence of overt CVD, we not only found 47% ($P < .005$) decrease in PON-1 activity, but also for the first time, 30% ($P = .019$) decrease in HCTL activity in subjects with a prior coronary artery bypass surgery. There was corresponding decreased effectiveness of HDLs from diabetic groups (with and without CVD) in protecting against LDL oxidation. Moreover, the PON-1 activity was significantly inversely correlated to the extent of intracoronary lesions determined at catheterization (ie, a high Gensini score). These decreases in PON-1 and HCTL activity were not due to any bias in preferential distribution of low-activity QQ homozygotes in the diabetic groups compared with the control group because QQ allele was equally distributed in all the experimental groups, whereas RR allele tended to increase in the diabetic subjects with coronary artery bypass surgery compared with the other groups. Therefore, clinical intervention to restore the impaired antiatherogenic activities of HDL should be considered an important goal in the treatment of persons with diabetes.

1. Introduction

Cardiovascular disease (CVD) is the predominant cause for morbidity and mortality in diabetes mellitus (DM). Several etiologic factors increase susceptibility to CVD in DM including insulin resistance, dyslipidemia, endothelial dysfunction, prothrombosis, and increased protein glycation. Of particular interest is the role of oxidative stress, which is exaggerated in DM [1] and considered a major contributor to the atherogenic process [2]. Hyperglycemia may promote oxidative modification of low-density lipoprotein (LDL) into oxidized LDL (OxLDL), which is considered to be the central component in the pathogenesis of atherosclerosis. Therefore, any mechanism that would decrease OxLDL should be antiatherogenic. The high-density lipoprotein (HDL) particle(s) is known to prevent the formation of OxLDL by means of the HDL-associated enzyme paraoxonase (PON); its antioxidant properties prevent the accumulation of lipid peroxides on LDL. For

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example, the absence of PON, via knockout, increases LDL oxidation and atherosclerotic lesions [3]. Paraoxonase activity, which is under both genetic and environmental influences, varies widely among individuals. Although several studies [4-8] have shown an association of the PON allele 192 Arg with CVD, there have been conflicting reports concerning polymorphism of other alleles and PON activity [9,10]. Several studies have shown decreased PON activity and/or concentration in both type 1 and type 2 DM [11-14]. Streptozotocin-induced DM also lowers PON activity in rats [15].

Paraoxonase is a multifunctional antioxidant enzyme that not only can destroy OxLDL but also can detoxify the homocysteine metabolite, homocysteine thiolactone, which can pathologically cause protein damage by homocysteinylation of the lysine residues, thereby leading to atherosclerosis [16]. Because of the key role played by this single HDL-associated multifunctional enzyme in destroying 2 vital cardiovascular risk factors, that is, OxLDL and homocysteine thiolactone, a systematic correlation of the status of PON as well as that of homocysteine thiolactonase (HCTL) with the severity of CVD assumes great importance. The present study demonstrates for the first time a combined negative correlation of serum PON and HCTL activity and the protective capacity of HDL against LDL oxidation with the degree of CVD in a diabetic population.

2. Materials and methods

The study subjects were patients with type 2 DM enrolled in the VA Medical Center (Washington, DC) diabetic clinic and 15 healthy individuals on the medical center staff. The study was approved by the Institutional Review Board of the VA Medical Center. The diabetic subjects were divided into the following 3 groups: DM − CVD, consisting of diabetic patients without overt CVD (n = 15); DM + CVD, consisting of diabetic patients with overt CVD (n = 19); and DM + CABG, consisting of diabetic patients who had undergone coronary artery bypass surgery (CABG) within the preceding 3 years (n = 21). The control subjects were nondiabetic subjects (n = 15) who were age matched to the diabetic subjects. CVD was assessed in DM − CVD and DM + CVD groups based on any or all of the following: exercise tolerance testing, nuclear stress testing, cardiac catheterization, and/or a myocardial infarction within 3 years of enrollment. Coronary angiography scoring was performed in the patients from DM − CVD and DM + CVD groups by using the modified Gensini score as has been described and validated previously [17]. This score provides an index of both the severity and the extent of coronary atherosclerosis. The majority of diabetics in this study (52 of 55) reported abstention from alcohol use (which is known to influence PON-1 activity) [18,19]. Similarly, the regular use of antioxidants such as vitamin E was noted in a minority of patients (Table 1). In all aspects, the patients studied were receiving routine subspecialty care. At the time of evaluation, routine fasting blood was drawn between 7 and 8 AM for lipoprotein profile, glycosylated hemoglobin (HbA_{1c}), and SMA-7 electrolytes determined by the in-hospital–based multichannel analyzer.

Table 1

Parameter	DM − CVD group	DM + CVD group	DM + CABG group
n	15	19	21
Age (y)	55 ± 9	62 ± 7	62 ± 8
DM (y)	14 ± 3	14 ± 4	16 ± 5
OHA (%)	60	40	60
Insulin (%)	70	60	75
Statin (% use)	30	70	82
Mean statin dose (mg)	11.6	35.7	52.2
Antioxidant (% use)	0	0	8
FPG (mg/dL)	126 ± 80	178 ± 38	170 ± 45
Total cholesterol (mg/dL)	179 ± 33	178 ± 38	170 ± 43
Triglyceride (mg/dL)	170 ± 81	190 ± 96	207 ± 160
HDL (mg/dL)	46 ± 12	42 ± 8	38 ± 11
LDL (mg/dL)	105 ± 33	98 ± 31	94 ± 34
HbA_{1c}) (%)	7.97 ± 1.8	7.66 ± 1.3	8.17 ± 2.2

OHA indicates oral hypoglycemic agent; FPG, fasting plasma glucose.

2.1. Preparation and assay of serum PON activity

Blood was collected without any additives and serum was obtained by centrifugation and stored at −80°C until PON activity determination. Paraoxonase activity was assayed by using established procedures [19,20]. Briefly, PON activity was determined in a 10-μL aliquot of serum using 1 mmol/L paraoxon as the substrate in 50 mmol/L Tris-HCl buffer (pH 8.0) by measuring the increase in the absorbance at 412 nm (SpectraMAX 190 microplate reader, Molecular Device, Sunnyvale, CA) due to the formation of 4-nitrophenol. Blanks were included without the added serum to correct for the spontaneous hydrolysis of paraoxon. PON activity is expressed as units per deciliter. One unit is defined as 1 nmol of 4-nitrophenol formed per deciliter of serum per minute under the above assay conditions.

2.2. Assay of serum HCTL activity

Serum HCTL activity was determined essentially as described previously [21]. Briefly, [^{35}S]homocysteine thiolactone was synthesized from [^{35}S]methionine based on prior methods [22,23]. A 50-μL aliquot of the serum was incubated with purified [^{35}S]homocysteine thiolactone (5 mmol/L) in 0.1 mol/L potassium phosphate-HEPES buffer, pH 7.4, 2 mmol/L $CaCl_2$ for 1 hour, and the radioactivity in labeled homocysteine formed was determined by thin-layer chromatography. HCTL activity is expressed as nanomoles of homocysteine formed per deciliter of serum per minute under the above assay conditions.

2.3. Preparation of HDL from plasma

Blood samples from all subjects were collected separately and processed for preparation of HDL by heparin/Mn

followed by dextran sulfate precipitation methods [24,25]. Briefly, 0.1 vol of heparin-$MnCl_2$ (1 vol of solution contained 0.226 vol of 10,000 USP units/mL heparin [Elkin Sinn, Cherry Hill, NJ] in 1.0 mol/L $MnCl_2$) solution was added to 1.0 vol of plasma. After 30 minutes incubation at room temperature, the samples were centrifuged for 30 minutes at 600*g* at 4°C. The supernatant solutions were processed for total HDL precipitation by addition of dextran sulfate (50,000 MW, in 0.15 mol/L NaCl, 0.65% final concentration) and $MnCl_2$ (0.2 mol/L final concentration). After thorough mixing, the samples were left at room temperature for 20 minutes and then centrifuged for 30 minutes at 600*g* at 4°C. The pellets containing total HDL were redissolved in 0.1 mol/L sodium citrate and, after removing the undissolved dextran-$MnCO_3$, dialyzed extensively against 10 mmol/L phosphate-buffered saline (pH 7.4). Total protein was estimated by the Bradford method [26] using bovine serum albumin as standard.

2.4. Assessment of antiatherogenic capacity of HDL in inhibiting LDL oxidation kinetics

In vitro LDL oxidation was performed using a modification of a previously described procedure [20,27]. Purified LDL (100 μg/mL), obtained by 2 successive flotations, was incubated in phosphate-buffered saline (pH 7.4) with freshly prepared 10 μmol/L $CuSO_4$ in a total volume of 1 mL at 37°C. Each serum sample was processed to precipitate very low-density lipoprotein (VLDL)/LDL [24] and an aliquot of the supernatant solution (HDL-rich serum) was used to test its ability to inhibit LDL oxidation. The LDL oxidation was carried out both in the absence and in the presence of 100 μg apoprotein A-1 protein equivalent of VLDL/LDL-free serum per milliliter from each group. The kinetics of LDL oxidation was monitored spectrophotometrically at 234 nm using a quartz 96-well microplate in a SpectraMAX 190 microplate reader (Molecular Device) for 5 hours. The change in absorbance over time was divided into 3 consecutive phases: lag phase, propagation phase, and decomposition phase. The lag time (in minutes) of each kinetics was then determined.

2.5. PON genotyping

The genotyping of PON was determined as described by us [19] and others [28]. Initially, genomic DNA was extracted from whole blood by using a salting-out DNA extraction kit (Eppendorf, Westbury, NY). Allele-specific primers previously described [29] to encompass the polymorphism affecting position 192 were used to amplify the polymorphic region of the gene. The polymerase chain reaction contained approximately 100 to 500 ng of DNA template, 0.1 μmol/L of each primer, 0.2 mmol/L of the 4 dNTPs, 1 U *Taq* DNA polymerase, and 1.5 mmol/L $MgCl_2$. After denaturing the DNA for 5 minutes at 95°C, the reaction mixture was subjected to 46 cycles of denaturing for 1 minute at 94°C, 1 minute of annealing at 61°C, and 1 minute extension at 72°C. The nucleotide substitution corresponding to position 192 (Gln-Arg) creates an *Alw*I restriction site. Allele Q (glutamine) corresponded to a 99–base pair (bp) fragment, whereas allele R (arginine) corresponded to 65- and 34-bp fragments. The polymerase chain reaction product was restriction digested using *Alw*I at 37°C for 3 hours. The digested products were then subjected to 3.0% agarose gel electrophoresis for 45 minutes at 90 V and visualized with 0.1% ethidium bromide staining.

3. Statistical analysis

The results are expressed as mean ± SD. The significance was established using the Sigma Plot software (Systat Software, Point Richmond, CA) program including Student *t* test, regression analysis, and by a 1-way analysis of variance followed by the Tukey test [30].

4. Results

Table 1 summarizes the clinical and metabolic information obtained for the patients in this study. There were no significant differences in any of the lipid or diabetes parameters among various groups except for a slightly lower mean age in the DM − CVD group compared with the DM + CVD and DM + CABG groups ($P < .05$). Serum PON-1 activities in the various groups are shown in Fig. 1. Compared with the control group, serum PON-1 activity was significantly reduced by 30% ($P < .05$) in the DM − CVD group, by 30% ($P < .05$) in the DM + CVD group, and by 47% in the DM + CABG group ($P < .005$ vs DM − CVD group and $P < .04$ vs DM + CVD group).

Genotyping of blood samples for genotyping revealed that the QQ allele showed no statistically significant difference in the distribution pattern among various groups, whereas QR and RR alleles in the DM + CABG groups were significantly different ($P < .05$) from the DM − CVD

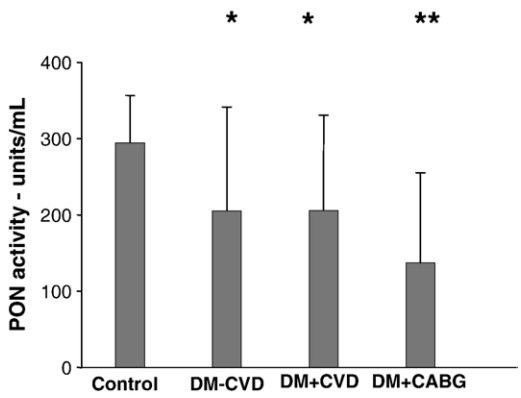

Fig. 1. PON activity in control and diabetic groups. Blood serum was collected from the study subjects in various groups and serum PON activity was determined as described in Materials and methods. DM − CVD: *$P < .05$ vs control. DM + CVD: *$P < .05$ vs control. DM + CABG: **$P < .005$ and $P < .04$ vs DM − CVD.

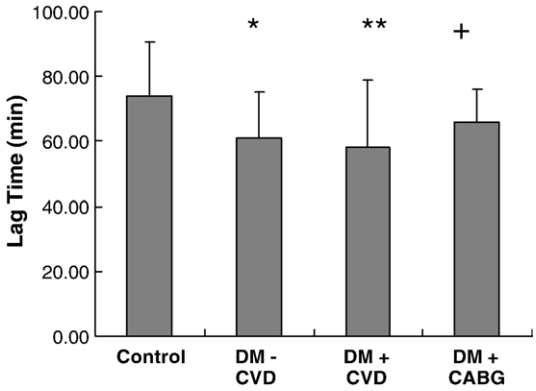

Fig. 2. Lag time of LDL oxidation in the presence of HDL from various diabetic groups. LDL/VLDL-free serum was collected from each study subject and its ability to inhibit copper-induced LDL oxidation was determined as described in Materials and methods. DM − CVD: $*P < .05$ vs control. DM + CVD: $**P < .005$ vs control. DM + CABG: $^{+}P = .05$ vs control.

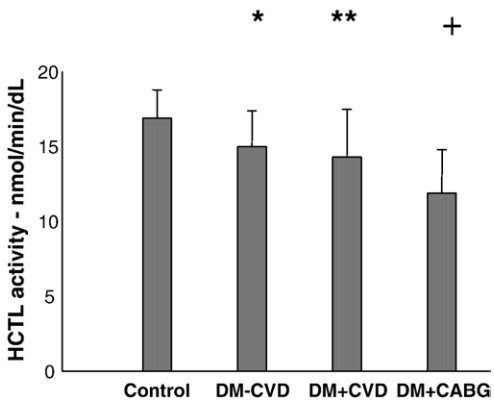

Fig. 4. Homocysteine thiolactonase activity in controls and diabetic patients. Serum HCTL activity was determined in each group as described in Materials and methods. DM − CVD: $*P < .05$ vs control. DM + CVD: $**P < .03$ vs control. DM + CABG: $^{+}P = .019$ vs control.

and DM + CVD groups (DM − CVD, 50% QQ, 33% QR, 17% RR; DM + CVD group, 50% QQ, 33% QR, 17% RR; DM + CABG group, 50% QQ, 17% QR, 33% RR). Predictably, PON-1 activity was lowest in the QQ group (162 ± 101 U/dL), intermediate in the QR group (210 ± 78 U/dL), and the highest in the RR group (311 ± 86 U/dL).

The antiatherogenic effects of the HDL preparations from various groups, as measured by the mean lag time of LDL oxidation, are presented in Fig. 2. It can be seen that compared with the controls, the mean lag time of LDL oxidation was significantly reduced by 24% ($P < .05$) in the DM − CVD group, by 32% ($P < .005$) in the DM + CVD group, and by 18% ($P = .05$) in the DM + CABG group.

In Fig. 3, correlation of serum PON activity with the extent of coronary lesions (the Gensini score) is plotted in those patients in the DM − CVD and DM + CVD groups who had undergone cardiac catheterization (n = 19). The results show a significantly progressive decline in PON-1 activity with increased atherosclerosis ($r = 0.640$, $P < .008$).

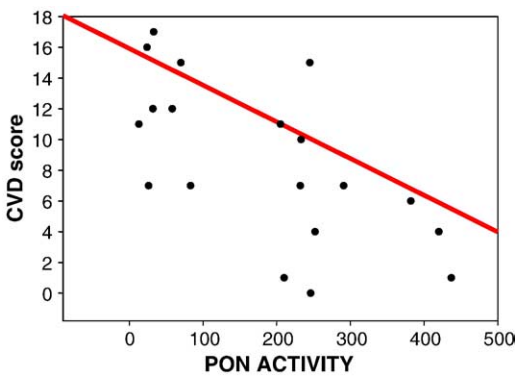

Fig. 3. The Gensini score obtained from cardiac catheterization in 19 patients from DM − CVD and DM + CVD groups plotted against their respective PON-1 activities. The correlation coefficient was 0.640 and the r^2 was 0.410 ($P < .008$).

As shown in Fig. 4, HCTL activity exhibited a similar relationship to the degree of CVD as observed for PON-1 activity. There was 11% decrease in the DM − CVD group ($P < .05$) compared with controls, 16% decrease in the DM + CVD group ($P < .03$) compared with controls (and $P = .019$ vs DM − CVD group), and 30% decrease in the DM + CABG group ($P < .0001$) compared with controls (and $P = .0015$ vs DM + CVD group). Regression analysis revealed no significant correlation of either the PON-1 activity or the HCTL activity with HDL, LDL, total cholesterol, triglycerides, fasting blood glucose, or HbA_{1c}) in any of the groups (data not shown).

5. Discussion

Augmented levels of oxidative stress, as in states of dyslipidemia, aging, and smoking, have been associated with decreased PON activity. Our data confirm previous observations of low PON-1 activity and higher lipid peroxidation in patients with type 2 DM [12-15] in contrast to which one study [31] failed to find this association. In the present study we found that type 2 diabetic patients have lower serum PON-1 activity compared with controls (Fig. 1). Indeed, patients with insulin resistance and the metabolic syndrome also have impaired PON activity [32]. When we subdivided the diabetic patients according to their CVD status, it became evident that diabetic patients without overt CVD have relatively higher PON-1 activity compared with those with established CVD. This trend was most pronounced in those diabetic patients who had already undergone CABG. Because we did not observe any significant difference in the PON genotype distribution, the decreased PON-1 activity is most likely due to the diabetic pathophysiology rather than to the PON polymorphism. Furthermore, increased angiographic atherosclerotic activity (ie, the Gensini score) was associated with decreased PON-1 activity (Fig. 3).

The lag time of LDL oxidation kinetics reflects the antioxidant potential of HDL preparations from various groups. That the mean lag time progressively decreased with increasing severity of CVD in diabetic patients compared with the controls (Fig. 2) implies that the atherogenic process is accelerated concomitantly with the progression of diabetic pathology. The only exception was the group consisting of those patients with CABG, who had a mean lag time greater than that found in the DM − CVD and DM + CVD groups. This could be attributed to other factors that affect the antioxidant potential of HDL distinctly different from its PON-1 activity. For example, those diabetic patients who had undergone CABG were more aggressively treated than were DM − CVD and DM + CVD groups, receiving a higher mean dose of statins, which might have contributed to additional antioxidant potential. Although the stimulation of PON-1 activity by statins is in dispute [33,34], it has been reported that statins may have a direct protective effect on the LDL oxidation process [35]. It should also be pointed out that although HDL has antioxidative properties, some recent reports have pointed out that purification of PON protein leads to the loss of its intrinsic ability to destroy products of lipid peroxidation [36-38]. The level of glycemia may also impact differently on PON-1 as compared with the antiatherogenic potential of HDL against LDL oxidation. It is noteworthy that both hyperlipidemia and hyperglycemia have been shown to induce ROS generation and lipid peroxidation [39-41].

The low PON-1 activity could be responsible for the diminished protective capacity of the HDL to inhibit LDL oxidation as evidenced by the shorter lag time in diabetic patients compared with controls (Fig. 2). This phenomenon occurred across the spectrum of CVD and may be indicative of early increased lipid peroxidation, which subsequently results in foam cell formation and atherosclerosis.

Our data on serum HCTL activity also correlate well with the severity of CVD in diabetic patients (Fig. 4). Significantly, this is the first time a direct negative correlation between serum HCTL activity and the severity of CVD has been demonstrated. Thus, the decreased activity of HCTL in diabetic patients could account for the increased homocysteinylation of proteins, which in turn leads to atherosclerosis [4,42]. In support of this concept, it has been shown that homocysteinylation of human HDL decreases its PON activity [43]. Furthermore, long-term infusion of homocysteine thiolactone has been shown to cause atherosclerosis in rabbits and baboons [44,45] by increasing plasma cholesterol, LDL, and VLDL. More recently, it has been shown that HCTL can homocysteinylate LDL leading to its aggregation and enhanced uptake by endothelial cells in vitro [46].

Based on all of the above, our present data support the concept that, in addition to the well-accepted inverse correlation between PON activity and incidence of CVD, the HCTL activity of HDL may be another important independent antiatherogenic property that negatively correlates with the severity of CVD in diabetic patients. Clinical intervention to restore the impaired antiatherogenic activity of HDL in diabetic patients would be an effective goal for the treatment of these patients.

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