Modulation by Blood Glucose Levels of Activity and Concentration of Paraoxonase in Young Patients With Type 1 Diabetes Mellitus

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Paraoxonase (PON) is a high-density lipoprotein (HDL)-associated esterase, which may prevent the transformation of low-density lipoproteins (LDL) into biologically active, atherogenic particles. PON concentration and activity are affected by PON1 gene polymorphisms and found to be altered in type 2 diabetes patients with retinopathy. We investigated serum PON concentration, in vitro activity and polymorphism at position 54 (L/M, Leu-Met54) in 193 Caucasian adolescents and young adults (88 males, 105 females) with type 1 diabetes mellitus, as well as its relationship to the presence of retinopathy. An inverse linear correlation was found between blood glucose levels and both serum PON concentration ($r = -.20$, $P = .017$) and its activity ($r = -0.17$, $P = .037$). Patients with elevated blood glucose values (≥10 mmol/L) had significantly lower levels of both PON concentration ($P = .003$) and activity ($P = .028$) than those with lower glucose levels. After adjusting for blood glucose and diabetes duration, PON activity was significantly higher in patients with different stages of retinopathy compared with those without retinopathy ($P = .003$). The L/L genotype was closely associated with the presence of retinopathy ($P < .0001$). These data show that young people with type 1 diabetes and the L/L polymorphism at position 54 of PON1 gene are more susceptible to retinal complications. However, the role of serum PON concentration and activity as a possible marker for monitoring late microvascular complications in these patients has to be established.

HUMAN SERUM PARAOXONASE (PON) is a calcium-dependent high-density lipoprotein (HDL)-associated ester hydrolase, which has been implicated in enzymatic removal of lipid peroxides by limiting the accumulation of lipid oxidation products in low-density lipoproteins (LDL)[1] and preventing the transformation of LDL into biologically active, atherogenic particles.[2] Because oxidation of LDL with consequent injury to retinal endothelia and pericytes[3] may be an important mechanism contributing to diabetic retinopathy, the PON gene and its product has attracted the interest of investigators concerned with the development of diabetes microvascular complications.[4] Polymorphisms of PON1 gene and its promoter region have been shown to have the most marked impact on serum concentration and in vitro activity of this enzyme.[5,6]

In a previous study, we showed that there is a clear association between the genotype Leu-Leu (L/L) of PON1 at position 54, but not of Gln-Arg at position 192 and the development of early retinopathy in young patients with type 1 diabetes mellitus.[7] In the present study, we investigated serum concentration of PON and its activity in a second cohort of young patients with type 1 diabetes, because reduced PON activity has been reported in patients with type 2 diabetes and retinopathy.[4,8]

PATIENTS AND METHODS

Patients

The study cohort consisted of 193 Caucasian adolescents and young adults (88 males, 105 females; median age, 15.9 years [interquartile range, (IQR), 13.8 to 20.3]) with type 1 diabetes mellitus (diabetes duration, 8.8 years [IQR, 4.3 to 13.4]). All patients attended the Diabetes Complication Assessment Service (DCAS) of Royal Alexandra Hospital for Children in Sydney, NSW, Australia. Informed consent was given by all participants, and the study was approved by the Hospital's Ethics Committee.

Screening for Diabetic Retinopathy

Retinal examinations were performed annually using stereoscopic fundal photography of 7 standard fields. Retinal staging was performed using an adaptation of the Airlie House system.[9] Early background retinopathy was defined as any microaneurysm or hemorrhage in at least 1 eye (stage 21/10), while the next stage was characterized as at least grade 21 in 1 eye and at least 31 in the other eye. The latter was used for defining "clinical retinopathy" in the Diabetes Control and Complications Trial.[10] Retinal photography was not gradable in 3 (1.6%) patients. In addition to the retinal examination, blood was taken for measurement of glycosylated hemoglobin A_{1c} (HbA_{1c}, Diamat Biorad, Munich, Germany), blood glucose, and cholesterol (both of them at postprandial conditions).

PON1 Met-Leu 54

This polymorphism was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using a slightly modified procedure primarily described by Humbert et al.[11] Briefly, 100 ng of DNA were denatured at 94°C for 12 minutes and then amplified for 35 cycles using the PCR primers as described by Humbert[11]: each cycle comprised denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension time of 5 minutes. The 170-bp PCR product was digested with *Hsp* 92 II (Promega, Madison, WI) at 37°C for 2 hours. Digested products were separated by polyacrylamide gel (10%) electrophoresis and stained by ethidium bromide. The PON1 Leu54 (L) allele corresponded to the presence of a nondigested fragment of 170 bp, while the PON1 Met54 (M) allele corresponded to 2 digestion fragments of 126 bp and 44 bp.

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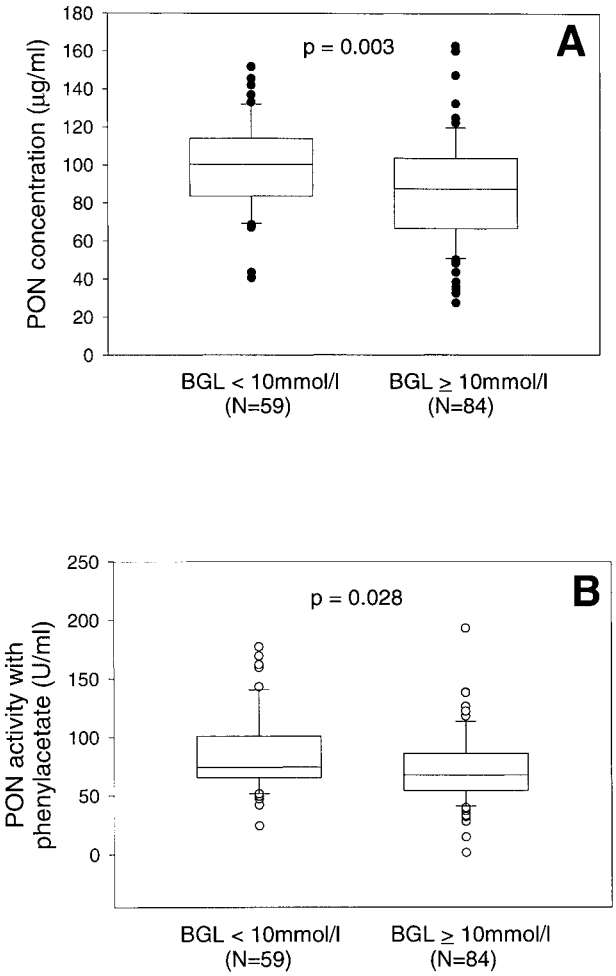

Fig 1. Concentrations (A) and in vitro activity (B) of PON in diabetes patients with different blood glucose levels (BGL). *P* value according to Mann-Whitney *U* test. Whiskers of the box plots correspond to 10th and 90th percentile, respectively.

PON Activity and Concentration

All measurements were performed in Geneva, Switzerland (RW.J.). PON enzyme activities were assayed in serum samples using both phenylacetate and paraoxon as substrates according to previously described methods.[12] PON concentrations were measured using a competitive enzyme-linked immunosorbent assay (ELISA).[12]

Statistical Analysis

Data were analyzed using the SPSS 9.0.1 software (SPSS, Chicago, IL). Group differences for continuous variables were tested using Mann-Whitney *U* test (2 independent groups) or Kruskal-Wallis test (3 or more independent groups). Differences of frequencies for categorical variables were tested by the χ^2 test, and Z-test was used to assess difference in proportions. Analysis of covariance was used to examine serum PON levels and in vitro activity in patients with different stages of retinopathy, adjusting for other biologic variables. Normally distributed data are presented as mean ± SD, otherwise as median and IQR ranges.

RESULTS

An inverse linear correlation was found between blood glucose levels and both serum concentrations of PON (Pearson correlation coefficient $r = -.20$, $P = .017$) and its activity ($r = -.17$, $P = .037$). Moreover, patients with ambient blood glucose values below 10 mmol/L had significantly higher levels of both PON concentration ($P = .003$) and activity ($P = .028$) than those with elevated (≥10 mmol/L) blood glucose (Fig 1). However, these parameters did not correlate with HbA_{1c} reflecting longer-term glucose levels in diabetes patients.

For patients carrying different genotypes for PON1 54, no statistical differences were found for PON serum concentrations (L/L, 92.0 ± 28.3 μg/mL; M/L, 90.4 ± 25.6 μg/mL; M/M, 89.2 ± 24.3 μg/mL, $P = .570$) and enzyme activity with phenylacetate as substrate (L/L, 74.9 U/mL [IQR, 61.9 to 91.1]; M/L, 69.4 U/mL [IQR, 54.9 to 86.0]; M/M, 64.5 U/mL [IQR, 53.4 to 86.6], $P = .172$).

In multiple regression analysis, an independent effect on PON serum concentration was found only for ambient blood glucose levels (standardized coefficient beta, −0.83 [95% confidence interval (CI), −1.51 to −0.15], $P = .017$) accounting for 4% of the variation of PON levels. Similarly, glucose levels (standardized coefficient beta, −0.86 [95% CI, −1.66 to −0.06], $P = .036$) and presence of retinopathy (standard coefficient beta, 13.27 [95% CI, 2.48 to 24.10], $P = .016$) independently influenced the PON activity (adjusted $R^2 = .06$, $P = .004$). In both models, PON 54 genotype, HbA_{1c}, cholesterol levels, diabetes duration, and age of patients did not have a significant effect on PON concentration and in vitro activity.

As in the previous study, patients with PON1 54 L/L genotype more frequently had retinopathy ($P < .0001$) compared with those with M/L or M/M genotypes. Also, among patients with clinical background retinopathy, the L/L genotype was the dominant one (Table 1). Further significant differences between patients with different stages of retinopathy were found for age ($P < .0001$) and diabetes duration ($P < .0001$), but not for ambient blood glucose ($P = .913$), HbA_{1c} ($P = .212$) or cholesterol levels ($P = .909$).

Interestingly, PON activity was significantly different in patients with different grades of retinopathy (no retinopathy, 67.2 U/mL [IQR, 53.6 to 80.7]; early background retinopathy, 80.2 U/mL [IQR, 59.1 to 98.3]; clinical background retinopathy, 67.8 U/mL [IQR, 54.9 to 83.8], $P = .026$). However, after adjusting PON activity for ambient glucose levels and diabetes duration, enzyme activity was significantly higher in patients with early or clinical retinopathy compared with those without retinopathy (Fig 2), while PON1 54 polymorphisms did not

Table 1. Distribution of PON1 Genotypes at Position 54 in Young Diabetic Patients With and Without Retinopathy

PON1 Genotype	No Retinopathy (%)	Early Background Retinopathy (%)	Clinical Background Retinopathy (%)
M/M	26 (22)	1 (2)*	2 (7)†
M/L	60 (51)	12 (28)†	13 (43)
L/L	31 (27)	30 (70)*	15 (50)†
Total	117 (100)	43 (100)	30 (100)

*$P < .001$; † $P < .05$ compared with patients without retinopathy.

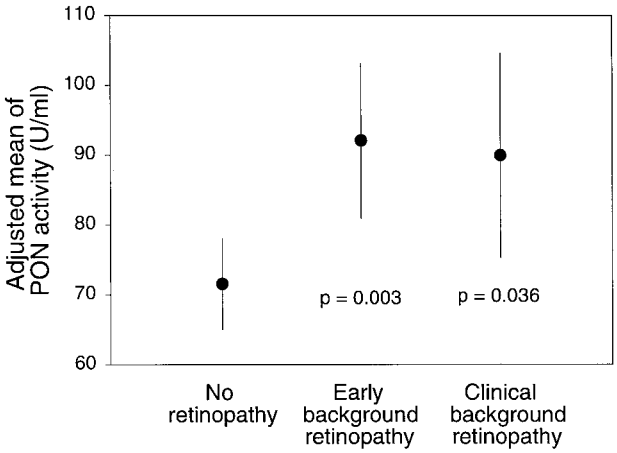

Fig 2. Mean and 95% CI of PON in vitro activity in type 1 diabetes patients with different stages of retinopathy after adjusting for ambient blood glucose and diabetes duration. *P* values show significant differences from no retinopathy.

have a significant effect. Serum PON concentrations did not differ between patients with different stages of retinopathy.

DISCUSSION

These data support and extend our previous report[7] underlying the association between the L/L polymorphism of PON1 gene at position 54 and the development of retinopathy in young patients with type 1 diabetes mellitus.

In contrast to previous studies,[5] we could not confirm higher PON concentration and/or enzyme activity in individuals carrying the L/L genotype at position 54 compared with those with L/M or M/M genotype. One possible explanation for this deviation may be differences between populations,[13] as they have been reported even when subjects were compared according to their PON genotype.[12] Moreover, patients participating in this study were markedly younger compared with those in previous reports.[8,12] To our knowledge, there are no data available concerning PON serum concentrations and activity at very young ages, particularly age-related changes based on different levels of gene expression. In very recent studies, Leviev and James[6,14] showed that polymorphisms at the promoter region of PON1 gene have a more profound effect on serum concentration and in vitro activity of PON than the PON1 54 genotypes. Thus, the distribution of such polymorphisms has to be studied in this patient cohort. Interestingly, ambient blood glucose levels correlated inversely with PON concentration and in vitro enzyme activity in this cohort. Patients with elevated blood glucose had lower levels of PON and lower enzyme activity with phenylacetate as substrate independent of their genotype. In a very recent report by Hedrick et al,[15] glycation of HDL under in vitro conditions lead to a 65% reduction of PON enzymatic activity. That study did not report on HbA_{1c} and PON. One possible explanation for an association between PON and glucose, but not between PON and HbA_{1c} may lie in turnover rates. As is well known, glycosylated hemoglobin reflects glycemic control over a period of weeks or months due to the half-life of hemoglobin. We have no data on PON metabolism but, given its tight links with HDL, we could reasonably postulate that turnover will be similar to that of HDL. Turnover of HDL is on the order of days, considerably shorter than that of hemoglobin. Thus, acute changes in glucose would have a greater short-term impact on PON than HbA_{1c}.

PON activity toward exogenous substrates was significantly different among patients with different stage of retinopathy and, in particularly, after adjusting for confounding factors, ie, glucose levels, it was higher in those patients with retinopathy compared with those without this complication. On the other hand, a greater percentage of subjects with "high-expressor" genotype (L/L) was present in the group with retinopathy. This result is in contrast with studies reporting significantly lower levels of PON activity, for example in patients with type 2 diabetes mellitus and more severe stages of retinopathy,[4,8] but in accordance with those reporting L54 allele as a risk factor for microvascular complications in patients with diabetes.[5] The first question is whether it is the increased or relatively reduced activity of the enzyme that determines susceptibility for macro- and microvascular complications. It has been shown that a higher LDL cholesterol-to-PON concentration ratio may indicate a reduced capacity of PON to limit LDL oxidation[14]; unfortunately, data on LDL cholesterol concentration were not available in the present study. Although there is evidence of an association between different PON1 genotypes and enzyme activities measured with these substrates, we still do not know the physiologic relevance of the activity polymorphism. Furthermore, there is presently no published evidence that the PON 54 polymorphism affects enzyme stability or hydrolytic efficiency. For example, specific activities (enzyme activity/unit mass enzyme) are the same for L and M 54 isoforms, suggesting equivalent hydrolytic efficiency. Studies have focused to a greater extent on the 191 polymorphism, which is known to affect activity toward exogenous substrates. The situation with potential endogenous substrates (ie, oxidized LDL) is less clear, as there are conflicting reports that the B 191 isoform is less efficient, and on the other hand, of similar efficiency as the A 191 isoform in protecting LDL from oxidation. Interestingly, James et al[14] showed recently that lower expression defined by polymorphisms at the promoter region ie, −107T allele, can moderate the significance of the presence of a "high-risk" allele (for example R191 for cardiovascular disease). In this context, we also have to evaluate possible implications of the PON promoter polymorphisms in retinopathy. In conclusion, these data show, that not only patients with type 2 diabetes, a metabolic disorder including more frequently lipid abnormalities, but also young people with type 1 diabetes carrying the L/L genotype at position 54 of PON1 are more susceptible to retinal complications. Furthermore, we showed that high blood glucose levels influence in vivo the concentration of PON and its in vitro activity. However, the role of serum PON concentration and activity as a possible marker for monitoring late microvascular complications in patients with type 1 diabetes is yet to be established.

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