Low paraoxonase activity in type II diabetes mellitus complicated by retinopathy

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ABSTRACT

Human serum paraoxonase 1 (PON1) is located on high-density lipoprotein and has been implicated in the detoxification of organophosphates, and possibly in the prevention of lipid peroxidation of low-density lipoprotein. PON1 has two genetic polymorphisms, both due to amino acid substitutions: one involving glutamine (Q genotype) and arginine (R genotype) at position 192, and the other involving leucine (L genotype) and methionine (M genotype) at position 55. We investigated the effects of these polymorphisms, and of a polymorphism of the PON2 gene at position 310 (Cys/Ser; C and S genotypes respectively), on serum PON1 activity and concentration, plasma lipids and lipoproteins and glycaemic control in 93 individuals with type II diabetes with no complications and in 101 individuals with type II diabetes with retinopathy. Serum PON1 activity in the group with no complications [median 164.1 nmol min$^{-1}$ ml$^{-1}$ (range 8.0–467.8)] was significantly higher than in the group with retinopathy [113.4 nmol min$^{-1}$ ml$^{-1}$ (3.0–414.6)] ($P < 0.001$), but the serum PON1 concentration was not different between the groups. The gene frequencies of the PON1-55 and PON1-192 polymorphisms and of the PON2-310 polymorphism were not different between the study populations. The PON1-55 and PON1-192 polymorphisms affected PON1 activity in the way described in a previous study of a control group and subjects with type II diabetes. The PON2-310 polymorphism also significantly affected serum PON1. PON1 activity was significantly higher in individuals with the PON2-310 CC genotype in both groups with type II diabetes, and the PON1 concentration was significantly higher in PON2-310 CC homozygotes with no complications than in the group with retinopathy. Neither the PON1-55 nor the PON1-192 polymorphism was correlated with the serum lipid or lipoprotein concentration in either group. In the group with retinopathy (but not the group with no complications), all three PON polymorphisms were correlated with glycaemic control, which was worse for the PON1-55 genotypes in the order MM > LM > LL ($P = 0.0032$), for the PON1-192 genotypes in the order RR > QR > QQ ($P = 0.011$) and for the PON2-310 genotypes in the order CC > CS > SS ($P = 0.010$). Low serum PON1 activity in retinopathy may be related to an increased tendency for lipid peroxidation. Our findings thus raise the possibility that, in retinopathy, the PON2 gene may influence PON1, and that an inter-relationship between the PON1 and PON2 genes may influence glycaemic control in subjects with type II diabetes complicated by retinopathy.

INTRODUCTION

The oxidative modification of low-density lipoprotein (LDL) in the artery wall is currently believed to be central to the pathogenesis of atherosclerosis [1]. Oxidized LDL has also been shown to be cytotoxic to retinal capillary endothelial cells and pericytes, and could provide an important mechanism contributing to the

Key words: diabetes mellitus, paraoxonase, polymorphisms, retinopathy.

Abbreviations: ANOVA, analysis of variance; apo B (etc.), apolipoprotein B (etc.); HbA$_{1c}$, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PON, paraoxonase.

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development of retinopathy in diabetes [2]. Therefore mechanisms that prevent the oxidation of LDL have received increasing attention in recent years. One such mechanism is the prevention of LDL oxidation by high-density lipoprotein (HDL) [3]. HDL appears to decrease the accumulation of lipid peroxides on LDL by a mechanism that is, at least in part, enzymic [4]. We have subsequently shown that the HDL-associated enzyme, paraoxonase 1 (PON1), is one of the enzymes of HDL responsible for its ability to prevent the accumulation of lipid peroxides on LDL [5,6]. These findings have since been confirmed by others [7,8]. PON1 has received most attention because it is the enzyme present in the serum of mammals (as opposed to birds and insects) which is responsible for resistance to organophosphate toxicity [9]. Serum PON1 activity is decreased in subjects who have had a myocardial infarction [10], and in subjects with type I or type II diabetes [11,12]. Also, streptozotocin-induced diabetes results in a progressive decrease in serum PON activity [13]. This evidence has given rise to speculation that decreased PON1 activity is associated with the increased lipid peroxidation found in diabetes and could therefore contribute to excess mortality from coronary heart disease, and that it might be a factor determining predisposition to complications of diabetes, perhaps because lipid peroxidation is also important in the development of such complications.

PON1 activity is genetically determined by two alleles at a single autosomal locus [14,15]. However, the PON1 gene has two polymorphisms: one at position 192 [glycine (Q) to arginine (R) substitution] and a second at position 55 [leucine (L) to methionine (M) substitution]. The polymorphisms affect the hydrolytic activity of the PON1 isoenzymes with respect to certain substrates, such as paraoxon and lipid peroxides [16,17]. The dominant effect on activity is exerted by the PON1-192 polymorphism. However, the PON1-55 polymorphism also exerts a smaller, but significant, effect on activity [16,17].

In some case-control studies the PON1-192 RR and PON1-55 LL genotypes were found to be present with an increased frequency in both patients with type II diabetes and non-diabetic patients that had coronary heart disease [18–21], although in other studies such an association was not found [22–26]. Recently we have shown that HDL isolated from RR homozygotes, although most active in the hydrolysis of paraoxon, is much less effective at protecting LDL against lipid peroxidation than HDL from either QQ homozygotes or QR heterozygotes [16,27]. The purified PON1 Q alloenzyme also hydrolyses phospholipid and cholesteryl ester hydroperoxides more efficiently than the R alloenzyme [8].

PON1 is part of a multi-gene family also comprising the PON2 and PON3 genes, which are also located on chromosome 7 [28]. Polymorphisms of the PON2 gene have been associated with glycaemic control [29] and susceptibility to coronary artery disease [30].

Recently, Kao et al. [31] have reported an increase in the frequency of the PON1-55 L allele in subjects with type I diabetes with retinopathy compared with those without this complication. The LL genotype was strongly associated with the development of retinopathy; however, no association was found with the PON1-192 polymorphism, and the effects of PON2 polymorphisms were not investigated. This has prompted us to examine the relationship between the polymorphisms of the PON1 and PON2 genes, PON1 activity and mass with the occurrence of retinopathy in patients with type II diabetes.

METHODS

Subjects

The group with type II diabetes comprised 194 unselected patients who were attending the Manchester Diabetes Centre. Diabetes mellitus was diagnosed according to the 1980 World Health Organisation Expert Committee Report [32]. Of these patients, 93 individuals were free of diabetic complications, and 101 individuals had retinopathy (of which 89 had peripheral neuropathy, 65 had nephropathy and 23 had coronary artery disease). Retinopathy was assessed by fundus examination through dilated pupils using an ophthalmoscope. The minimum requirement for a diagnosis of retinopathy was the presence of background retinopathy classified as microaneurysms, haemorrhages, exudates or venous beading. In the group with no retinopathy, 51 patients were receiving no drug therapy, five were receiving insulin and 37 were receiving oral hypoglycaemic agents (seven on metformin and 30 on sulphonylurea drugs). In the group with retinopathy, 42 patients were receiving insulin therapy and 52 were receiving oral hypoglycaemic agents (eight on metformin and 44 on sulphonylurea drugs). Seven patients were receiving combination therapy (four on insulin plus sulphonylureas, one on sulphonylureas, β-blocker therapy and aspirin, and two on this combination plus insulin). None of the patients with type II diabetes were receiving lipid-lowering drug therapy.

This study was approved by the Central Manchester Health Authority Research Ethics Committee. Written informed consent was obtained from all subjects included in the study.

Blood sampling and DNA extraction

Venous blood was obtained from the subjects with diabetes between 9.00 and 10.00 hours after an overnight fast. Serum and EDTA-plasma were obtained by low-speed centrifugation (3000 g for 15 min at 4 °C). Plasma was used immediately to separate HDL by ultra-
centrifugation in an LM 55 ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.) [33]. Serum and HDL were stored at −20 °C before further analysis. DNA was extracted from white blood cells present at the interface of the plasma and red blood cells using a Split-Second Kit (Boehringer Mannheim, E. Lewes, Sussex, U.K.). After denaturing the DNA for 5 min at 95 °C, the reaction mixture was subjected to 46 cycles of 1 min of extension at 72 °C for the polymorphism at position 192. The 99 bp PCR product was digested with 8 units of AluI restriction endonuclease (New England Biolabs, Cambridge, MA, U.S.A.) overnight at 37 °C, and the digested products were separated by electrophoresis on a 3 % (w/v) agarose gel and visualized using ethidium bromide. The R genotype (arginine) contains a unique AluI restriction site which results in products of 66 and 33 bp, whereas the Q genotype (glutamine) will not be cut, allowing the PON1-192 genotype to be determined.

For the PON1-55 polymorphism, the PCR reaction and cycling were the same as described above, except that 30 cycles were carried out. The PCR product (170 bp) was digested with NalIII (New England Biolabs) in the presence of BSA (37 °C, overnight), and the digested products were separated and identified as above. The L genotype (leucine) does not contain an NalIII site, whereas the M genotypy (methionine) does contain an NalIII site, giving rise to products of 126 and 44 bp.

For the PON2-310 polymorphism (sense primer, 5’TTCCTCAGGGATCTCGCAGGTTGGG 3′, antisense primer, 5′ ACAGAATCTCTTGGAGAA-CAGACCCATTG 3′), the PCR reaction and cycling were the same as for the PON1-192 polymorphism. The PCR product (102 bp) was digested with DdeI (New England Biolabs) and the digested products were separated as above. The DdeI site is unique for the PON2-310 polymorphism, whereas the M genotype (methionine) does contain a DdeI site, whereas the S genotype (serine) does contain a DdeI site, giving rise to products of 68 and 36 bp.

In each case, the genotype was assigned by two people independently with no knowledge of the samples.

Statistical analysis
Statistically significant differences between parameters with a Gaussian distribution (age, body mass index, serum cholesterol, HDL cholesterol, apo B and apo A1) were tested by Student’s unpaired t test. Variables with a non-Gaussian distribution (serum triacylglycerols, PON1 activity, concentration and specific activity) were compared using the Mann–Whitney U test. The Chi-squared test was used to determine the significance of differences in allele frequency. Analysis of variance (ANOVA) was used to test for differences in parameters between genotypes. Probabilities of ≥ 0.05 were considered significant.

RESULTS
Demographic details of the study populations
The group with retinopathy were significantly older and had worse glycaemic control than the group with no
Serum PON1 activity
The median values for serum PON1 activity and specific activity in the group with retinopathy were significantly lower than in the group with no complications (both \( P < 0.001 \)) (Table 1); however, PON1 mass was not different between the groups. We could find no correlation between glycaemic control and PON1 activity [glycated haemoglobin (HbA\(_\text{c} \)) versus PON1 activity: + retinopathy, \( r = 0.163, P = 0.129 \); no complication, \( r = 0.065, P = 0.541 \)] or between PON1 activity and age (+ retinopathy, \( r = 0.075, P = 0.474 \); no complication, \( r = 0.0006, P = 0.995 \)) in either population.

PON1 and PON2 polymorphisms in the study populations
There were no significant differences between the population with retinopathy and that with no complication for
any of the polymorphisms studied (Table 2). Although in the population with retinopathy the gene frequencies of the PON1-192 Q, PON1-55 L and PON2-310 C genotypes were all increased, none of these differences reached statistical significance.

Effects of PON polymorphisms on serum PON1 activity

The effects of the PON1-55 and PON1-192 polymorphisms on serum PON1 activity were the same for both subjects with retinopathy and those with no complications as reported previously by our laboratory [37] and others for normal subjects and those with type II diabetes. Thus QQ homozygotes have the lowest activity and RR homozygotes the highest, with QR heterozygotes having intermediate activity (Figure 1a). MM homozygotes had significantly lower PON1 activity than LM heterozygotes, who in turn had lower activity than LL homozygotes (Figure 1b). However, the major difference between the groups was due to a highly
Effects of the PON1-192 (a) and PON1-55 (b) polymorphisms and the PON2-310 polymorphism (c) on glycaemic control in the study populations

The values presented are medians. Significant difference from QQ and QR genotypes: * \( P = 0.011 \); significant difference from LL and LM genotypes: ** \( P = 0.0032 \); significant difference from SS and CS genotypes: *** \( P = 0.010 \).

Figure 3 Effects of the PON1-192 (a) and PON1-55 (b) polymorphisms and the PON2-310 polymorphism (c) on glycaemic control in the study populations

The values presented are medians. Significant difference from QQ and QR genotypes: * \( P = 0.011 \); significant difference from LL and LM genotypes: ** \( P = 0.0032 \); significant difference from SS and CS genotypes: *** \( P = 0.010 \).

significant decrease in activity for the LL genotype in the group with retinopathy \( [126.4 \ (13.9-414.6) \ \text{nmol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1} \ \text{serum \ (median \ (range))}] \) compared with the group with no complications \( [229.2 \ (58.2-467.8) \ \text{nmol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1} \ \text{serum}] \ (P < 0.001) \). As we have reported previously [37], neither PON1 polymorphism affected PON1 mass, and PON1 specific activity showed the same pattern of distribution as activity (results not shown).

The presence of the PON2-310 polymorphism was also related to differences in serum PON1 activity. In both groups of study subjects, serum PON1 activity increased in the order SS < CS < CC \( (P < 0.001 \) for subjects with no complications; \( P = 0.003 \) for subjects with retinopathy) (Figure 2). It occurred to us that this finding could be due to an enhanced presence of RR individuals within the CC genotype. This was certainly true for the group with no complications, in which only two individuals had the CC genotype, both of whom were RR. However, this was not the case for the individuals in the group with retinopathy, where the PON1-192 gene frequency in the CC genotype was 0.72 Q and 0.28 R, no different from the population as a whole. The serum PON1 concentration was significantly higher for the CC genotype in the group with no complications \( (P = 0.034) \) compared with the SS and CS genotypes; however, there were no differences in PON1 concentration between genotypes in the group with retinopathy (Figure 2). PON1 specific activity was not different between the PON2-310 genotypes in the retinopathy group, but did differ in the group with no complications \( (P = 0.0073) \) (Figure 2).

**Effects of PON polymorphisms on glycaemic control and plasma lipoproteins**

In the group of subjects with type II diabetes and no complications, there was no apparent effect of any of the PON polymorphisms on glycaemic control (Figure 3). However, in the group with retinopathy, glycaemic control was significantly worse in the PON1-55 genotypes in the order MM > LM > LL \( (P = 0.0032) \) (Figure 3) (i.e., subjects with genotype MM show the worst control), significantly worse in the PON1-192 genotypes in the order RR > QR > QQ \( (P = 0.011) \) (Figure 3) and significantly worse in the PON2-310 genotypes in the order CC > CS > SS \( (P = 0.010) \) (Figure 3). There were no significant effects of the PON1 or PON2 polymorphisms on plasma lipids or lipoproteins (results not shown).

**DISCUSSION**

These results provide further evidence for low PON1 activity in subjects with type II diabetes, particularly when diabetes is associated with microvascular complications. We and others have shown previously that PON1 activity is lower in individuals with type II diabetes than in controls, and lower still in diabetic subjects with neuropathy and nephropathy [11,12]. In the present investigation, PON1 activity was lowest in association with the presence of retinopathy (Figure 4). We could find no significant differences in the pro-
PON1 is therefore a major component of HDL causing its anti-inflammatory properties. Several HDL components have been found in the retina [36], including PON1 (B. Mackness, unpublished work). The low PON1 activity that we have found associated with retinopathy may result in an increased amount of oxidized LDL in the retina, leading to an increased rate of development of retinopathy.

One of the more surprising findings of the present investigation is the correlation of the PON2-310 CC genotype with higher PON1 activity in both of the populations with type II diabetes. Such a relationship has not been found previously. The reasons for this association are unclear, but were not due to an over-representation of the high PON hydrolysing isof orm (R), and does not appear to be a function of increased PON1 mass. The protein product of the PON2 gene is presently unknown [28]; it is feasible that this product may be an activator/inhibitor of PON1 and that the polymorphism at position 310 may affect the interaction with PON1.

We also found a correlation between glycaemic control and all three polymorphisms studied, with the PON1-192 RR, PON1-55 MM and PON2-310 CC genotypes all having significantly worse glycaemic control in the group with retinopathy, but not in the one with no complications. To our knowledge this is the first time a correlation has been found between PON1 polymorphisms and glycaemic control in a diabetic population with microvascular disease. An association between a polymorphism of the PON2 gene at position 148 and glycaemic control has been shown previously in Canadian Inuit [29]; however, the mechanism behind this association is unknown. It is possible, however, that PON2 plays a role in glucose metabolism, perhaps at the cellular level.

In conclusion, low serum PON1 activity in subjects with retinopathy as a complication of type II diabetes may be related to an increased tendency for lipid peroxidation. Our findings also raise the possibility that, in retinopathy, the PON2 gene may influence PON1, and that an inter-relationship between the PON1 and PON2 genes may influence glycaemic control in subjects with type II diabetes complicated by retinopathy.
ACKNOWLEDGMENTS

This work was supported by the Medical Research Council (U.K.).

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Received 26 July 1999/27 October 1999; accepted 9 December 1999.