Effect of the human serum paraoxonase 55 and 192 genetic polymorphisms on the protection by high density lipoprotein against low density lipoprotein oxidative modification

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Abstract Human serum paraoxonase (PON1) associated with high density lipoprotein (HDL) has been postulated to have a role in protecting low density lipoprotein (LDL) against oxidative modification, which has led to the proposal that PON1 is an anti-atherogenic, anti-inflammatory enzyme. PON1 has two genetically determined polymorphic sites giving rise to amino-acid substitutions at positions 55 (L→M) and 192 (R→Q) and therefore 4 potential alloenzymes. We have examined the effects of these molecular polymorphisms on the ability of HDL to protect LDL from oxidative modification. HDL protected LDL from oxidative modification, whatever the combination of PON1 alloenzymes present in it. However, HDL from QQ/MM homozygotes was most effective at protecting LDL while HDL from RR/LL homozygotes was least effective. Thus after 6 h of co-incubation of HDL and LDL with Cu^{2+} PON1-QQ HDL retained 57 ± 6.3% of its original ability to protect LDL from oxidative modification, while PON1-QR HDL retained less at 25.1 ± 4.5% ($P < 0.01$) and PON1-RR HDL retained only 0.75 ± 0.40% ($P < 0.005$). In similar experiments HDL from LL and LM genotypes retained 21.8 ± 7.5% and 29.5 ± 6.6% (P = NS), respectively, of their protective ability, whereas PON1-MM HDL maintained 49.5 ± 5.3% ($P < 0.01$). PON1 polymorphisms may affect the ability of HDL to impede the development of atherosclerosis and to prevent inflammation.

Key words: Paraoxonase; Lipid peroxide; Low density lipoprotein; High density lipoprotein; Atherosclerosis

1. 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]. Therefore, mechanisms that prevent the oxidation of LDL could be anti-atherogenic. One such mechanism which has received increasing attention in recent years is the prevention of LDL oxidation by high density lipoprotein (HDL) [2]. We were the first to show that HDL decreased the accumulation of lipid peroxides on LDL by a mechanism that was at least partly enzymatic in nature [3]. We have subsequently shown that the enzyme paraoxonase (PON1), located on HDL, is largely responsible for HDL's ability to metabolise lipid peroxides on LDL [4,5]. These findings have subsequently been confirmed and extended by others [6,7].

Serum PON1 activity is decreased in coronary heart disease (CHD) [7,8] and in both insulin dependent and non-insulin dependent diabetes (IDDM and NIDDM), particularly when peripheral neuropathy is present [9]. These findings have led to the theory that PON1 may impede lipid peroxidation of LDL and perhaps nerve membranes, which are themselves related to atherogenesis and neuropathy.

PON1 activity towards paraoxon is genetically determined by two polymorphisms within the alleles acting at a single autosomal locus. The first of these pairs reported involved an amino acid substitution at position 192, giving rise to two alloenzymes with low activity (glutamine at position 192) and high activity (arginine at position 192) towards paraoxon [10,11]. This polymorphism has been found to be substrate specific; the alloenzyme activity is the opposite way round to that of paraoxon with certain substrates such as diazoxon [12]. There also exists a second polymorphism of the human PON1 gene affecting amino acid 55, giving rise to a leucine (L-allele) substitution for methionine (M-allele). This has also been found to modulate activity towards paraoxon [13,14].

Four recent case control studies have indicated the PON1–192 polymorphism is related to CHD [15–18] while three others have not shown this relationship [18–20]. In the positive case control studies it was the R-allele (with high activity towards paraoxon) which was associated with CHD. This led to the hypothesis that the R-alloenzyme was less able to prevent the accumulation of lipid peroxides on LDL, i.e. this activity of PON1 might resemble the differential activity towards diazoxon rather than paraoxon. One study has suggested that the PON1–55 L-allele is a risk factor for CHD in NIDDM [13]. However, nothing is known regarding the effect of the 55 polymorphism on the ability of PON1 to hydrolyse lipid peroxides.

In order to answer these questions we have determined the effect of the two PON1 polymorphisms on the ability of HDL to prevent the accumulation of lipid peroxides on LDL.

2. Methods

2.1. Subjects

Thirty-six healthy, unrelated volunteers (18 men) aged 22–60 years participated in the study which was approved by Central Manchester Area Health Authority Clinical Ethical Committee. Volunteers were either staff of the Manchester Royal Infirmary or attending for a routine health check at a local GP surgery or were staff of two local factories. All subjects were free of ischaemic heart disease, as defined by a lack of history of angina and no previous myocardial infarction. Subjects with peripheral vascular disease, diabetes mellitus, renal disease, hepatic disease and hyperlipidaemia or hypertension or receiving any medication were also excluded.

2.2. Blood sampling and DNA extraction

Venous blood was obtained from the subjects between 9 and 10 a.m. after a 12-h fast. Serum and EDTA plasma were obtained by low-speed centrifugation. Serum was stored at −20°C before further analysis. DNA was extracted from white blood cells left at the inter-

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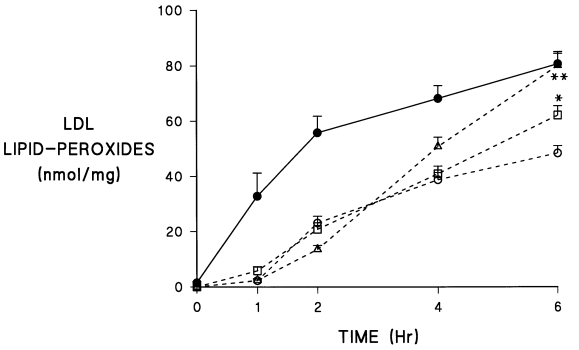

Fig. 1. Effect of the PON-192 polymorphism on the ability of HDL to protect LDL against oxidative modification. LDL and HDL were isolated by ultracentrifugation. 1.5 mg of LDL and 1.5 mg of HDL protein were co-incubated in 1 ml of PBS at 37°C. Oxidation was initiated by the addition of 5 μM $CuSO_4$. Sub-samples were withdrawn after 0.5, 1, 2, 4 and 6 h of incubation and LDL lipid peroxides determined as described [3]. Data are mean ± S.D. * Significantly different from QQ-HDL, $P < 0.01$. ** Significantly different from QQ-HDL, $P < 0.005$. ●—●, LDL alone; ○- - -○, LDL+QQ HDL; □- - -□, LDL+QR HDL; △- - -△, LDL+RR HDL.

face of the plasma after centrifugation using a Split-second kit (Boehringer-Mannheim).

2.3. Analysis of paraoxonase activity

PON1 activity was measured by adding serum to 1 ml Tris-HCl buffer (100 mmol/l, pH 8.0) containing 2 mmol/l $CaCl_2$ and 5.5 mmol/l paraoxon (0,0-diethyl-0-*p*-nitrophenylphosphate; Sigma Chemical Co.). The rate of generation of *p*-nitrophenol was determined at 405 nm, 25°C, with the use of a continuously recording spectrophotometer (Beckman Du-68).

2.4. Paraoxonase genotype determination

PON1 genotypes were determined following PCR according to previously published protocols [10,11].

Genotype was assigned by two people independently with no knowledge of the source of the samples.

2.5. Effect of paraoxonase genotype on ability of HDL to prevent LDL lipid peroxidation

LDL (d 1.019–1.063 g/ml) was prepared by sequential ultracentrifugation [21]. HDL (d 1.063–1.21 g/ml) was prepared by sequential ultracentrifugation in the presence of 5 mM $CaCl_2$. Under these conditions 70–80% of PON1 remains associated with HDL and no differences in the loss of different alloenzymes from HDL are found [22]. LDL (1.5 mg) or autologous HDL (1.5 mg) or LDL+HDL (1.5 mg each) were incubated in a total volume of 1 ml of EDTA-free PBS at 37°C. Oxidation was initiated by the addition of 5 μM $CuSO_4$ and the generation of lipid peroxides in LDL determined using cholesterol-iodide reagent, at various time intervals up to 6 h, as described previously [3–5].

2.6. Statistical analysis

Statistical analysis was conducted using the SPSS statistical package for Windows 95. Differences in parameters between genotype was sought by Student's *t*-test for variables with a gaussian distribution and by the Mann-Whitney U-test for variables with a non-gaussian distribution.

3. Results

In the 36 study subjects the distribution of the PON1 genotypes was 39% QQ, 47% QR and 14% RR for the PON-192 genotype (gene frequency = 0.62 A; 0.38 B) and 33% LL, 47% LM and 20% MM for the PON-55 genotype (gene frequency = 0.57 L; 0.43 M. The effect of the two PON1 polymorphisms on the serum hydrolysis of paraoxon in the 36 subjects in this study was similar to our previous findings in

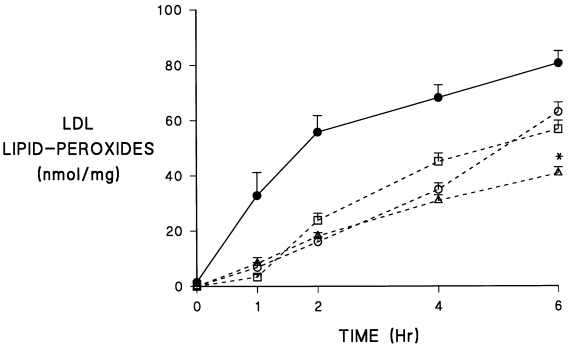

Fig. 2. Effect of the PON-55 polymorphism on the ability of HDL to protect LDL against oxidative modification. LDL and HDL were isolated and incubated as described in the legend to Fig. 1. Data are mean ± S.D. * Significantly different from LL and LM HDL, $P < 0.01$. ●—●, LDL alone; ○- - -○, LDL+LL HDL; □- - -□, LDL+LM HDL; △- - -△, LDL+MM HDL.

a much larger healthy population of 279 individuals [14] (result not shown), indicating the study population to be representative of the general population.

The ability of HDL to protect LDL against oxidative modification was similar regardless of the PON1 alloenzymes it contained for at least the first 2 h of incubation (Figs. 1 and 2). In the case of the PON1–192 polymorphism, HDL from RR homozygotes appeared less effective in decreasing LDL lipid peroxide accumulation at around 4 h (Fig. 1). After 6 h, significant differences between HDL from the different PON1–192 genotypes were apparent. They were expressed as the proportionate decrease in LDL lipid peroxides when HDL was present as opposed to when it was absent. HDL from PON1 homozygotes for the QQ alloenzyme retained 57.0 ± 10.4% (mean ± S.D.) of its ability to prevent LDL lipid peroxide generation. However, HDL heterozygous for the Q and R alloenzymes retained less protective ability at 25.0 ± 9.5% ($P < 0.01$ compared to QQ-HDL) and HDL from homozygotes for the PON-RR alloenzyme retained only 0.75 ± 0.1% of its original ability to protect LDL against oxidative modification ($P < 0.005$ compared to AA-HDL) (Fig. 3a).

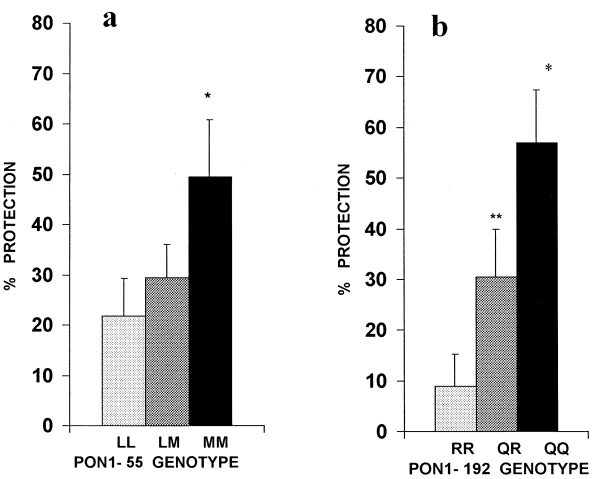

Fig. 3. Effect of the PON-55 (a) and 192 (b) polymorphisms on the ability of HDL to protect LDL against oxidative modification. LDL and HDL were isolated and incubated as described in the legend to Fig. 1. Bars represent the mean value with standard error bars.

The PON-55 polymorphism also affected the ability of HDL to protect LDL against oxidative modification (Fig. 2). There were no significant differences between the genotypes at 1, 2 and 4 h. However, at 6 h a significant difference between HDL from the MM homozygotes and LM and LL genotypes was evident (Fig. 3b). Thus after 6 h of incubation HDL containing LL and LM alloenzymes retained $21.8 \pm 7.5\%$ and $29.5 \pm 6.6\%$ of their ability to protect LDL against oxidation, respectively (P = NS). Whilst HDL containing MM alloenzymes maintained $49.5 \pm 11.4\%$ of its ability to protect LDL against oxidation ($P < 0.01$ compared to both LM and LL HDL).

4. Discussion

PON1 is important in the detoxification of both OP insecticides and nerve gases [10–12] and pro-atherogenic phospholipid hydroperoxides generated during the oxidative modification of LDL [4–7]. The results presented here indicate a critical role for the amino acid substitutions at both positions −55 and −192 in determining the activity of PON1 towards lipid peroxides. We have previously shown that individuals homozygous for the AA/MM genotypes have greatly reduced ability to detoxify paraoxon [14]. The present series of normal volunteers were typical in this respect.

The inverse relationship between plasma HDL concentration and CHD has been known from epidemiological studies for many years [23]. The mechanism by which HDL might confer protection from CHD has been the subject of much debate, but has generally been considered to be due to the central role of HDL in reverse-cholesterol transport. More recently a second mechanism has been proposed; the protection of LDL against oxidative modification by HDL [2]. Our initial observation that PON1 isolated from HDL was extremely potent in protecting LDL against oxidation [4,5], has subsequently been confirmed and extended by others [6,7]. More recently it has been reported that immunostaining for PON1 in the human artery wall increases as atheroma progresses, possibly in response to the increasingly pro-oxidant environment [24]. PON1 is present in human tissue fluid [25] and could potentially therefore protect LDL even after it has crossed the vascular endothelium and perhaps cell membranes from oxidation.

In the present investigation we have shown that the polymorphisms of PON1 due to amino acid substitutions at positions 55 and 192 can greatly modify the efficacy of HDL in protecting LDL against lipid peroxidation. With regard to the 192 polymorphism PON1 QQ-HDL was most efficient at protecting LDL against oxidative modification and PON1 RR-HDL least efficient. This may explain why in some case control studies the PON1 R-allele is associated with CHD [15–18]. LDL is believed to be sequestered in the artery wall where it becomes oxidised and pro-atherogenic [1]. A protective mechanism retaining its effectiveness over several hours might therefore be crucially important in determining the rate of atherogenesis. HDL containing the PON1-Q alloenzyme in the present study retained 6 times more protective effect over 6 h than the R-alloenzyme. The −55 polymorphism also affected the efficiency with which HDL protected LDL against lipid peroxidation. PON1 MM-HDL retained twice as much ability to protect LDL after 6 h as did HDL from either LM heterozygotes or LL homozygotes. HDL from those individuals who are homozygous for both the Q and M polymorphisms is the most efficient at protecting LDL, and these individuals should according to our hypothesis be least susceptible to developing CHD. HDL from individuals homozygous for the R and L polymorphisms was least protective and these individuals should be more susceptible to CHD development, as suggested by the only case control study of this polymorphism thus far reported [13].

The reasons why the polymorphisms have such a dramatic effect on substrate specificity are unknown. However, with regard to lipid peroxides, the effect on the polymorphism resembles the hydrolysis of diazoxon rather than of paraoxon. Whilst the results presented here suggest that the PON1 alloenzyme located on HDL is a major determinant of its ability to protect LDL against oxidation, other HDL-associated enzymes, such as platelet-activating factor acetylhydrolase and lecithin/cholesterol acyltransferase, have been shown to metabolise LDL phospholipid hydroperoxides [26,27], and it remains possible that PON1 exerts its effect in protecting LDL against oxidative modification in conjunction with other enzymes present in HDL.

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