

# Decreased stability of the M54 isoform of paraoxonase as a contributory factor to variations in human serum paraoxonase concentrations

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**Abstract** There are considerable variations in serum concentrations of the high density lipoprotein (HDL)-associated enzyme, paraoxonase (PON), which is an important determinant of the antioxidant capacity of HDL. The present study examined the hypothesis that differences in the stability of isoforms arising from the coding region L54M polymorphism could contribute to such variations. A model system was developed using transfected Chinese hamster ovary cells to secrete recombinant PON corresponding to human L or M isoforms. The recombinant peptides exhibited the molecular properties of human serum PON. They formed complexes with lipoproteins in culture medium, notably binding to apolipoprotein A-I-containing particles. The enzymatic properties of the recombinant isoforms were comparable to those of human serum PON. The recombinant M isoform lost activity more rapidly and to a greater extent than the recombinant L isoform [ $26.0 \pm 3.0\%$  vs.  $14.0 \pm 1.0\%$  (phenylacetate substrate) and  $36.1 \pm 2.0\%$  vs.  $19.3 \pm 2.0\%$  (paraoxon substrate) over 96 h ( $P < 0.01$ )] in medium containing fetal calf serum or PON-free human serum. Addition of a protease inhibitor resulted in retention of activity by both isoforms. Parallel results were obtained in incubation studies of human serum from donors homozygous LL or MM for the L54M polymorphism. Enzyme activity was lost more rapidly and to a greater extent from MM than LL sera ( $P < 0.01$ ). A parallel loss of PON peptide mass was also observed, with a significantly greater loss from MM homozygotes ( $P < 0.001$ ). It corresponded to the appearance of a smaller molecular mass band on SDS-PAGE analysis. Direct analysis of the proteolytic effect using HDL isolated from homozygotes and incubated with purified kallikrein confirmed the greater loss of activity from MM homozygotes and the protective effect of proteolysis inhibitor. The results provide evidence for lesser stability of the M54 isoform of PON, apparently involving greater susceptibility to proteolysis. It provides one mechanism to explain variations in serum levels of PON and has implications for the antioxidant capacity of HDL.—Leviev, I., S. Deakin, and R. W. James. Decreased stability of the M54 isoform of paraoxonase as a contributory factor to variations in human serum paraoxonase concentrations. *J. Lipid Res.* 2001. 42: 528–535.

**Supplementary key words** oxidative stress • HDL • polymorphism • coronary disease • recombinant protein

Paraoxonase (PON) is a serum enzyme entirely complexed to high density lipoprotein (HDL) (1). Clinical interest in the enzyme derived initially from its ability to neutralize highly toxic exogenous derivatives, notably pesticides (2), thus highlighting its potential importance as a protective agent against environmental poisoning. Such derivatives also include nerve gases (2, 3), further underlining the potential protective benefits of serum PON activity. Recent clinical interest in the enzyme has arisen from the hypothesis that PON protects low density lipoproteins (LDL) from lipid peroxidation (4, 5). Peroxidation can induce substantial changes to the behavior of LDL, modifying its metabolism and, equally important, its ability to influence gene expression (6, 7). The latter is suggested to be the basis for the inflammatory effect of oxidized LDL (6, 8) and, more generally, the strong association between LDL and risk of vascular disease. The activity of PON thus confers an anti-inflammatory/antioxidant function on HDL (5, 9). Consequently, factors that diminish the PON content of HDL will affect this protective role. Animal models provide support for this contention, in which lower serum levels of PON are associated with greater susceptibility of LDL to oxidation and increased risk of atheroma (10, 11). Thus, factors that influence serum PON are of particular clinical relevance.

There are considerable interindividual variations in serum concentrations of PON (12, 13). In our attempts to identify factors involved, we have focused both on its serum transport vehicle, HDL (1, 12, 14), and genetic influences. Our earlier studies demonstrated that a coding region polymorphism affecting amino acid 54 (L54M) was associated with highly significant variations in serum con-

Abbreviations: apo, apolipoprotein; CHO, Chinese hamster ovary; HDL, high density lipoprotein; LDL, low density lipoprotein; PON, paraoxonase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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centrations of PON. A second common polymorphism at amino acid position 191 (Q191R) was not associated with concentration (13). Subsequent studies showed differences in the levels of expression of L- and M-type mRNA by human liver biopsies, consistent with observed differences in serum PON concentrations between the isoforms (15). This clearly indicated a genetic basis to variations in serum PON levels. Most recently, we identified polymorphisms in the promoter region of the *PON1* gene, which appear to have a predominant effect on serum concentrations of the enzyme (16). There is also strong linkage disequilibrium between the promoter and coding region L54M polymorphisms. However, our analyses indicated that the promoter polymorphisms do not entirely account for the association between the L54M polymorphism and variations in serum PON concentrations. The present study was undertaken to attempt to explain this observation. We examined the hypothesis that differences in the stability of L- and M-type PON isoforms could contribute to variations in serum concentrations and activities of the enzyme.

## METHODS

### Analysis of PON gene polymorphisms, activities, and concentrations

Serum samples analyzed in the present study were obtained from subjects recruited at the university hospital and medical research center in Geneva. Participants gave informed written consent and the study was conducted according to the ethical requirements of the medical faculty. DNA was extracted from blood lymphocytes and the promoter and coding region polymorphisms genotyped as described (13, 16, 17). PON was quantified by a competitive enzyme-linked immunosorbent assay (ELISA), as described previously in detail (12), and enzyme activities were determined with paraoxon (PON activity) and phenylacetate (arylesterase activity) (12).

Single- and two-dimensional polyacrylamide gel electrophoresis (PAGE) of culture medium conditioned by Chinese hamster ovary (CHO) cells secreting human PON were performed as described previously (1, 18). Profiles were electrotransferred to nitrocellulose sheets and processed by immunoblotting with anti-PON antibody (1).

### Expression of PON isoforms

PON cDNA was obtained by reverse transcription of RNA from human liver (heterozygous LM for the 54 polymorphism; homozygous AA for the 191 polymorphism), using the oligonucleotide primer (antisense) CCTCGGAATATGGCAAGCGG complementary to nucleotides from 1116 to 1135 of human PON mRNA. The cDNA was amplified by polymerase chain reaction (PCR), using oligonucleotide primers CAAAAGCTTATGGCGAAGCTGATTGCGC TCACCC (sense) complementary to positions from 1 to 25 of *PON1* mRNA and ATGGCATGGGTGCAAATCCG (antisense) complementary to positions from 1073 to 1092. The design of primers was based on the human *PON1* cDNA sequence obtained by Hassett et al. (19); numbers are from A of the initiation codon. Thermally stable *Pfu* polymerase (Stratagene) was used for amplification. PCR products were cloned into a plasmid containing an expression cassette with the SV-40 promoter. The entire cDNA was subsequently sequenced to ensure *i*) the presence of the desired nucleotides at the positions coding for amino acids 191 and 54, and *ii*) the absence of other alterations in cDNA.

CHO cells were grown in Iscove's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco). (The PON activity of fetal calf serum was negligible.) Cells were transfected with 5  $\mu$ g of plasmid DNA containing either the L- or M-type alleles and 15  $\mu$ l of Superfect reagent (Qiagen). Conditioned medium was removed 24 h after transfection for measurement of activities and used nonconcentrated in studies.

### Isolation of lipoproteins

Lipoproteins were isolated from fasting human serum samples by sequential gradient ultracentrifugation (20) (LDL,  $d = 1.019$ – $1.063$  g/ml; HDL,  $d = 1.063$ – $1.21$  g/ml). Total lipoprotein was isolated by ultracentrifugation at  $d = 1.23$  g/ml.

Immunoaffinity depletion of human apolipoprotein (apo) A-I from culture medium was performed as described previously (18). Briefly, culture medium containing 10% PON-free human serum [PON was removed by immunoaffinity chromatography (1)] was conditioned by PON-transfected CHO cells. An aliquot was passed onto an anti-apoA-I column, incubated 1 h, and washed to remove unbound protein. The nonbound eluant was collected and concentrated.

### Electrophoresis

Human serum and culture medium containing isolated human HDL were subjected to agarose gel electrophoresis using the Sebia hydragel system (Sebia; Issy Les-Moulineaux, France). They were also analyzed by single-dimensional SDS-PAGE under nonreducing conditions and immunoprobed with anti-apoA-I antibodies after electrotransfer to nitrocellulose sheets (1).

### PON stability

To test PON stability, culture medium was conditioned by transiently transfected cells, filtered under sterile conditions, then incubated at 37°C. Studies were performed with conditioned media containing the same PON enzyme activity levels per milliliter of medium for the L and M isoforms. Aliquots were removed at intervals under sterile conditions to test PON. In some studies, fetal calf serum of the culture medium was replaced with 10% human serum from which endogenous PON had been removed as described above. In other studies, the protease inhibitor aprotinin was added to the conditioned medium (final concentration 28  $\mu$ g/ml) prior to incubation. In studies where the protease kallikrein (Sigma) was used, an aliquot was removed prior to addition of kallikrein to establish the baseline PON activity/concentration. Protease activity in aliquots removed after timed intervals was inhibited with aprotinin (10-fold excess). In control incubations, aprotinin was added to the HDL samples prior to adding kallikrein.

## RESULTS

**Table 1** shows serum concentrations and activities of PON in a population that was selected to be homozygous for polymorphisms of the promoter region of the *PON1* gene (haplotype CGT-CGT, reflecting nucleotides at the -907, -824, -107 polymorphic sites) (16). There were significant differences in concentrations between subgroups defined by genotypes of the L54M polymorphism. Carriers homozygous for the L allele had significantly higher concentrations of PON than MM homozygotes; heterozygotes had intermediate values. This was paralleled by similar differences in enzyme activity using phenylacetate or paraoxon as substrates. To eliminate the possible confounding effects

TABLE 1. Serum concentrations and activities of PON as a function of the L54M coding region polymorphism

	L54M Genotype			ANOVA
	LL	LM	MM	
n	47	140	98	
Concentration ( $\mu\text{g/ml}$ )	$93.1 \pm 21.7$	$82.3 \pm 20.9$	$74.9 \pm 14.0$	<0.0001
Arylesterase (U/ml)	$76.8 \pm 18.4$	$67.4 \pm 19.9$	$63.2 \pm 15.8$	0.0002
Paraoxonase (U/ml)	$324.8 \pm 129.9$	$219.2 \pm 110.5$	$91.0 \pm 30.2$	0.0001
Paraoxonase (U/ml) (QQ homozygotes)	$152.5 \pm 76.9$	$115.6 \pm 61.8$	$91.0 \pm 30.2$	0.0001
Specific activity	$0.84 \pm 0.17$	$0.84 \pm 0.26$	$0.86 \pm 0.21$	ns

Values are means  $\pm$  SD. Subjects were homozygous for promoter CGT-CGT polymorphisms. For paraoxonase activity with QQ homozygotes, LL: n = 9; LM: n = 53; MM: n = 98. Specific activity was determined with arylesterase. ANOVA, analysis of variance; ns, not significant.

of the Q191R polymorphism on paraoxon hydrolytic activity, the latter was also analyzed in subjects homozygous QQ for the 191 site. As shown in Table 1, differences in activity between L54M genotypes remained highly significant. Specific activity did not differ between the genotypes (Table 1), indicating that the effect concerns protein mass rather than the enzymatic site of PON.

In a first series of studies, we analyzed L and M isoforms that had been produced by transiently transfected CHO cells. Examples of immunoblots of single- and two-dimensional gel profiles of PON (L at position 54 and Q at the 191 polymorphic site) secreted by transfected cells are shown in Fig. 1. For comparison, profiles of human serum PON are also given. The two profiles were very similar. In single-dimensional SDS-PAGE gels, the recombinant pro-

tein (Fig. 1A, lane 2) showed the presence of major and minor bands corresponding to human serum PON (Fig. 1A, lane 1), as we previously reported (1). The molecular size of recombinant PON (43–46 kDa) corresponds to that of human serum PON. The two-dimensional profiles (Fig. 1A, images 3, 4) were again quite comparable and typical of glycosylated proteins. Analyses of the relative enzyme activities with paraoxon and phenylacetate [activity (U/ml) with paraoxon in 1 M NaCl]/[activity (U/ml) with phenylacetate] were  $2.94 \pm 0.11$  and  $2.98 \pm 0.07$  (n = 3 for both) in two different preparations of conditioned medium. This mirrors the relative ratios of activities measured in human serum samples ( $2.85 \pm 1.19$ ; n = 462) and to literature values for PON isoforms homozygous for the A allele at position 191 (21). Fast protein liquid chro-

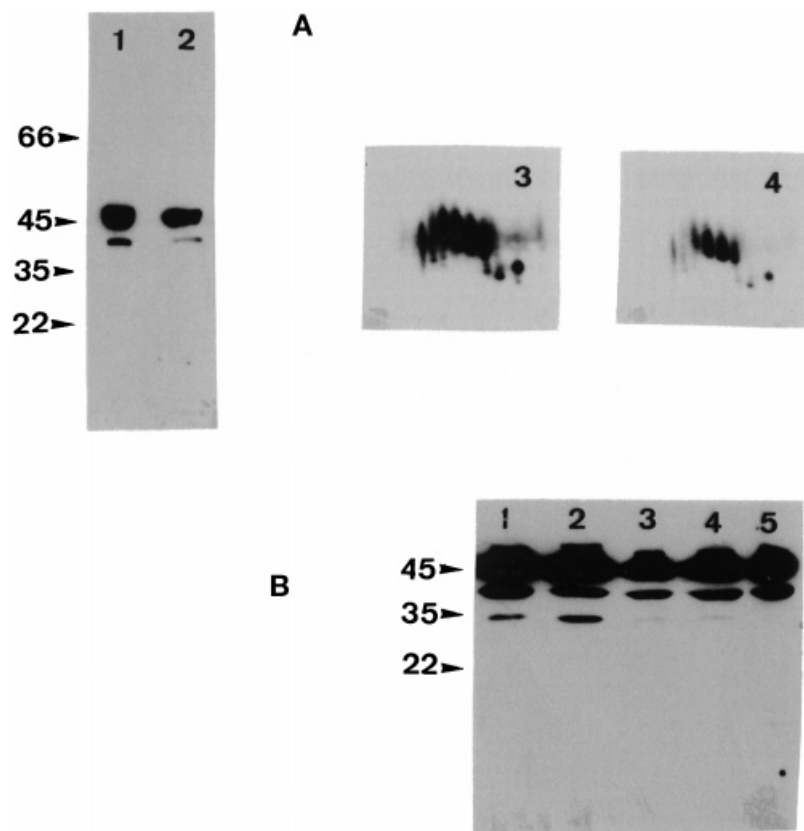
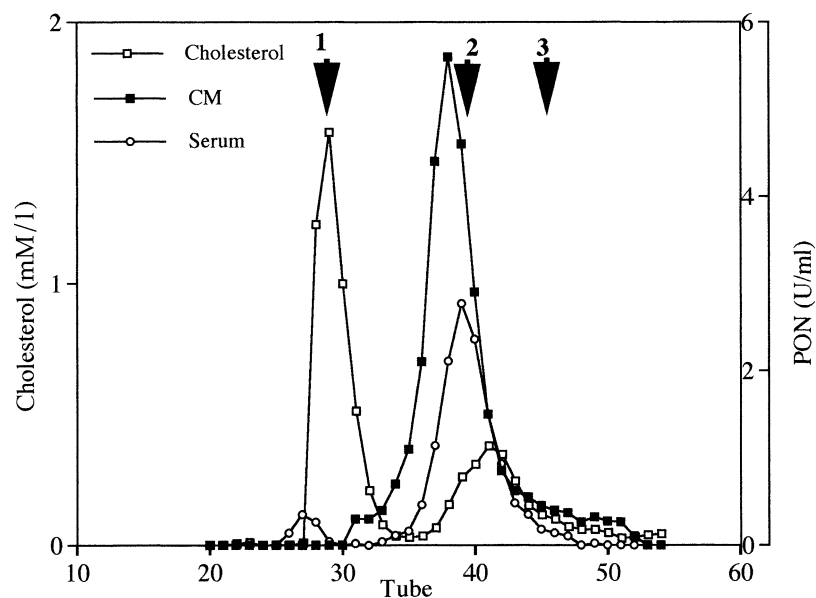


Fig. 1. A: Immunoblots of single-dimension SDS-PAGE (lanes 1, 2) and two-dimensional PAGE (lanes 3, 4) profiles of human serum (lanes 1, 3) and recombinant (lanes 2, 4) PON. The arrows are molecular mass markers of, respectively from bottom to top, 22, 35, 45, and 66 kDa. B: Immunoblots of single-dimension SDS-PAGE of sera from AAMM (lanes 1, 2) and AALL (lanes 3, 4) subjects after incubation for 3 days at 37°C. Lane 5 is fresh serum. The arrows are molecular mass markers of, respectively, from bottom to top, 22, 35, and 45 kDa. Immunoblots were developed with monoclonal anti-PON antibody (1).



**Fig. 2.** Fast protein liquid chromatographic analysis of human serum (0.5 ml) and culture medium (0.5 ml) conditioned by PON-secreting CHO cells. The serum eluants were analyzed for total cholesterol and PON activity. The culture medium eluants were analyzed for PON activity. Western blot analysis confirmed that PON protein was present only in tubes showing enzyme activity. The arrows show the peak elution tubes for 1: VLDL/LDL, 2: HDL, 3: albumin. CM, culture medium PON activity; serum, serum PON activity.

matographic (FPLC) analysis (1) revealed that PON in conditioned medium had the molecular size of human HDL. As shown in **Fig. 2**, PON in conditioned culture medium co-eluted with serum PON, and both were in the higher molecular mass range of the HDL peak. No PON protein could be detected by Western blot in tubes not containing enzyme activity. Culture medium was also prepared containing human serum from which the PON-containing fraction had been removed by specific immunoaffinity chromatography. This procedure (1) removes less than 10% of HDL apoA-I. The preparation was used to confirm the association of PON with HDL after incubation with PON-secreting cells by two means. Ultracentrifugation of the culture medium showed the majority of recombinant PON activity (approximately 85%) to be associated with the  $d < 1.23$  g/ml fraction for both isoforms. This compares with 91% of human serum PON associated with the  $d < 1.23$  g/ml fraction (**Table 2**). Second, when the culture medium was passed through an anti-human apoA-I affinity column, the majority of PON activity was removed for both isoforms (**Table 2**). In a previous study, we observed that human apoA-I immuno-

absorption removed 89% of serum PON (1). There were no significant differences between the L and M isoforms with respect to the percentages associated with the lipoprotein fractions defined by ultracentrifugation or immunoaffinity absorption.

Medium containing fetal calf serum conditioned either with the L or M type isoforms of PON was incubated at 37°C, and PON enzymatic activity followed. The M isoform lost activity more rapidly than the L isoform. Over a 96-h period, the M isoform lost a significantly greater percentage of enzyme activity than the L isoform when measured as arylesterase activity (**Fig. 3A and B**;  $P < 0.01$ ,  $n = 6$ ). A similar result was obtained with paraoxon as substrate ( $P < 0.01$ ,  $n = 6$ ) (**Fig. 3B**). To analyze the effect of human serum, the study was repeated with culture medium containing 10% PON-free human serum. The M isoform again lost activity more rapidly and to a greater extent than the L isoform (**Fig. 3C**). One potential explanation for the observations is loss of activity due to proteolysis. To examine this possibility, complementary studies analyzed loss of activity during incubation with and without the proteolysis inhibitor, aprotinin. Under these conditions (**Fig. 3B and C**), both isoforms retained >90% of initial activities over a 96-h incubation period. Similarly, when samples were frozen for the duration of the incubation period in the absence of protease inhibitor, no loss of activity was evident (results not shown).

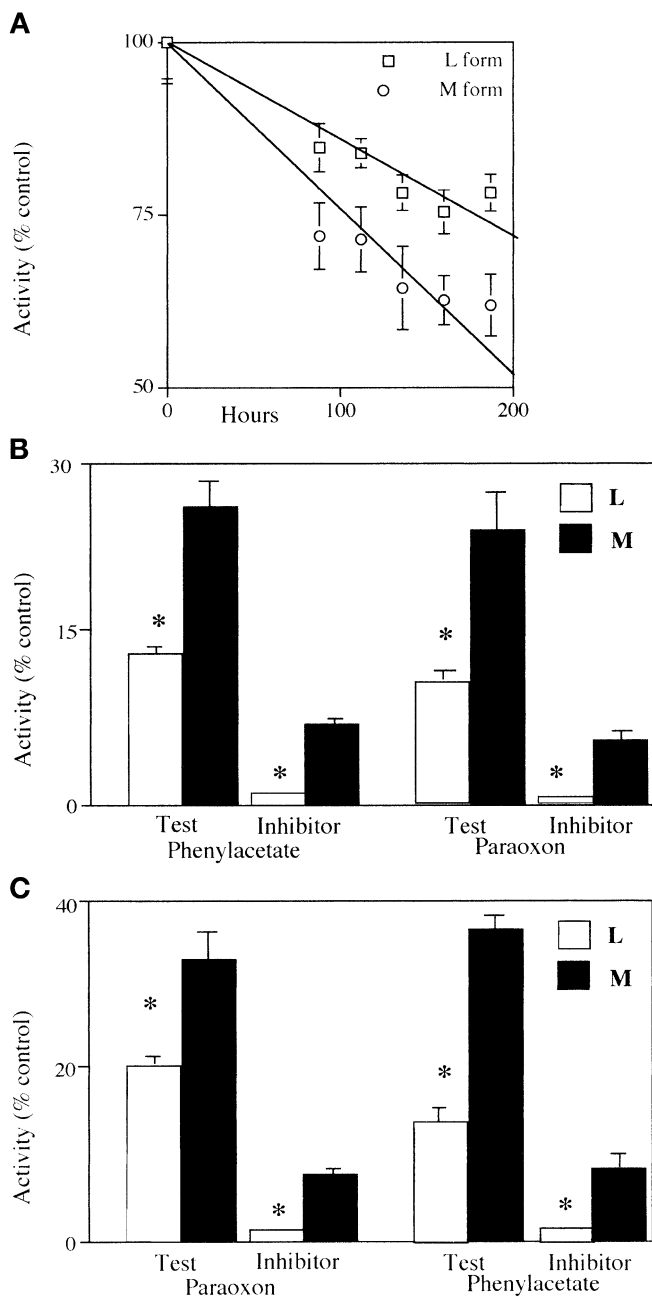
The relevance of the observations to serum PON was examined in a third series of studies. Serum samples were obtained from subjects who were homozygous LL or MM at the L54M polymorphic site and homozygous QQ at the Q191R polymorphic site. **Figure 4** shows enzyme activities and concentrations of PON after incubation of the serum samples at 37°C. There was a gradual decrease in PON (**Fig. 4A**) and arylesterase (**Fig. 4B**) enzyme activities. However, the decrease was more pronounced in the sera from the MM homozygotes who lost PON significantly more rapidly than the LL samples ( $P < 0.01$ ). Serum concentra-

**TABLE 2.** Association of recombinant paraoxonase with the lipoprotein fraction

Sample	Activity (%)
Present in $d < 1.23$ g/ml	
Culture medium: isoform L	84.6 ± 2.2
Culture medium: isoform M	82.3 ± 3.5
Human serum	91.0 ± 3.3
Removed by anti apo AI column	
Culture medium: isoform L	85.2 ± 3.5
Culture medium: isoform M	83.3 ± 3.8

The values are the means ± SD of triplicate analyses and represented the percentage of arylesterase activity found in the  $d < 1.23$  g/ml fraction after centrifugation or retained by the anti-apoA-I column. Densities were adjusted by adding sodium bromide to conditioned medium (isoforms L and M,  $n = 3$ ) or to human serum ( $n = 10$ ).





**Fig. 3.** Enzyme activities of recombinant forms of PON. A: Activity toward phenylacetate in conditioned medium incubated at 37°C. Results ( $n = 4$ ) are expressed as the percentage of activity at time 0 (when the medium was removed from the cells). B: Percentage loss of phenylacetate and paraoxon hydrolytic activities after incubation (96 h, 37°C) of conditioned medium ( $n = 6$ ). C: Percentage loss of paraoxon and phenylacetate hydrolytic activities after incubation (96 h, 37°C) of conditioned medium ( $n = 6$ ) containing 10% human serum. \*  $P < 0.01$  for L versus M under same incubation conditions. Test, no inhibitor; Inhibitor, containing aprotinin.

tions of PON were also analyzed in this study (Fig. 4C). These confirmed a decrease in measurable PON protein mass, complimenting the observations on enzyme activities. To determine whether proteolytic fragments were detectable, the samples were also analyzed by SDS-PAGE (Fig. 1B). This revealed the presence of a molecular mass band of approximately 35 kDa, which was distinct from

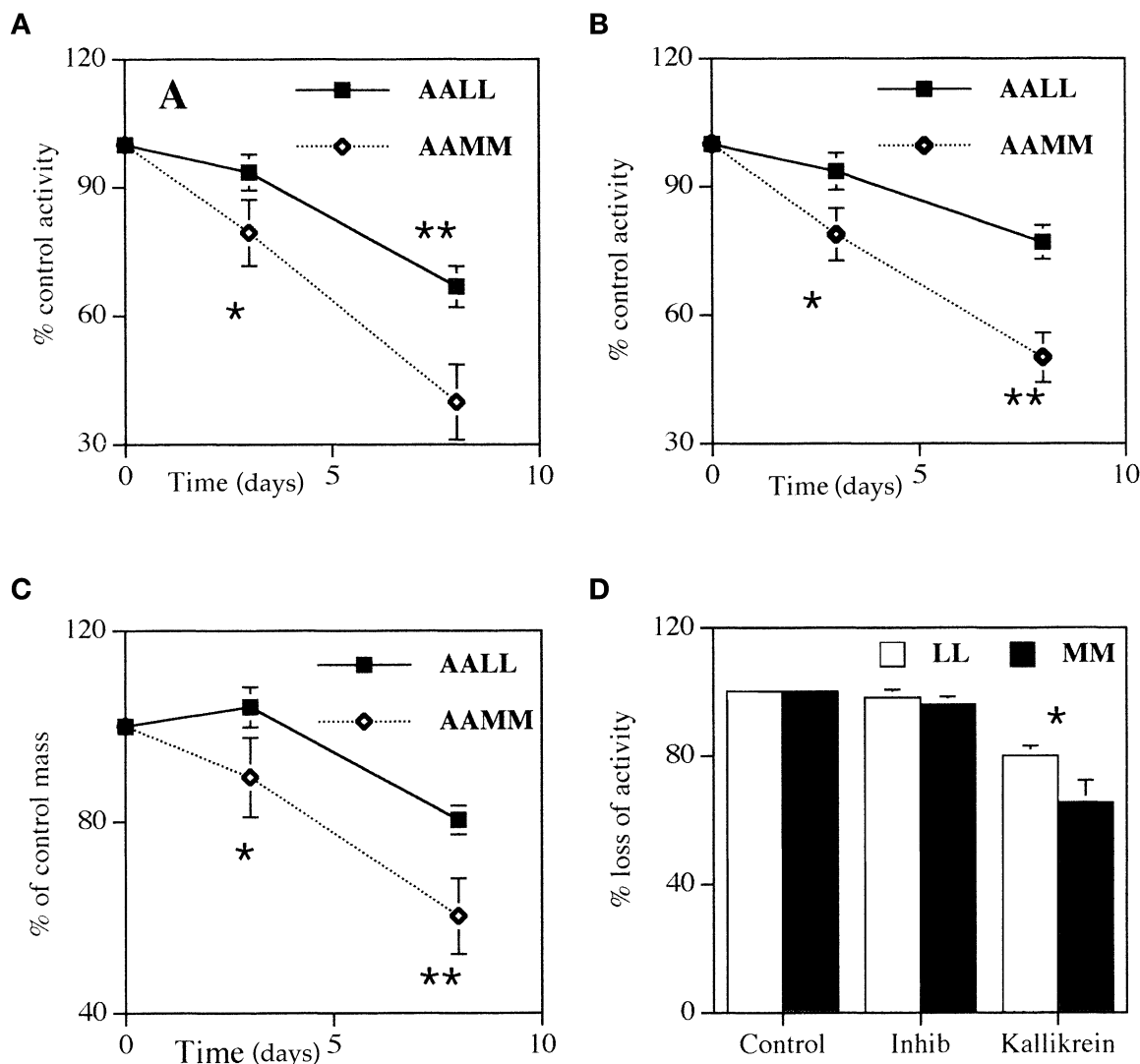
the 42–46 kDa (Fig. 1B, lane 5) of intact PON. The 35-kDa band was more evident in the samples from M allele carriers (Fig. 1B, lanes 1, 2) than L allele carriers (Fig. 1B, lanes 3, 4). No bands of smaller molecular mass could be detected (Fig. 1B).

The effects of the incubation conditions on HDL were also examined by agarose gel electrophoresis and SDS-PAGE under nonreducing conditions. **Figure 5A** shows agarose gel profiles of serum (lanes 1, 2) and culture medium containing human HDL (lanes 3, 4). Compared to serum maintained at 4°C (lane 1), serum incubated at 37°C (lane 2) showed negligible changes in HDL electrophoretic mobility, although there was an increase in migration of LDL. Incubation of HDL at 37°C in culture medium (lane 4) did not modify its electrophoretic mobility compared with control HDL (lane 3). These results concur with those of SDS-PAGE analyses of apoA-I (Fig. 5B). Immunoblotting showed the predominant band to be monomeric apoA-I in culture medium maintained at 4°C (lane 1) or 37°C (lane 2), or serum at 4°C (lane 3) or 37°C (lane 4). There was no evidence for extensive cross-linking to form higher molecular mass complexes. These results suggest no extensive modifications to HDL lipid or protein components during the incubation periods at 37°C.

To examine more directly the influence of proteolysis on PON activity, HDL was isolated from MM and LL sera and incubated at 37°C together with the protease kallikrein, with and without the inhibitor, aprotinin. The results (Fig. 4D) paralleled those obtained with conditioned medium and whole serum. The M isoform lost activity to a greater extent than the L isoform ( $P < 0.01$ ) in the presence of kallikrein, whereas inhibition of the protease prevented loss of activity from both samples.

## DISCUSSION

Data from *in vitro* studies and animal models provide firm support for the contention that variations in the PON content of HDL influence the ability of the lipoprotein to protect LDL from oxidative modifications. There is considerable within-population variation in serum PON levels. Our recent studies have demonstrated that there is undoubtedly a strong genetic component to these variations (16), but it cannot entirely explain the interindividual differences. Nongenetic factors appear to play a role. A significant positive correlation between serum HDL and PON concentrations (12) suggests an influence of the lipoprotein on PON levels, confirmed by our recent study showing substantial decreases in PON activities and concentrations in patients with HDL deficiencies (14). The present study focused on isoforms arising from the coding region polymorphism L54M. In initial studies, we linked the polymorphism to variations in serum concentrations of the enzyme (13); our more recent observations suggested that it was due to linkage disequilibrium with promoter polymorphisms (16). In subsequent analyses, however, we found that the L54M polymorphism remained significantly associated with variations in serum PON independently of



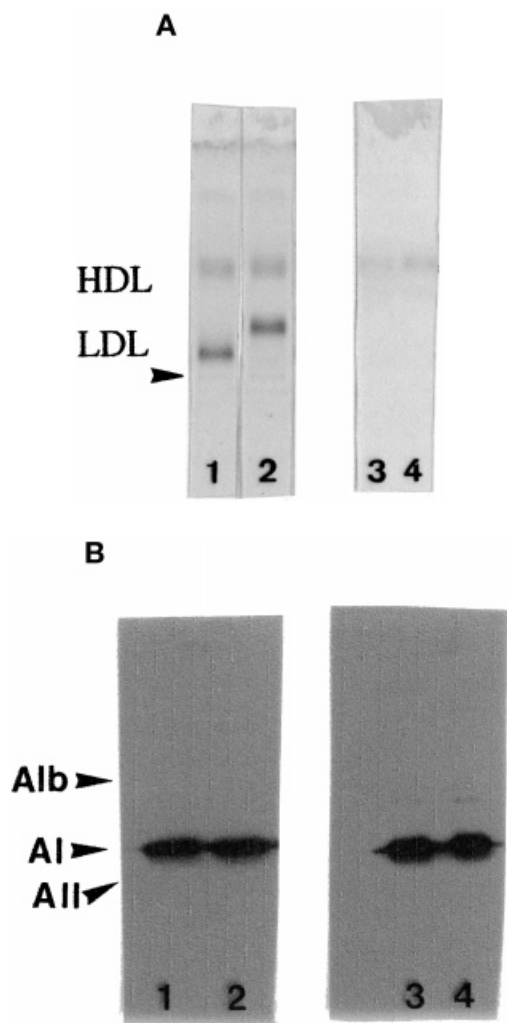
**Fig. 4.** PON enzyme activity with paraoxon (A), phenylacetate (B), and PON mass concentration (C) in sera from LL and MM homozygotes incubated under at 37°C. Results ( $n = 3$  subjects) are expressed as the percentage of activity at day 0. D: Enzyme (PON) activity in HDL isolated from LL and MM homozygotes after incubation at 37°C for 24 h. Results are means  $\pm$  SD ( $n = 3$  subjects). Control, activity before incubation; inhib, activity after incubation for 24 h with aprotinin + kallikrein; kallikrein, activity after incubation for 24 h with kallikrein. \*  $P < 0.01$ , \*\*  $P < 0.001$ .

promoter polymorphisms, as revealed in this study. It led us to examine the relative stability of the L and M isoforms as one possible explanation for this observation.

In the first instance, we adopted a strategy whereby the isoforms were secreted into a standardized medium, thus ensuring a uniform background against which the studies were performed. This eliminated potential confounding factors arising from the use of serum as a source of PON. Interindividual variations in serum composition and, more importantly, the composition of the serum transport vehicle, HDL, could independently influence PON isoform stability. We wished also to avoid PON purified from serum, as it necessitates vigorous procedures that denature the enzyme to a certain extent. The present study employed (for the initial studies) recombinant PON corresponding to the L and M isoforms with the same nucleotide at a second coding region polymorphic site (191), which is known to affect en-

zyme activity. The single- and two-dimensional PAGE profiles of the recombinant PON strongly resembled those of human serum PON, indicating that processing of the enzyme, notably with respect to glycosylation, followed that of the native peptide. The recombinant peptides were enzymatically active toward phenylacetate and paraoxon, showing relative activities similar to that of human serum PON, and were recognized by a monoclonal antibody raised against human PON. Finally, PON formed complexes with HDL in the conditioned medium, as shown by FPLC and confirmed by immunoaffinity chromatography.

The data from the initial study quite clearly show that the M isoform lost activity more rapidly and to a greater extent than the L isoform. This was a consistent observation and gave rise to an approximately 2-fold greater loss of activity after incubation. Human serum was employed in some studies to give a medium more closely corresponding to na-



**Fig. 5.** A: Lipid-stained agarose gel profiles of human serum (lanes 1, 2) and culture medium containing human HDL (lanes 3, 4) after incubation at 4°C (lanes 1, 3) or 37°C for 4 days (lanes 2, 4). The arrow indicates the origin. B: Immunoblots of culture medium containing human HDL (lanes 1, 2) and human serum (lanes 3, 4) after incubation at 4°C (lanes 1, 3) or 37°C for 4 days (lanes 2, 4). After SDS-PAGE (nonreducing conditions), the profiles were electrotransferred to nitrocellulose and probed with anti-apoA-I antibodies. The band corresponds to monomeric apoA-I. The arrows show the locations of AII: apoA-II, AI: apoA-I, and Alb: albumin.

tive conditions; in particular, the presence of human HDL. Even in this experimental setting, the M isoform was less stable than the L isoform. As one possible explanation for the observations, we examined the relative susceptibilities of the two isoforms to proteolysis. To investigate this possibility, studies were repeated in the presence of a protease inhibitor. Under these conditions, loss of PON enzyme activity was largely abolished for both isoforms. This strongly suggested that the basis for the differential loss of enzyme activity is greater susceptibility of the M isoform to proteolysis by enzymes present in the culture medium.


To examine more precisely this possibility, we made use of serum PON in HDL isolated by ultracentrifugation from donors who were homozygous LL or MM. This enabled us to analyze directly the impact of the serum proteolytic en-

zyme, kallikrein. The results confirmed those outlined above. Namely, the M54 isoform was more susceptible to loss of activity in the presence of kallikrein. The specific effect of the proteolytic enzyme was confirmed by showing full retention of PON activity when kallikrein was first inhibited by preincubation with aprotinin prior to addition to HDL-PON. In light of these results, we examined the stability of PON isoforms in whole serum. This provided a third approach whereby ultracentrifugation and formation of PON-HDL complexes under *in vitro* conditions were eliminated. It is possible that these nonphysiological manipulations could be responsible for the greater susceptibility of the M isoform to proteolysis. The results were consistent with those obtained using recombinant PON and HDL-PON. The M54 isoform lost activity more rapidly and to a greater extent than the L isoform. In addition, we demonstrated that loss of activity was accompanied by loss of measurable PON protein mass, consistent with the action of a proteolytic enzyme. Further analysis revealed the appearance of a smaller molecular mass band than intact PON, consistent with proteolysis of the enzyme.

The nucleotide polymorphism at the 54 position induces a methionine-leucine interchange. This is a conservative alteration, which would not be expected to alter susceptibility to proteolysis at this site. It may, however, induce conformational changes elsewhere in the peptide, making it more amenable to proteolytic degradation perhaps by modifying its ability to complex with HDL.

One important consideration is the potential physiological relevance of the observations. In the present study, we did not compare the antioxidant capacity of HDL after proteolysis. There appear to be several mechanisms by which HDL can protect LDL from oxidation (22). Proteolysis could also affect these mechanisms and, thus, it is not possible to attribute any decreased antioxidant effect unambiguously to the effect on PON. However, there is strong evidence in the literature to support the contention that modulating the PON content of HDL influences the antioxidant function of the lipoprotein. In animal models, the level of serum HDL-PON has been positively correlated with the degree of protection of LDL from oxidation (10, 11, 23). *In vitro* studies have shown that the ability to prevent oxidation of LDL correlates with the PON content of HDL (5, 9, 24), whereas Aviram et al. (25) reported that lipid peroxidation induced in whole serum and isolated HDL correlated negatively with the PON level. Several studies have also shown a dose-dependent inverse correlation between PON (as measured by enzyme activity) and the levels of lipid peroxides (9, 25, 26). Finally, in a recent study (27), we demonstrated specifically that varying the PON content of HDL modulated its protective role. We observed incremental decreases in copper-induced oxidation of LDL with increases in the PON content of HDL. The latter study also showed that variations in PON concentrations of 10–20% (similar to that due to proteolysis in the present study) were sufficient to decrease the antioxidant capacity of HDL. A second consideration is whether the time-course for the proteolytic effect is sufficient to influence serum PON concentrations *in vivo*. There are at present no data on PON turnover. However, the residence

time of HDL in serum, determined from apoA-I kinetics, is 4–5 days (28), whereas we observed a significant impact of proteolysis on PON within 4 days. These time scales are compatible with the suggestion that proteolysis could have an influence on serum PON concentrations in vivo.

In conclusion, the results are consistent with the hypothesis that differences in serum levels of PON linked to the L54M polymorphism are due to reduced stability of the M isoform arising from its greater susceptibility to proteolysis. The observations have several implications. First, they provide a further explanation for the wide range of PON activities observed in human sera. Second, they indicate that knowledge of both the L54M and Q191R genotypes is of clinical and functional relevance. Third, the results have implications for the antioxidant capacity of HDL that is of importance in the cardiovascular field, given the reported association of PON with risk of vascular disease. Finally, the observations are relevant to the toxicology field, as PON offers a measure of protection against various environmental poisons. 

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