HDL subfraction distribution of paraoxonase-1 and its relevance to enzyme activity and resistance to oxidative stress

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Abstract The subfraction distribution of HDL-associated peptides has implications for their functions and the impact of pathological modifications to lipoprotein metabolism on these functions. We have analyzed the subfraction distribution of paraoxonase-1 (PON1) and the consequences for enzyme activity and stability. HDL subfractions were defined by the presence (LpA-I,A-II) or absence (LpA-I) of apolipoprotein A-II (apoA-II). PON1 was present in both subfractions, although increased concentrations of HDL were associated with significantly increased PON1 in LpA-I. ApoA-II did not modify the capacity of native human HDL or reconstituted HDL to promote PON1 secretion from cells or to stabilize enzyme activity, nor did apoA-II decrease PON1 activity when added to rabbit serum normally devoid of the apolipoprotein. LpA-I,A-II particles isolated from human serum or reconstituted HDL (LpA-I,A-II) showed a significantly greater capacity than HDL(LpA-I) to stabilize secreted PON1 and purified recombinant PON1 added to such particles. PON1 associated with apoA-II-containing particles showed greater resistance to inactivation arising from oxidation. ApoA-I, apoA-II, and LpA-I,A-II, but not LpA-I, were independent determinants of serum PON1 concentration and activity in multivariate analyses. PON1 is at least equally distributed between LpA-I and LpA-II,A-II HDL particles. This dichotomous distribution has implications for PON1 activity and stability that may impact on the physiological role of the enzyme.—Moren, X., S. Deakin, M-L. Liu, M-R. Taskinen, and R. W. James. **HDL subfraction distribution of paraoxonase-1 and its relevance to enzyme activity and resistance to oxidative stress. *J. Lipid Res.* 2008.** 49: **1246–1253.**

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HDLs show considerable compositional heterogeneity with respect to both lipid and apolipoprotein components (1). This reflects, in part, functional heterogeneity, with apolipoproteins and other HDL-associated peptides conferring specific functions on discrete lipoprotein particles. A major subclassification is defined by the presence or absence of apolipoprotein A-II (apoA-II) (2). All HDL particles contain apoA-I (LpA-I), whereas a variable fraction [40–70%, (2, 3)] contain, in addition, apoA-II (LpA-I,A-II).

The overall impact of apoA-II on HDL metabolism and function is unclear. It has been variously reported to have beneficial and detrimental influences on these parameters (as reviewed in Refs. 4, 5). Likewise, there is no consensus on the relative clinical impacts of LpA-I and LpA-I,A-II, with suggestions that both particles, or predominantly the LpA-I particles, are protective against vascular disease (4, 5). Animal models have indicated that overexpression of apoA-II is detrimental for atherosclerosis (6, 7), although the interpretation of such models is open to question. ApoA-II exhibits greater hydrophobicity than apoA-I, binds more tightly to lipids, and appears to stabilize the HDL complex (8). Its hydrophobic nature may be a factor in the influence of apoA-II on HDL-associated enzyme activity, by modulating interactions of enzymes with the lipoprotein.

Paraoxonase-1 (PON1) is a serum enzyme predominantly associated with HDL (9). The subfraction distribution of the enzyme has not been clearly established, although correlations of serum PON1 with apoA-I and apoA-II have been noted (10). Data from an early preliminary study led us to suggest that it was primarily associated with LpA-I-type HDL particles (11). Our subsequent and more-recent studies (12, 13) have prompted us to question our initial conclusion that little PON1 was associated with apoA-II-containing HDL particles in human

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Abbreviations: AAPH, 2,2'-azobis (2-amidinopropane) hydrochloride; apoA-II, apolipoprotein A-II; ARE, arylesterase; CHO, Chinese hamster ovary; PON1, paraoxonase-1.

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serum. Given that apoA-II can have an important impact on the activity and function of HDL-associated enzymes, we have reexamined the subfraction distribution of PON1 in human serum, with respect to LpA-I and LpA-I,A-II particles. We have subsequently addressed the question of whether the lipoprotein association affects enzyme activity and stability.

METHODS

Immunofractionation of LpA-I and LpA-I,A-II

Fasting serum samples (n = 23, 14 males, 9 females; triglycerides, 0.60–1.56 mmol/l; HDL-cholesterol, 0.98–1.84 mmol/l; cholesterol, 3.94–6.41 mmol/l) were obtained from healthy subjects. All studies employing human subjects/samples were authorized by the ethics commissions at Geneva University Hospital or Helsinki University Central Hospital. Serum was fractionated by passage through a mouse monoclonal anti-human apoA-II immunoaffinity column, as described previously in detail (14). Briefly, serum (0.75 ml) was incubated with an immunoaffinity column containing immobilized monoclonal anti-apoA-II antibody (4°C, 1 h). The nonbound fraction was eluted and concentrated (Vivaspin ultrafiltration; Vivascience, Stonehouse, UK). The nonbound fraction contains LpA-I; the absence of apoA-II from this fraction was ascertained by Western blotting using a polyclonal rabbit anti-human apoA-II antibody (Milan Analytica; Basel, Switzerland) The bound fraction (LpA-I,A-II) was subsequently eluted with 3 M thiocyanate solution and rapidly dialyzed prior to lyophilization. The elution procedure inactivates the enzyme, and also appears to interfere with the ELISA assay of (PON1) mass. Thus, the distribution of PON1 between LpA-I and LpA-I,A-II was determined by measuring enzyme activities (3) [arylesterase (ARE) with phenylacetate and basal paraoxonase (PONb) with paraoxon] and mass (15) in nonfractionated serum and the LpA-I (nonbound) fraction; LpA-I,A-II-associated PON1 was determined by subtracting LpA-I values from those for nonfractionated serum. To check the fractionation procedure, serum from which LpA-I,A-II had been previously removed by immunofractionation was repassed through the anti-apoA-II column. The yield of activity in the nonbound fraction was 93.4% (n = 3).

A second fractionation procedure [comparable to Lamarche et al. (16)] was also developed for rapid analysis of smaller volumes of serum or HDL. Briefly, anti-apoA-II affinity gel (1.0 ml packed gel) in mini-columns was incubated with 0.05 ml serum (or 0.3 mg HDL protein). Nonbound HDL was eluted with 2.0 ml wash buffer prior to elution of bound HDL with the same volume of elution buffer.

Human serum (0.25 ml) was also fractionated on an anti-human PON1 immunoaffinity column (11), using a monoclonal mouse antibody isolated in the laboratory. Bound and nonbound fractions were probed with polyclonal anti-human apoA-II or monoclonal anti-human PON1 (of a differing specificity from that used for the immunoaffinity column).

Subfractions were also isolated from total human HDL purified by ultracentrifugation [d 1.063–1.21 g/ml; (17)]. In this case, the fraction nonbound to the anti-apoA-II affinity column is termed HDL(LpA-I), and the bound fraction is identified as HDL(LpA-I,A-II).

Cell culture

Cell culture studies employed the previously described (12) Chinese hamster ovary (CHO) model with cells stably transfected with human PON1 (CHO-hPON1). This model was used to analyze PON1 secretion as a function of HDL subfraction type or to condition HDL subfractions with PON1. In the latter case, HDL was incubated overnight with cells, and the recovered medium was usually concentrated (Vivaspin ultrafiltration) prior to use.

Recombinant PON1 and reconstituted HDL

Recombinant PON1 (rePON1) was expressed in *Escherichia coli* and isolated as described previously (18, 19). Isolated rePON1 was stored in Tris buffer (Tris 50 mM, pH 7.8, $CaCl_2$ 20 mM) containing Tergitol, 0.05% (v/v).

Reconstituted HDL (rHDL) was prepared by the cholate dialysis method (20). Human apoA-I and apoA-II were isolated from delipidated HDL by repeated filtration chromatography. Phosphatidylcholine was purchased from Sigma (Buchs, Switzerland). rHDLs were prepared containing apoA-I [rHDL(A-I)] and apoA-I plus apoA-II [rHDL(A-I,A-II)] (12). For rHDL(A-I,A-II), the A-I:A-II molar ratio of was 2:1.

Oxidation of HDL and whole serum

rHDL subfractions (0.1 mg protein) conditioned with PON1 by incubation with CHO-hPON1 cells (16 h) were harvested, concentrated (Vivaspin), and subjected to oxidation by using 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH; 1.0 mM, 37°C) as described previously (19). Enzyme activity (n = 6) was measured at various time points after initiating oxidation. Results are one of three analyses.

Serum (1.0 ml) was incubated (37°C, 18 h) without or with AAPH (50 mM), dialyzed (TBS; Tris 10 mM, pH 8.0, NaCl 0.15 M, $CaCl_2$ 5 mM) then fractionated (0.75 ml) on the anti-apoA-II immunoaffinity columns as described above. ARE activity (n = 6) was analyzed in prefractionated serum and in the nonbound fractions (equivalent to LpA-I). LpA-I,A-II-associated PON1 activity was calculated as the difference between the two values. Total HDL (1.0 mg protein) was subjected to the same analysis, except that it was oxidized with 1.0 mM AAPH for up to 3 h at 37°C.

Addition of apoA-II to rabbit serum

Fresh rabbit serum was incubated (3 h, room temperature) with purified human apoA-II to give molar ratios of apoA-I:apoA-II of 2:1 and 1:2 [assuming a rabbit apoA-I concentration of 0.5 mg/ml (21)]. PON1 enzyme activity was then analyzed. Aliquots of rabbit serum containing no apo A-II or apoA-II at a 2:1 molar ratio to apoA-I were also fractionated on an anti-human apoA-II affinity column as described above.

RESULTS

Correlations between serum PON1 and HDL apolipoproteins and particles

The correlations between apoA-I, apoA-II, HDL subfractions, and PON1 were analyzed in two distinct populations. **Figure 1A, B** show the correlations between the enzyme and apoA-I or apoA-II in a group of healthy subjects recruited in Geneva (22). There were highly significant correlations (apoA-I, $r = 0.35$; apoA-II, $r = 0.39$; both $P < 0.001$) between both variables and PON1 activity (ARE). **Table 1** shows multivariate analyses of variables that were associated with serum PON1 activity. Both apoA-I and apoA-II were independent determinants of

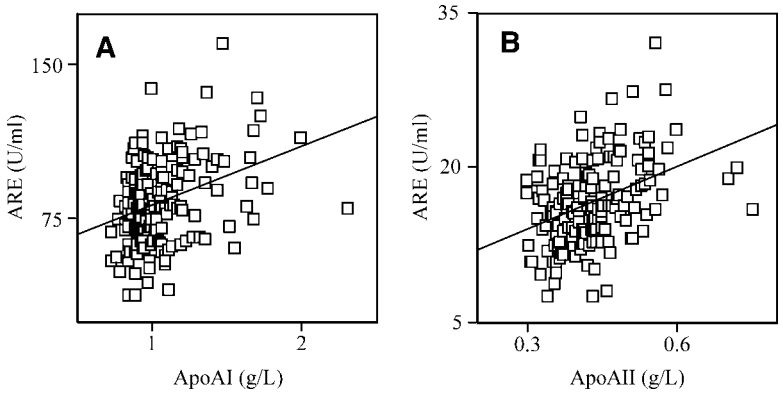

Fig. 1. Serum paraoxonase-1 (PON1) arylesterase (ARE) activity as a function of serum concentrations of apolipoprotein A-I (apoA-I) (A) or apoA-II (B). The population of healthy volunteers is that described in detail in a previous publication (22).

activity. However, only apoA-II was an independent determinant of PON1 mass. We confirmed these observations in a second, independent population (23) in which apoA-I and apoA-II were associated with PON1 mass ($r = 0.19$, $P = 0.03$ and $r = 0.27$, $P = 0.002$, respectively) and ARE activity ($r = 0.28$, $P = 0.001$ and $r = 0.34$, $P < 0.001$, respectively). We were also able to analyze the correlations between apoA-II-defined HDL subfractions and PON1 in this population. PON1 mass and ARE activity were significantly correlated with LpA-I,A-II (mass, $r = 0.208$, $P = 0.014$; ARE, $r = 0.392$, $P < 0.001$). Conversely, LpA-I was not significantly correlated with either PON1 parameter (mass, $r = 0.068$, $P = 0.43$; ARE, $r = 0.01$, $P = 0.91$). Consequently, as shown in **Table 2**, the LpA-I,A-II particle was shown to be an independent determinant of serum PON1 activity and mass in multivariate analyses, whereas LpA-I did not enter either model.

PON1 and apoA-II are associated in complexes isolated with specific antibodies

To provide definitive proof of an association between PON1 and apoA-II, whole serum was immunofractionated on affinity columns containing antibodies specific for PON1 or apoA-II. Bound and nonbound fractions were subsequently immunoprobed with anti-PON1 or anti-apoA-II antibodies. Analyses of the bound fraction of the anti-apoA-II column revealed the presence of PON1 (**Fig. 2A**, lane 3). The enzyme was also present in the nonbound fraction (Fig. 2A, lane 4), in accordance with its association with LpA-I particles (see **Table 3**). Western blots showed that the anti-PON1 column retained all serum PON1 [no signal in the nonbound fraction (Fig. 2B)]. The bound fraction also showed a positive signal for apoA-II (Fig. 2B, lane 2) confirming its presence within complexes containing PON1. ApoA-II was also present in the nonbound fraction (Fig. 2B, lane 3), indicating the existence of LpA-I,A-II particles lacking PON1.

The above results were confirmed by analysis of the distribution of PON1 activity and peptide mass (by ELISA) between LpA-I and LpA-I,A-II (Table 3). The majority of enzyme activity was associated with the LpA-I,A-II particle, both for ARE and PONb. The distribution difference was accentuated for PON1 peptide (Table 3), also in favor of LpA-I,A-II. Conversely, enzyme-specific activity was significantly higher in the LpA-I particle for both ARE and basal PON1.

The distribution of PON1 between LpA-I and LpA-I, A-II was also analyzed as a function of serum concentrations of HDL in healthy subjects (n = 12). There were positive correlations between serum HDL-cholesterol and *i*) total serum PON1 activity ($r = 0.33$, $P = 0.012$) and *ii*) activity associated with LpA-I ($r = 0.62$, $P < 0.01$). The percentage of total activity associated with LpA-I increased as serum HDL concentrations rose ($r = 0.75$, $P < 0.001$).

Fast-protein liquid chromatography of immunoabsorbed serum

Previous studies have established that LpA-I and LpA-I,A-II particles differ in molecular size (2, 3). The latter was examined in terms of associated PON1, by analyzing the molecular size distribution of enzyme activity in whole serum and the LpA-I fraction. **Figure 3** shows the elution profile from a fast-protein liquid chromatography column, using ARE activity to identify PON1 (Western blots confirmed the presence of PON1 protein; data not shown). To allow comparisons of the elution profiles, activity per tube is expressed as a percentage of total activity eluted from the column. The elution profile for LpA-I-associated PON1 is shifted to a higher molecular size

TABLE 1. Multivariate analysis of variables associated with serum PON1 activity and mass in healthy subjects

Variable (n = 157)	Activity		Mass	
	β	*P*	β	*P*
Prom 107	0.510	<0.001	0.270	<0.001
ApoB	0.474	<0.001	−0.452	<0.001
Cholesterol	−0.328	0.001	0.314	0.016
ApoA-II	0.255	<0.001	0.176	0.031
ApoA-I	0.234	<0.001	0.131	0.109
Gender	−0.174	0.002		
Cod 191	0.153	0.007	0.137	0.069
Triglycerides	0.131	0.037		
Cod 55			0.342	<0.001
Adjusted r^2	0.620	<0.001	0.334	<0.001

Age and body mass index were tested but did not enter either model. β, standardized coefficient; Prom 107, PON1 promoter polymorphism C(-107)T; ApoB, apolipoprotein B; β, standardized regression coefficient; Cod, coding region polymorphism (at positions 55 and 191). The population was previously described in detail (19).

TABLE 2. Multivariate analysis of determinants of serum PON1 activity and mass in affected and unaffected members of families with familial combined hyperlipidemia

Variable (n = 150)	Activity		Mass	
	β	*P*	β	*P*
Age			−0.190	0.015
BMI	−0.207	0.018		
Cholesterol			0.368	0.004
HDL	0.114	0.283		
LpA-I:A-II	0.297	0.003	0.164	0.022
ApoB			−0.201	0.100
Cod 55	−0.270	0.001	−0.587	<0.001
Adjusted r^2	0.235	<0.001	0.475	<0.001

Triglycerides, gender, LpA-I, and the coding region polymorphism 191 were also tested but did not enter either model. The population is that described in detail in a previous publication (23). BMI, body mass index; β, standardized regression coefficient; PON1, paraoxonase-1.

compared with whole serum. This is in accord with data showing that LpA-I particles have higher (and lower) molecular weights than LpA-I,A-II particles. In contrast, the calculated elution profile for the LpA-I,A-II particles shows PON1 to be associated with lower molecular weight complexes. The data are consistent with the suggestion that PON1 exists on at least two types of distinct, HDL particles that can be differentiated by apoA-II. The minor, leading peak of the elution profile is PON1 associated with VLDL-sized particles, as we previously reported (24). Its presence in the LpA-I fraction shows that it was not removed by the anti-apoA-II column.

HDL subfractions and PON1 secretion

The next series of studies attempted to determine whether PON1 metabolism was influenced by its subfraction association. HDL was isolated by cumulative flotation ultracentrifugation, PON1 was removed by immunoabsorption, and then HDL was fractionated on the anti-apoA-II immunosorbent column. The unbound HDL(LpA-I) and bound HDL(LpA-I,A-II) fractions were extensively dialyzed against TBS before their capacity to promote PON1 secretion from CHO-hPON1 cells was analyzed. **Figure 4A, B** show typical PON1 secretion curves (one of four analyses). Comparable dose-response curves were observed for PON1 release as a function of HDL concentration, with similar affinity constants for secretion of PON1 (apparent K_m values: LpA-I, 3.87 ± 1.0 μg HDL protein/ml; LpA-I,A-II, 3.09 ± 0.6 μg HDL protein/ml; n = 4, not significant). Studies were also performed using rHDL, where possible differences in lipid and peptide composition between LpA-I and LpA-I,A-II are eliminated apart from the presence or absence of apoA-II. The dose-response curves for PON1 release were also comparable when rHDLs containing apoA-I or apoA-I,A-II were analyzed (Fig. 4B). The presence of apoA-II does not appear to be detrimental to HDL-stimulated PON1 release from cells.

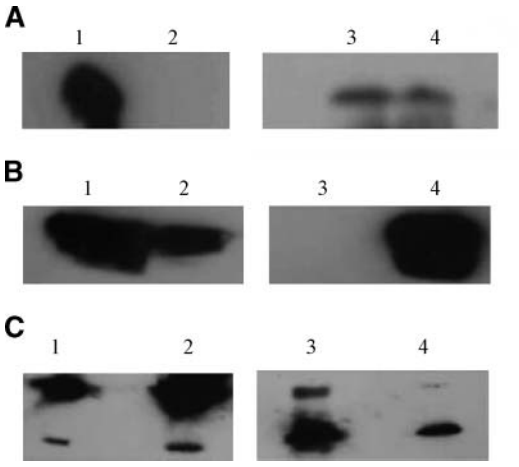

Fig. 2. Immunoblot analyses of fractions (from 0.75 ml of processed serum) retained by anti-PON1 and anti-apoA-II immunoaffinity columns. A: Fractions bound (lanes 1, 3) and nonbound (lanes 2, 4) by anti-apoA-II column and immunoprobed with anti-apoA-II (lanes 1, 2) or anti-PON1 (lanes 3, 4). B: Fractions nonbound (lanes 1, 3) and bound (lanes 2, 4) by anti-PON1 column and immunoprobed with anti-apoA-II (lanes 1, 2) or anti-PON1 (lanes 3, 4). C: Serum (lanes 1, 4) and purified HDL (lanes 2, 3) fractionated by SDS-PAGE and immunoblotted with anti-apoA-II antibodies; nonreduced (lanes 1, 2) and reduced (lanes 3, 4). For all fractions, the equivalent of 4 μl of serum was analyzed.

HDL-associated PON1 activity and stability

As shown independently by a number of groups (12, 25–27), binding of PON1 to HDL can stabilize and improve the activity of the enzyme. This was analyzed with respect to HDL subfractions. In the first series of studies, rePON1 was used with rHDL. Isolated rePON1 is stable in the presence of detergent, but rapidly loses activity when the detergent concentration is lowered [(25); (**Fig. 5A**)]. Enzyme activity was examined when rePON1 was diluted into buffer alone, or buffer containing rHDL. As shown in Fig. 5A, rePON1 rapidly lost activity in buffer alone. Conversely, rHDL(A-I) and rHDL(A-I,A-II) stabilized and increased PON1 ARE activity, with the effect evident over a 24 h period. rHDL(A-I,A-II) had a small but significantly greater ($P < 0.01$) stimulatory and stabilizing influence than rHDL(A-I) over the same period.

A second approach used both rHDL and subfractions isolated from human HDL by immunoaffinity chromatography. Here the subfractions were conditioned with human PON1 by CHO-hPON1 cells, then removed from the cells and incubated overnight at 37°C. Compared with HDL-free culture medium, HDL subfractions stabilized PON1 activity. Although total loss of activity was small, the differences were significant between apoA-II-containing and apoA-II-free HDL. For both native HDL and rHDL, a significantly higher level of activity was retained by the apoA-II-containing HDL subfraction (Fig. 5B).

It has been suggested that apoA-II is detrimental to PON1 activity (7, 28). To examine further the effect of the apolipoprotein on serum PON1 activity, the enzyme was examined in rabbit serum. Rabbit serum contains no apoA-II (21). However, addition of human apoA-II to rabbit serum gives rise to HDL particles containing apoA-I and apoA-II (21). Purified human apoA-II was added to rabbit serum to give apoA-I:apoA-II molar ratios of respectively 2:1 and 1:2. PON1 activities (arbitrary units

TABLE 3. Distribution of PON1 activity and mass between LpA-I and LpA-I,A-II

Parameter	Serum	LpA-I	LpA-I,A-II	*P*
Activity ARE (U/ml)	91.3 ± 17.6	40.8 ± 7.9	50.5 ± 12.6	<0.01
Activity (%)	—	46.5 ± 9.1	53.5 ± 9.0	<0.001
Specific activity (U/μg)	0.92 ± 0.2	1.33 ± 0.2	0.74 ± 0.3	<0.001
Activity PONb (U/ml)	7.10 (3.1, 15.5)	2.78 (1.6, 5.6)	4.24 (1.5, 10.4)	<0.01
Activity (%)	—	42.8 ± 7.8	57.2 ± 7.5	<0.001
Specific activity (U/μg)	0.090 (0.033, 0.150	0.116 (0.04, 0.16)	0.067 (0.024, 0.155)	<0.001
Mass (μg/ml)	100.4 ± 26.6	31.4 ± 8.4	69.0 ± 20.1	<0.001
Mass (%)	—	31.1 ± 5.0	68.9 ± 5.2	<0.001

Values for Lp(A-I,A-II) were calculated as total activity (nonfractionated serum) − LpA-I-associated activity (fraction nonbound by anti-apoA-II immunoaffinity column). ARE, arylesterase (mean ± SD); PONb, basal paraoxonase activity (median 95% CI); mass, mean ± SD.

[AU]/μl) measured in normal rabbit serum (17.1 AU ± 0.2) did not differ significantly from those with added apoA-II (17.5 AU ± 0.3 at 2:1 molar ratio, 17.3 AU ± 0.8 at 1:2 molar ratio, n = 6 for all). When subsequently fractionated on the anti-apoA-II column, 92.1% (n = 2) of activity was recovered in the nonbound fraction when rabbit serum alone was fractionated. In the presence of apoA-II, 61.3% (n = 2) was recovered in the nonbound fraction, suggesting that 38.7% (n = 2) of PON1 activity was retained on the column owing to the presence of apoA-II in the HDL-PON1 complex.

Oxidation of whole serum

PON1 activity is susceptible to oxidative stress. The possible impact of apoA-II on loss of PON1 activity resulting from oxidation was examined under several different conditions.

In the first study, rHDL(A-I) and rHDL(A-I,A-II) were conditioned with PON1 by incubation with CHO-hPON1 cells. The HDL-PON1 complexes were then subjected to oxidation in the presence of AAPH, and residual ARE activity was analyzed. As shown in **Fig. 6**, there was a gradual loss of activity as the HDL lipids were oxidized (data not shown). There was a significantly greater ($P < 0.05$) loss of activity from rHDL(A-I) than from rHDL(A-I,A-II).

The second set of analyses examined whether PON1 already associated with apoA-I could be protected by the addition of apoA-II. Human HDL-containing active PON1 was fractionated on the anti-apoA-II affinity column, and the nonbound fraction was retained [HDL(A-I)]. The latter was subjected to oxidation in the presence or absence of added purified human apoA-II (A-I:A-II molar ratio of 2:1). The consequences of oxidation for PON1 activity are shown in Fig. 6B. Again, loss of activity was less pronounced ($P < 0.01$ at 3 h) from PON1 associated with apoA-II-containing HDL.

Final studies were performed to determine whether the observations could be reproduced in whole serum. Figure 6C–E show the results for three serum samples (sample E corresponds to a pool of four sera). The distribution of ARE activities between HDL subfractions in nontreated sera were variable [percentage activity in LpA-I of 43.1 (A), 34.8 (B), and 50.3 (C)] and correspond to those observed previously. AAPH caused a variable loss of activity, ranging from 39% to 51%. With respect to HDL subfractions, oxidation induced a significantly greater loss of activity from LpA-I compared with LpA-I,A-II (68.7 ± 4.3% vs. 22.7 ± 11.0%, n = 3, $P < 0.05$).

DISCUSSION

The subfraction distribution of HDL-associated peptides has implications for their functions and the impact of pathological modifications to lipoprotein metabolism on these functions. It is thus important to characterize the lipoprotein association of peptides and determine the consequences, if any, of their activities. The present study modifies our understanding of the HDL association of PON1. It clearly demonstrates the presence of PON1 in HDL particles containing apoA-II. The peptide can be identified immunochemically in serum fractions retained by anti-apoA-II, and the latter removes a substantial proportion, but not all, of PON1 activity from serum. Correspondingly, apoA-II can be detected in the serum fraction retained by an anti-PON1 immunoaffinity column. The association with LpA-I,A-II HDL particles, in addition to

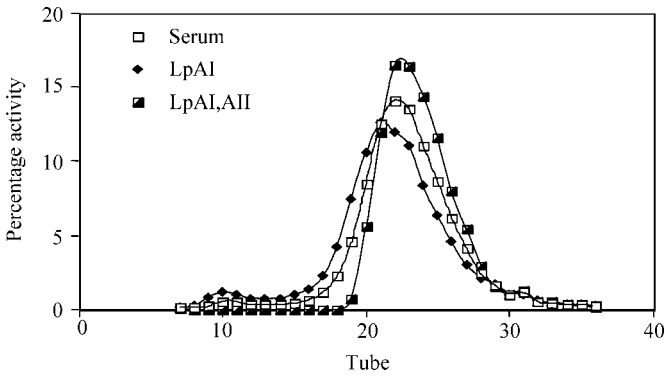

Fig. 3. Fractionation of whole serum (0.1 ml) and LpA-I (isolated from 0.1 ml serum) by fast-protein liquid chromatography (24). Activity associated with LpA-I,A-II was calculated by subtracting LpA-I activity from activity of the whole serum sample for each tube of the elution profile. Results per tube are expressed as percentage of total eluted activity for each sample. Results are from one of three individual analyses.

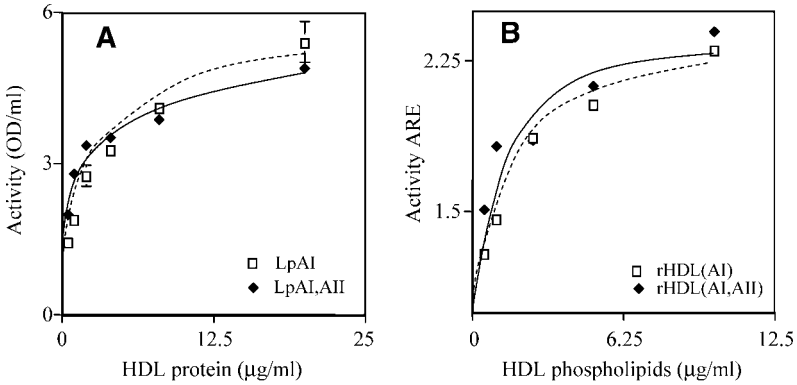

Fig. 4. Release of PON1 from Chinese hamster ovary (CHO)-hPON1 cells as a function of HDL protein (A) and phospholipid (B) concentration. Results (ARE activity) are means ± SD, n = 6. A: HDL subfractions isolated from human HDL; B: Reconstituted HDL.

LpA-I particles, would appear to have an impact on PON1 enzyme activity.

A number of observations had led us to question our preliminary conclusions concerning the association of PON1 essentially with HDL containing apoA-I but no apoA-II. These are indicated in Tables 1 and 2 and illustrated in part by Fig. 1, which suggest that apoA-II is a determinant of serum PON1 activity/mass that is superior to that of apoA-I. Several groups have previously reported on the positive correlation of apoA-II with serum PON1, which appears incongruous with an absence of the enzyme from apoA-II-containing particles. The present study indicates that not only is PON1 associated with apoA-II in LpA-I,A-II, but such particles may have a greater capacity to influence enzyme activity. Using HDL subfractions isolated from human serum, LpA-I,A-II stabilized PON1 activity to a greater extent than did LpA-I. Complementary studies with reconstituted HDL, in which lipid and protein composition can be strictly controlled, clearly indicate that apoA-II provides greater stabilization of enzyme activity in these in vitro studies. That the present results contrast with our previous conclusions may reflect the rather preliminary nature of the previous analysis, in which results of immunoblot analyses were not complemented by activity analyses.

Based on its overexpression in transgenic models, some studies have suggested that apoA-II may be detrimental to serum PON1 activity and function (7, 28, 29). The greater hydrophobicity of apoA-II is known to displace peptides from HDL and was suggested to explain the reduced serum PON1 activity of such models. However, apoA-II can also stabilize the HDL complex, and under more-physiological conditions of apoA-II production, this may be of greater relevance. Our studies of the impact of apoA-II on PON1 metabolism/activity are more consistent with the latter. First, release of PON1 from cells was stimulated to an equivalent degree by apoA-I- and apoA-I, A-II-containing HDL, whether reconstituted or isolated from serum. Second, addition of apoA-II to rabbit serum, normally devoid of the apolipoprotein, did not modify PON1 activity, despite the formation of HDL-A-II complexes containing PON1. Finally, addition of apoA-II to LpA-I isolated from human serum did not modify enzyme activity.

A major role attributed to PON1 is that of protection of lipoprotein lipids from oxidation. Oxidative stress can also inactivate the enzyme. Given our indications that apoA-II stabilized PON1 activity, we examined the effects of oxidative stress on PON1 activity of HDL subfractions. PON1 in rHDL-containing apoA-II offered greater resistance to inactivation during AAPH-induced oxidation than did PON1 associated with rHDL(A-I). More interestingly, a similar conclusion was reached when AAPH-induced oxidation of whole serum was examined. Greater loss of PON1 enzyme activity was observed from LpA-I than from LpA-I,A-II particles.

How apoA-II could stabilize PON1 activity and offer greater protection against oxidative stress is not immedi-

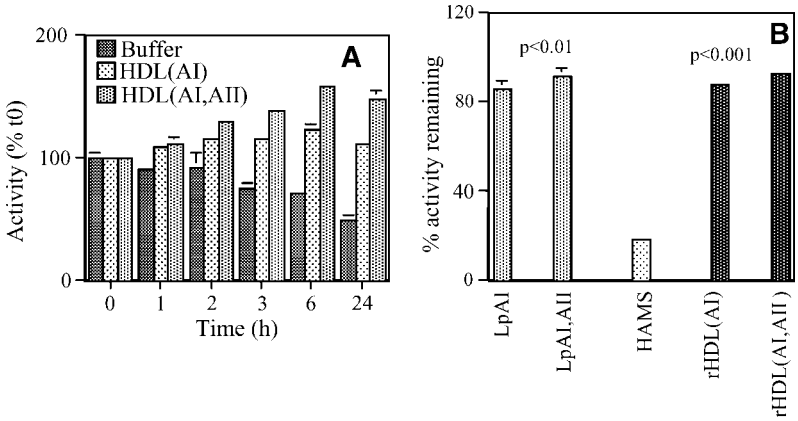

Fig. 5. Stabilization of PON1 activity as a function of HDL subfraction type. A: Recombinant PON1 was diluted into buffer alone, or buffer containing rHDL. PON1 activity was analyzed over a 24 h period and expressed as a function of activity at t0 (mean ± SD, n = 6). B: HDL subfractions isolated from human HDL or reconstituted were conditioned with PON1 by incubation with CHO-hPON1 cells. After removal from cells, activity was analyzed (t0) and again after incubation for 16 h at 37°C. Results are mean ± SD, n = 6.

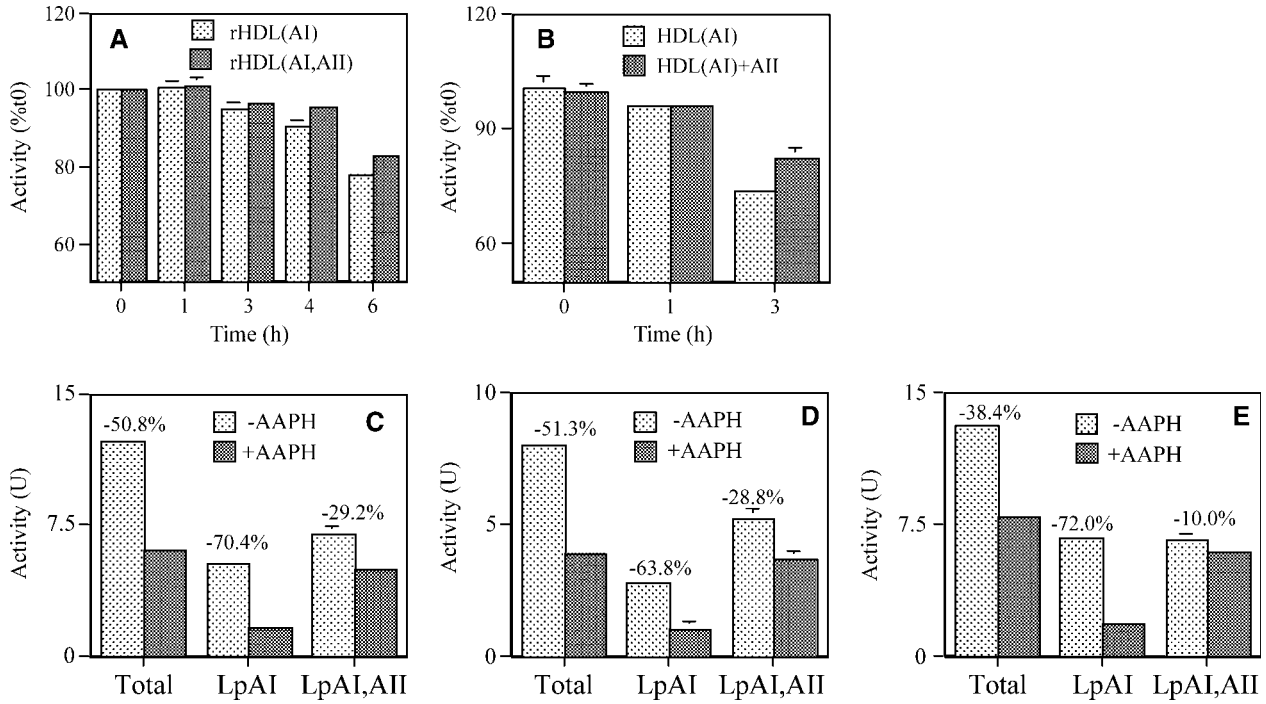

Fig. 6. Enzyme activity associated with HDL subfractions after oxidation with 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH). A: rHDL conditioned with PON1 by CHO-hPON1 cells. B: HDL(LpA-I) isolated from a total HDL preparation containing active PON1 was oxidized in the presence or absence of added, purified apoA-II. C–E: Whole serum was oxidized with AAPH then fractionated on the anti-apoA-II column. Activity was measured in whole serum and the nonbound fraction (LpA-I). Activity associated with LPA-I,A-II was calculated as serum activity−LpA-I activity.

ately evident. It has previously been shown by several groups that the interaction between apoA-I and PON1 is important for stabilizing enzyme activity and improving specific activity (12, 25–27). ApoA-II, through its ability to stabilize HDL structure (8), may complement the stabilizing influence of apoA-I, if not present at excess levels. In contrast, apoA-II alone has a negative impact on PON1 activity (25). Thiol groups are known to have anti-oxidant properties. Indeed, human apoA-I mutants carrying a cysteine residue show a greater anti-oxidant capacity than wild-type apoA-I (30). Human apoA-II carries a cysteine residue. Whereas this is normally cross-linked, giving rise to the human apoA-II homodimer, a fraction of the apolipoprotein may exist in a monomeric form [Fig. 2C and (31)]. An alternative, more-speculative explanation for the more-pronounced effect of oxidation on LpA-I-associated PON1 may be that it more readily participates in anti-oxidant reactions. This and other possible explanations await further investigation.

We have at present no explanation for the relative distribution of PON1 between the two HDL particles. Differences in the affinity of the particles for the cell surface would not appear to offer an explanation. Increased stability of PON1 subsequent to association with apoA-II-containing HDL may be a contributory factor. The distribution may reflect the relative concentrations of the two types of particle in serum, inasmuch as it approximates such a distribution (2). Supporting this proposition is the observation that increased HDL concentrations are associated with both increased PON1 in LpA-I-type-HDL and increased concentrations of serum LpA-I (3). The presence of PON1 in LpA-I,A-II particles may be of physiological relevance. This type of HDL particle is the principal acceptor of lipids shed from triglyceride-rich lipoproteins during lipolysis (32, 33). It could thus give PON1 greater access to alimentary lipids, allowing the enzyme to exert its hypothesized anti-oxidant influence on ingested lipids.

Our results differ from those that we previously reported with respect to the association of PON1 with apoA-II-containing HDL (11). We have no clear explanation for the differences. The previous analysis was somewhat preliminary and only focused on Western blots of the bound (HDL-A-I,A-II) fraction, with no confirmation by activity analyses. The sensitivity of Western blots may have been reduced by thiocyanate treatment necessary to elute the bound fraction, such that associated PON1 was less well recognized by our antibody.

There are several limitations to the present study. First, PON1 activity could not be directly analyzed in the apoA-II-containing fractions removed by the affinity column owing to inactivation during elution. We have, however, shown the presence of the PON1 peptide in such fractions in vitro, which confirms our findings from human studies of healthy subjects as well as familial combined hyperlipidemia patients (Tables 1, 2; Fig. 1). Second, the mechanism by which PON1 associates with HDL has not been fully clarified, but appears to play an important role in stabilizing the enzyme. The use of reconstituted HDL or addition of isolated rePON1 (with

physicochemical and enzymatic properties somewhat different from those of human PON1) to HDL may not fully reflect the in vivo conditions of association and stabilization. Again, where possible, we have used a variety of approaches and HDL fractions, including whole serum, and have obtained consistent results.

In conclusion, our study demonstrates an association of PON1 with the two major HDL subfractions, LpA-I and LpA-I,A-II. The association with two types of HDL particle would appear to be of relevance to PON1 enzyme activity and the response of the enzyme to increased oxidative stress. It is also likely to be of physiological relevance, given the functional differences between LpA-I and LpA-I,A-II particles.

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