Serum paraoxonase: effect of the apolipoprotein composition of HDL and the acute phase response

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Abstract **Genetic variations of paraoxonase (PON) correlate with HDL cholesterol and apolipoprotein A-I (apoA-I), suggesting antiatherogenic properties. Atherosclerosis occurs naturally in humans and rabbits but not in mice. We compared variations of PON arylesterase activity (PON AEase, phenylacetate substrate) in humans, rabbits, and mice. In humans and rabbits, >95% of PON AEase is HDL associated. In mice, about 30% of PON AEase is lipid poor. In the absence of apoA-I in mice, total PON AEase is reduced and >60% is lipid poor. PON AEase level and distribution is restored in apoA-I$^{-/-}$ mice injected with adenoviruses encoding human apoA-I and in transgenic mice expressing human apoA-I at a steady-state level. Thus, while apoA-I is not required for the HDL association of PON AEase, induced variations in apoA-I correlate with changes in HDL-associated, but not lipid-poor, PON AEase. PON AEase associates only with apoA-I- or apoE-containing HDL but not VLDL. In the absence of both apoA-I and apoE, PON AEase is all-lipid-poor. PON AEase is displaced from HDL by ultracentrifugation and following incubation with serum amyloid A. Variations in the PON distribution between HDL and lipid-poor fractions may have important consequences in its antioxidant activity and in atherogenesis.—**Cabana, V. G., C. A. Reardon, N. Feng, S. Neath, J. Lukens, and G. S. Getz. **Serum paraoxonase: effect of the apolipoprotein compostion of HDL and the acute phase response. *J. Lipid Res.* 2003.** 44: **780–792.**

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Paraoxonase (PON) [aryldialkylphosphatase (EC 3.1.8.1)] is a serum arylesterase that was initially identified by its hydrolysis of aromatic carboxylic esters and organophosphorus insecticides and nerve gases (1). Its name reflects its ability to hydrolyze paraoxon, a metabolite of the insecticide parathion. There are three members of the PON gene family: PON1, PON2, and PON3. They all possess antioxidant properties, share ~65% similarity at the amino acid level, and the genes are located in tandem on chromosome 7 in humans and on chromosome 6 in mice. It has been suggested that PON may play a protective role in the atherogenic process. PON1 and PON3 are mostly expressed in the liver and are carried in plasma bound to HDL (2). PON2 is ubiquitously expressed, but particularly in the cells associated with the artery wall and in macrophages, and may not be associated with lipoproteins (3).

PON1 is the most studied family member. The 45 kDa, 354-amino acid glycoprotein encoded by the *PON1* gene maps to human chromosome 7q21-22. The PON1 arylesterase and PON activities are calcium-dependent and can be totally and irreversibly inhibited by EDTA, while the protection of LDL against oxidation may not require calcium (4, 5). Deglycosylation of baculovirus-produced recombinant PON1 abolished its arylesterase and PON activities, but not its antioxidant properties (6). Thus, the antioxidative activity of PON and its arylesterase activity can be readily dissociated.

PON has been isolated from human plasma in association with apolipoprotein A-I (apoA-I) and with apoJ (7, 8). Using immunoaffinity chromatography, a subclass of HDL containing apoA-I, apoJ, and PON was obtained. This subfraction was relatively lipid poor, containing only 22% lipid (9). Not all of the PON was associated with apoJ. It is not clear from these studies whether apoJ, PON, and apoA-I form direct intermolecular complexes, or whether they are all harbored on a lipoprotein subclass whose lipid composition or particle size is the determinant of these associations. In Chinese hamster ovary cells transfected with human PON1, active PON was released in the presence of HDL, but not LDL or lipid-poor apoA-I (10).

The two common *PON1* gene coding polymorphisms, *PON1Q192R* and *PON1L55M*, have a correlative influence on the PON1 phenotype with HDL cholesterol (HDL-C)

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Abbreviations: apoA-I$^{-/-}$, apoA-I-deficient mice; apoE$^{-/-}$, apoE-deficient mice; E$^{-/-}$A$^{-/-}$, mice deficient for both apoA-I and apoE; FPLC, fast-phase liquid chromatography; LPS, lipopolysaccharide; PON, paraoxonase; PON AEase, paraoxonase measured as an arylesterase by the rate of hydrolysis of phenylacetate; SAA, serum amyloid A.

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and apoA-I levels (11), although some studies show that rather than genotype, phenotype may be a better predictor of vascular disease (12, 13). Its precise role in atherogenesis is not known. Among its functions, it may protect LDL from oxidation, oxidized LDL being a prime suspect in the development of atherosclerotic lesions (4, 7). Its involvement in the pathogenesis of atherosclerosis is suggested by the increasing PON1 immunoreactivity in the arterial wall as the atheroma advances (14). PON in human carotid and coronary plaques could be involved in the inactivation and removal of the lipid oxidation products (15). Mice genetically deficient in PON1 exhibit an increased susceptibility to the development of atherosclerosis (16). Overexpression of mouse PON1 protected the oxidation of LCAT and inhibited the formation of lipid hydroperoxide on HDL, (17) while overexpression of human PON1 decreased lesions in the atherosclerosis-prone apoE-deficient mice (apoE$^{-/-}$) (18).

Although serum PON1 activities and concentrations have been correlated with HDL-C and apoA-I levels, the relationship is not strong (4). Factors that influence HDL levels may not necessarily affect PON1 levels. A variety of nongenetic factors have been shown to influence serum PON1 levels. A cholesterol-rich diet fed to wild-type rabbits and those transgenic for human apoA-I (19) resulted in a significant reduction of PON1 activity. In humans, consumption of degraded cooking oil (20) lowered PON1 level while alcohol (21) and vitamins C and E (22) elevated it.

PON activity may also be altered as a part of the inflammatory response. Van Lenten et al. (23) showed that HDL became proinflammatory during the acute phase response, possibly due to loss of PON activity from HDL. Feingold et al. (24) showed a decrease of serum PON activity and hepatic PON mRNA in Syrian hamsters injected with lipopolysaccharide (LPS) to induce the acute phase response.

In this study, we have analyzed variations in the level and distribution of PON activity in the serum of three different species and different strains of mice with or without apoA-I expression. We have also analyzed the variations of PON activity during the acute phase response or following the incubation of serum with serum amyloid A (SAA). PON was measured as an arylesterase using phenylacetate as substrate since the arylesterase activity has been shown to be proportional to the PON1 enzyme protein concentration, whereas PON hydrolysis depends on the particular 192Q/R polymorphism (25, 26). Thus, in the remainder of this paper, we refer to what we have measured as PON measured as an arylesterase by the rate of hydrolysis of phenylacetate (PON AEase). Our results show that: *1*) PON AEase activity in human and rabbit serum is found only in the HDL fraction, while in the mouse it is associated with both the HDL and a lipid-poor fraction. In all the species studied, no PON AEase activity was detectable in the VLDL or IDL-LDL, even in those circumstances where VLDL contains apoA-I. *2*) PON AEase activity may associate with lipoproteins lacking apoA-I. *3*) PON AEase activity changes during the acute phase response concomitant with the acute phase response related changes in the SAA. *4*) Human and rabbit PON AEase activities are more tightly associated with HDL, while mouse PON AEase is more loosely bound and can be displaced from murine HDL particles. Given that the mouse is used as an experimental model for atherosclerosis studies, it is important to understand how the properties of molecules that influence atherogenesis differ between mouse and human.

MATERIALS AND METHODS

Serum sources

Human serum and lipoproteins were obtained from laboratory personnel who donated 10 ml of nonfasting blood. Rabbits purchased from commercial sources (Covance) were housed at the Animal Facility of the University of Chicago and fed rabbit chow ad libitum. Nonfasting blood was obtained from the central ear vessels. Mouse blood was collected by retroorbital or cardiac puncture of the animals anesthetized with Avertin [17.5 ml/g body weight, 2.5% in saline prepared from a stock solution of equal v/v tribromoethyl alcohol and tertiary amyl alcohol (Aldrich)]. C57BL/6 (C57), human apoA-I transgenic (AItgn) and apoA-I gene knockout (apoA-I$^{-/-}$) mice were purchased from Jackson Laboratories (Bar Harbor, ME). ApoE gene knockout (apoE$^{-/-}$) and apoE/A-I double gene knockout (E$^{-/-}$A$^{-/-}$) mice were bred and maintained in the animal facilities of the University of Chicago. To produce E$^{-/-}$A$^{-/-}$ mouse strain, breeder pairs of apoE$^{-/-}$ and apoA-I$^{-/-}$ single gene knockout mice backcrossed ten generations into the C57 strain were purchased from Jackson Laboratories. These mice were interbred, followed by brother-sister mating of the F1 offspring. The F2 animals were phenotyped by Western immunoblotting of sera separated by SDS-PAGE. For this procedure, 1 μl of serum was run in 4–20% precast Tris-Glycine gels (Invitrogen, Novex) and blotted using antibody against mouse apoA-I and rat apoE (cross reactive with mouse apoE) with C57 serum as a positive control. The sera of mice where the apoE and apoA-I bands were absent were taken as homozygous double knockout E$^{-/-}$A$^{-/-}$ progeny and were selected for use as breeders and as the source of sera for the PON assays.

All blood was collected without anticoagulant. To compare PON AEase activity in sera and heparinized plasma, the latter was obtained by collecting blood into Microtainer Brand tubes with lithium heparin (Becton Dickinson). The sera separated by centrifugation were immediately assayed for PON AEase activity as described below and fractionated by fast-phase liquid chromatography (FPLC) to analyze the distribution of the PON activity. Unused sera were stored at 4°C in the presence of the protease inhibitor phenylsulfonyl fluoride (0.001 M in methanol) and antibacterial agents (per milliter of plasma: 0.5 μg gentamicin sulfate, 50 μg NaN_3, and 1 μg chloramphenicol), and used within a week for other lipid and apolipoprotein analyses.

Acute phase sera were obtained from rabbits injected with croton oil (1% v/v mineral oil at multiple intramuscular sites) as described (27). Acute phase sera from mice were obtained following the intraperitoneal injection of 50 μg of bacterial LPS (*Escherichia coli* serotype 0127:B8, Sigma), as described (28). The sera were processed as described above. Adenovirus-expressing apoA-I was produced in the laboratory of Dr. V. I. Zannis by Dr. H-Y. Kan (29).

Serum fractionation

Serum lipoproteins were separated according to size by FPLC. Two hundred microliters of serum adjusted to 550 μl with saline

and centrifuged in a microcentrifuge to remove particulate matter were fractionated through two Superose 6 columns (Pharmacia) arranged in tandem using prefiltered and degassed phosphate buffered saline (20 mM phosphate, 50 mM NaCl) (pH 7.5). Seventy 0.4 ml fractions were collected and immediately analyzed for PON arylesterase activity. Because of limited lifetime, the columns were periodically replaced, which may result in slight differences in the fraction distribution; however, all experimental comparisons were obtained from the same column sets, e.g., baseline or acute phase, and incubations with SAA.

To analyze the density distribution of PON AEase activity, serum was fractionated by equilibrium density gradient ultracentrifugal flotation as previously described (27) using 200 μl of serum layered at the interface of a 3–20% NaBr solution in PON buffer (10 mM Tris, pH 8.0, with 1.0 mM $CaCl_2$), and centrifuged to equilibrium for 66 h at 38,000 rpm in a SW41 Ti rotor. Thirty 0.4 ml fractions collected using a gradient fractionator with tube piercing system (Brandel), and UV monitor and fraction collector (ISCO) were dialyzed against Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) and used for analyses.

PON assay

The PON AEase activity in the serum (2 μl) and serum fractions separated by FPLC or density gradient centrifugation (40 μl) were determined following the procedure described by Watson et al. (30) using phenyl acetate (Sigma) as substrate. Briefly, the samples prediluted in 900 μl of 10 mM Tris (pH 8.0) with 1.0 mM $CaCl_2$ were transferred to round-bottom snap cap polypropylene tubes. To the tubes was added 100 μl of 10 mM phenyl acetate (final concentration 1.0 mM), thoroughly mixed, and immediately transferred to quartz spectrophotometer cuvettes. Absorbance at 270 nm was taken every 15 s for 120 s or until a linear rate of change in optical density was established using a Beckman DU 65 spectrophotometer. The enzyme reaction rate was automatically calculated using a preprogrammed kinetics soft-pack module. Blanks were included to correct for the spontaneous hydrolysis of phenylacetate. Enzyme unit is expressed as μM phenyl acetate hydrolyzed/min/ml serum.

In vitro incubations with recombinant SAA

Lipid-free recombinant mouse SAA1.1 obtained from the pGEX-KG bacterial system has been described previously (31). Lipid-free recombinant human SAA1.2 [formerly SAA1b (32)] (A and V at positions 52 and 57 and R at position 71, respectively) was similarly produced using oligonucleotides (Integrated DNA Technologies, IA) designed as follows: 5′oligo GG GGTACC GGATCCGAAGCTTCTTTTCGTTC (the underlined bases corresponding to *Kpn*I and *Bam*HI restriction enzyme sites, respectively), and 3′ oligo GG TCTAGA GAATTC TCATAGCCAG GTCTCCTG (the underlined bases corresponding to *Xba*I and *Eco*RI restriction enzyme sites, respectively).

Typically, 500 μl of serum was incubated with 300 μg of the recombinant SAA, resulting in a final concentration of 600 μg SAA/ml serum, the approximate concentration of SAA in serum during the acute phase response. Incubation was for 1 h at room temperature, with occasional mixing. In some experiments, increasing amounts of SAA were used (up to 6 mg/ml serum). Following incubation, the mixture was fractionated by FPLC as described above and used in the PON AEase assay.

Lipid and protein quantitation

Total cholesterol was analyzed using commercially purchased enzymatic kits (Boehringer Mannheim). Phospholipids were quantitated by an enzymatic-colorimetric assay of choline-containing phospholipids (Wako). All lipoprotein analyses were performed by methods standardized against Centers for Disease Control and Prevention furnished standards. Protein was quantitated according to the procedure of Lowry et al. (33) with SDS to disrupt the lipid micelles (34) using bovine albumin as standard. ApoA-I was quantitated by radial immunodiffusion (28) after denaturation of the protein with 1% Triton-X100 using antibody against mouse apoA-I.

Statistical methods

Results are expressed as mean ± SD. Significance was tested using the paired Student's *t*-test. Data analyses were performed using the Minitab Statistical Software for Windows 95.

RESULTS

PON AEase level and lipoprotein distribution in normal sera

Baseline PON AEase activity was analyzed in human, rabbit, and mouse sera. Rabbits had extremely high levels of serum PON activity (308.5 ± 124.9 U/ml) compared with humans (114.3 ± 9.3 U/ml) and wild-type C57BL/6 (C57) mice (77.4 ± 5.1 U/ml) (**Table 1**). The distribution of PON AEase activity on lipoproteins and in lipid-poor fractions was determined following separation of sera via FPLC. The position of lipoprotein peaks was monitored by cholesterol and phospholipid concentrations in the fractions, and the elution of albumin was taken to represent the lipid-poor fraction. Human (**Fig. 1A**) and rabbit (Fig. 1B) sera showed a single peak of PON AEase activity, which coelutes with HDL. No PON AEase activity was detectable in VLDL, LDL, or the lipid-poor fractions. In this

TABLE 1. Paraoxonase activity measured as an arylesterase by the rate of hydrolysis of phenylacetate and apoA-I levels in human, rabbit, and mouse sera at baseline and during the acute phase response

Species/Strain	Number	Serum PON AEase		ApoA-I Baseline Level
		Baseline	Acute Phase Response	
		units[a]		*mg/dl*
Human	6	114.4 ± 9.3	nd	120–140[d]
Rabbit	2	308.5 ± 124.9	90.5 ± 0.35[b]	119.6 ± 21.3[e]
Mouse (C57)	6	77.4 ± 5.1	49.6 ± 9.8[c]	118.6 ± 7.8[f]

[a] Units, μM phenylacetate hydrolyzed/min/ml serum expressed as mean ± SD.
[b] Sixty-six hours post injection of croton oil, $P < 0.001$ versus baseline (see Fig. 3).
[c] Twenty-four hours post injection of lipopolysaccharide, $P < 0.001$ versus baseline.
[d] See reference 45.
[e] See reference 27.
[f] See reference 28.

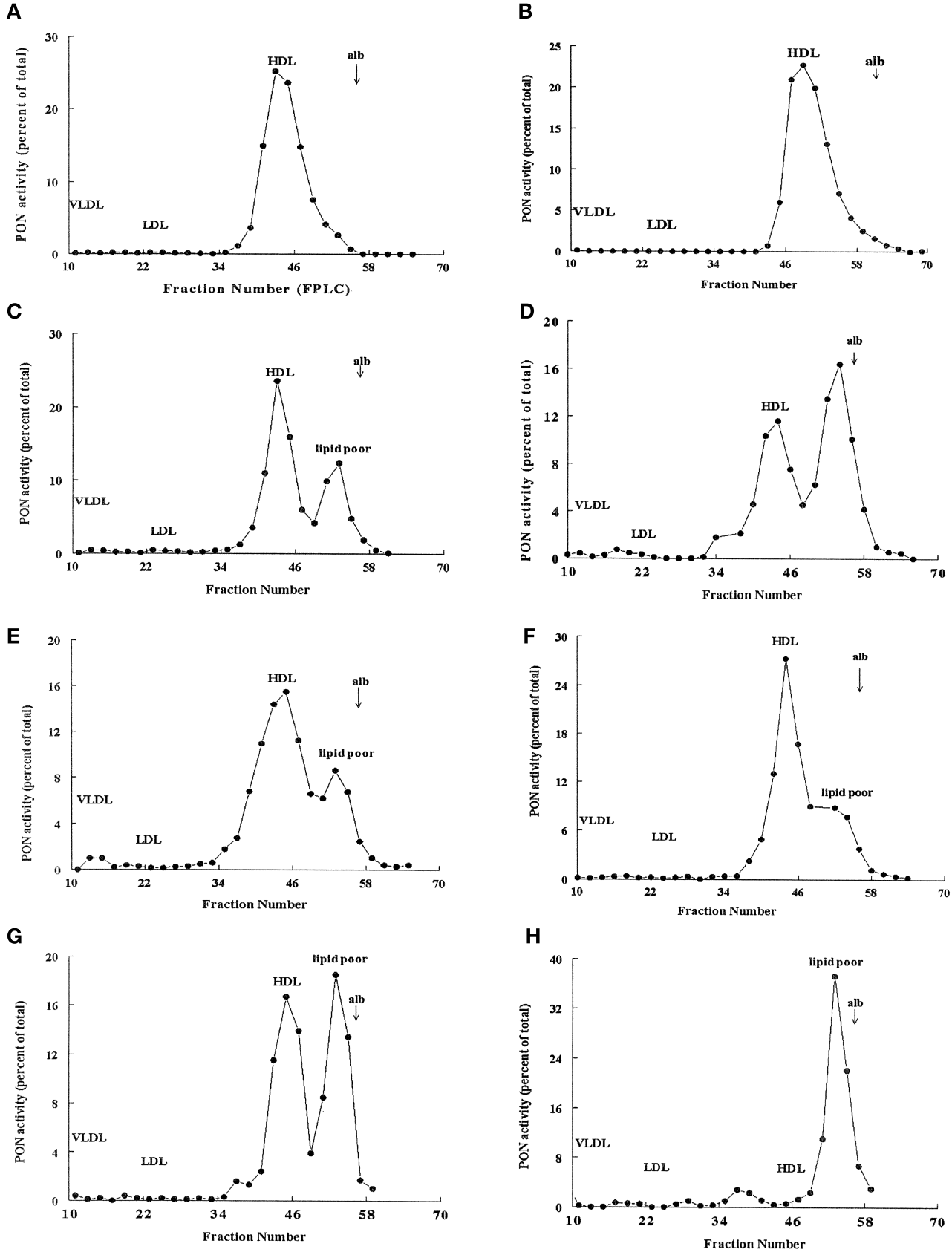

Fig. 1. Paraoxonase (PON) measured as an arylesterase by the rate of hydrolysis of phenylacetate (PON AEase) distribution in different species and in genetically altered mice. Two hundred microliters of sera were fractionated by fast-phase liquid chromatography (FPLC) as described in Materials and Methods. The results are expressed as percent of total PON AEase activity. A: Human (n = 6). B: Rabbit (n = 2). C: C57 (n = 6). D: ApoA-I$^{-/-}$ (n = 10). E: ApoA-I$^{-/-}$ 3 days following the injection of 5×10^8 plaque forming units of adenoviruses carrying a single copy of the wild-type human apoA-I gene (n = 2). F: Mice transgenic for human apoA-I (n = 3). G: ApoE$^{-/-}$ (n = 4). H: Mice deficient for both apoA-I and apoE (E$^{-/-}$A$^{-/-}$) (n = 6). alb, the peak of the elution of albumin used as the indicator for the lipid-poor fraction.

TABLE 2. Effect of the presence of apoA-I and apoE on the distribution of serum PON AEase activity in genetically modified mice

Mouse Strain	Number	Serum ApoA-I	Serum PON AEase	Lipid-poor PON AEase	
		mg/dl	*U/ml serum*	*% of total*	*U/ml serum*
C57	14	119 ± 7.8	76.9 ± 15.1	29.0	22.3
ApoA-I$^{-/-}$	10	0	38.2 ± 11.1[a]	56.1	21.4
ApoA-I$^{-/-}$ + adv-huAI[d]	6	323	61.2 ± 13.9[b]	28.7	17.6
HuA-Itgn[e]	3	271	86.6 ± 5.9	20.1	17.4
ApoE$^{-/-}$	6	71	71.1 ± 17.0	50.0	35.6
E$^{-/-}$A$^{-/-}$	16	0	55.6 ± 9.2[c]	87.8	48.8

[a] $P < 0.0001$ versus C57.
[b] $P < 0.0045$ versus apoA-I$^{-/-}$.
[c] $P < 0.027$ versus apoA-I$^{-/-}$; $P < 0.008$ versus C57.
[d] ApoA-I$^{-/-}$ mice injected via the retroorbital sinus with 5×10^8 pfu/mouse of adenoviruses carrying a single copy of the human apoA-I gene and bled 3 days post injection (see reference 29 for characterization of virus injection).
[e] Mice in the C57 background transgenic for the human apoA-I gene.

figure and in Fig. 4C, the rabbit sera were fractionated on a different column set (see note in Materials and Methods) than the human and mouse sera, probably accounting for the elution of HDL in different fractions. In contrast to what we have observed in human and rabbit sera, the mouse had two PON AEase peaks. In the C57 mice (Fig. 1C), about 70% of the total PON AEase activity was associated with HDL; the rest was found in the lpid-poor fraction (**Table 2**). No PON activity was observed in VLDL or LDL fractions.

Effect of the presence of apoA-I or absence of apoE and apoA-I on serum PON AEase activity and lipoprotein distribution in mice

ApoA-I is the major apolipoprotein of HDL and it could account for the association of PON AEase with HDL. Using genetically modified mice, we asked whether the level and distribution of PON AEase in serum was dependent on apoA-I in HDL. Table 2 reflects the effect of different levels of apoA-I expression on total PON AEase activity in the serum. In apoA-I$^{-/-}$ mice, there was a 40% reduction in total PON activity compared with wild-type C57 mice. The wild-type PON AEase level appeared to be restored when the apoA-I level was tripled by the administration of an adenovirus encoding human apoA-I to apoA-I$^{-/-}$ animals. The PON AEase activity was slightly elevated above this level in transgenic animals expressing human apoA-I at a steady-state level.

The distribution of PON AEase activity in these mice between HDL-sized particles and the lipid-poor fraction as separated by FPLC was then examined. In comparing the distribution of PON AEase activity (expressed as percentage of total PON AEase activity) in Fig. 1C–F, it is clear that in mice, the proportion of PON AEase activity in the lipid-poor fraction varied inversely with changes in the total serum activity. However, the absolute amount of PON AEase activity in the lipid-poor fraction showed little variation (Table 2), varying between 17 and 22 units in animals expressing either human or mouse apoA-I when apoE is present. Thus, in these animals the variation in total PON AEase activity in the serum is largely a reflection of variation in the HDL-associated enzyme.

To test whether PON AEase travels in lipoproteins containing apoA-I, regardless of the nature of the lipoprotein, we examined the distribution of PON AEase in apoE$^{-/-}$ mice. These mice have an increased apoA-I level in their VLDL, as previously shown by Plump et al. (35). In some mice, we found >40% of the total apoA-I in this fraction (data not shown). In spite of the high apoA-I level in VLDL of the apoE$^{-/-}$ mice, no PON AEase activity was detectable in the VLDL-IDL-LDL region (Fig. 1G), showing that PON AEase did not invariably partition with apoA-I-containing particles. In the apoE$^{-/-}$ mice, although the serum PON AEase level was comparable to wild-type C57 mice, the proportion of HDL-associated PON AEase (50%) was similar to that in the apoA-I$^{-/-}$ mice (56.1%) (Table 2). In addition, the absolute amount of lipid-poor PON AEase was higher (35.6 units) in these mice. When both apoE and apoA-I were absent, the serum PON AEase level was between that seen in the apoA-I$^{-/-}$ and apoE$^{-/-}$ single-gene knockout animals. However, almost all of the PON AEase activity in E$^{-/-}$A$^{-/-}$ mice was found in the lipid-poor fraction (Fig. 1H). To reinforce the designation of the nonHDL fraction as lipid poor, we have monitored the distribution of phospholipid and PON AEase activity in the same profiles (**Fig. 2A–D**). These distributions do, in fact, support the notion of a substantial proportion of PON AEase in the lipid-poor fraction. It is notable that these fractions are not completely devoid of phospholipid (Fig. 2A–D) and cholesterol (data not shown), except in the apoE$^{-/-}$ (Fig. 2C) and E$^{-/-}$A$^{-/-}$ (Fig. 2D) mice, in which the phospholipids were found mostly in the VLDL and IDL-LDL. In these animals, the PON AEase is found in fractions that contain little detectable lipid. Thus, PON AEase associates with either apoA-I- or apoE-containing HDL-sized particles, and in the absence of these apolipoproteins, PON AEase distributes largely to the lipid-poor fraction.

Changes in PON AEase activity and distribution during the acute phase response

Van Lenten and colleagues (23) have shown that, accompanying the acute phase response, HDL is converted from an antiinflammatory to a proinflammatory particle.

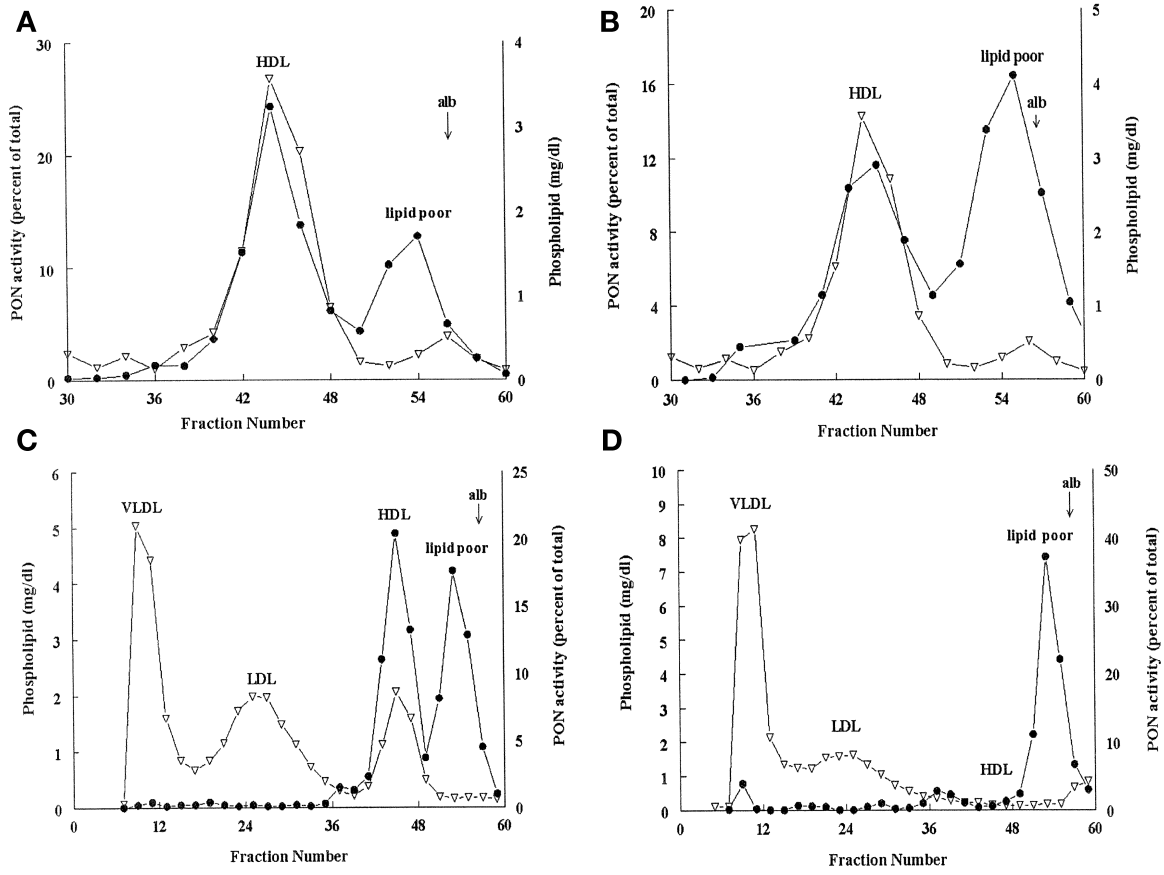

Fig. 2. Distribution of PON AEase (closed circle) and phospholipid (open triangle) in different mouse strain. A: C57 (n = 2). B: ApoA-I$^{-/-}$ (n = 4). C: ApoE$^{-/-}$ (n = 4). D: E$^{-/-}$A$^{-/-}$ (n = 4). Since the C57 and apoA-I$^{-/-}$ mice do not have significant amounts of lipids in the VLDL and LDL regions, only the HDL and lipid-poor fractions were included in A and B to highlight the position of the lipid-poor PON peak. ApoE$^{-/-}$ and E$^{-/-}$A$^{-/-}$ have significant amounts of VLDLs and LDLs, and these regions are included in C and D. The cholesterol followed the phospholipid profile, but for the sake of visual clarity was not included in the figure.

This transition could be related to a loss of PON AEase. Accordingly, we compared the changes in PON AEase level and distribution during the acute phase response in the rabbit and mouse. PON AEase levels during the acute phase response in human subjects were not assessed. In rabbits, following injection of croton oil, the levels decreased exponentially as the acute phase response progressed to a nadir of less than one third of the preinjection level at the time of sacrifice at 66 h post injection (Table 1 and **Fig. 3A**). Interestingly, this post injection level brings rabbit PON AEase level into the same range as is found in human and mouse sera. Even with the substantial decrease of PON AEase activity, there was no corresponding change of activity in the lipid-poor fraction (Fig. 3B). However, there is a tendency for the rabbit PON AEase to be associated with smaller particles by 54 h after the initiation of the acute phase response. We have previously shown (27) that HDL decreased in rabbits following induction of the acute phase response. These results suggest that PON AEase declines along with the reduction in HDL, perhaps concomitantly.

In C57 mice, following injection of LPS, PON AEase activity decreased by approximately one third at 24 h. However, unlike the rabbits, PON AEase activity in the mice returned to baseline within 72 h of the LPS injection (Fig. 3C). Although the total PON AEase activity declined during the acute phase response in the mouse, there were no changes in PON AEase distribution (Fig. 3D) up to 96 h post LPS injection. Therefore, at least in these two species, induction of the acute phase response does not change PON AEase distribution in the sera. The distribution of PON AEase does not track with SAA, which is found almost exclusively in the lipid-rich fractions (31), even in the E$^{-/-}$A$^{-/-}$ mice, which had <15% of their SAA in the lipid-poor fraction, even after density gradient centrifugation (data not shown).

While the absolute level of the lipid-poor PON in mice remains fairly constant regardless of the variation of HDL level as a function of apoA-I expression level (Table 2), this is not the case in the acute phase response. Although the HDL level does not fall in the acute phase response in the mouse (28), the PON AEase level does (Table 1). However, the decline in PON AEase (Table 1 and Fig. 3) is reflected in both the HDL and lipid-poor fractions so that the relative distribution remains unchanged. In the rabbits, there is a substantial decline in HDL and PON AEase during the acute phase response, and no appearance of lipid-poor PON AEase.

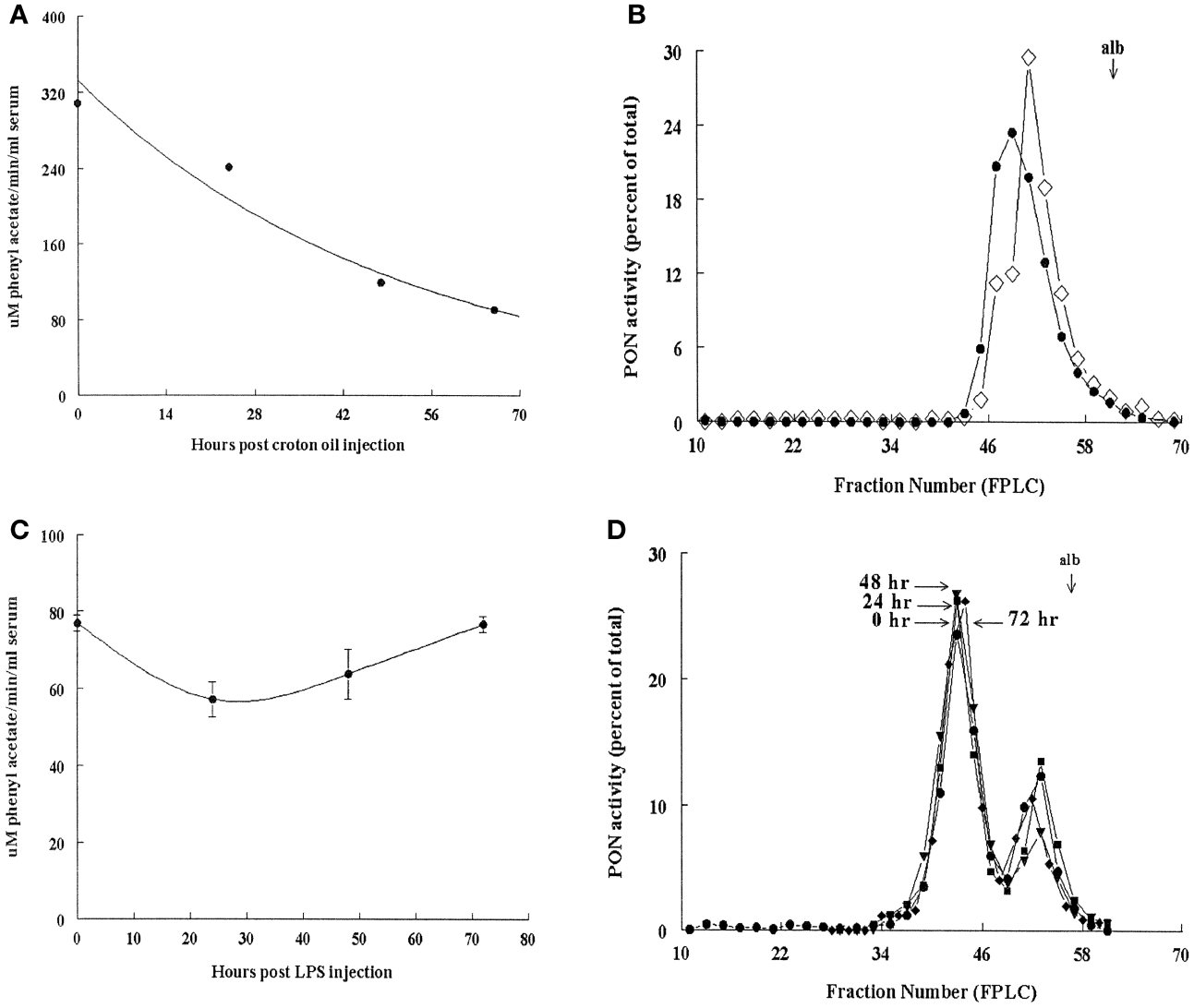

Fig. 3. Serum PON AEase activity following induction of the acute phase response. A: Changes of serum PON AEase levels in the rabbit following induction of the acute phase response by croton oil (n = 2). B: Distribution of PON AEase in rabbit serum (representative of n = 2) before (closed circle) and at 54 h (open diamond) following injection of croton oil. C: Changes in serum PON AEase levels in C57 mice (n = 2) following injection of lipopolysaccharide (LPS). D: Distribution of PON AEase in C57 serum (n = 2) before (circle) and at 24 h (square), 48 h (triangle), and 72 h (diamond) following the injection of LPS.

Effect of incubation with SAA upon the distribution of PON AEase activity

The distribution patterns of PON AEase activity in the three species studied here suggest that PON is less stably associated with HDL in the mouse than in either rabbits or humans. It has been shown that HDL becomes proinflammatory during the acute phase response, possibly by loss of PON from HDL (23). Acute phase HDL is enriched with SAA. Whether the increment in SAA in the acute phase response displaces PON AEase from HDL in vivo is not known. In this study, we have used recombinant SAA as an in vitro probe of a capacity to displace PON AEase from HDL fractions of the three species being examined here. Incubation of normal human serum (n = 3) with up to 1,200 μg of recombinant human SAA1.2 per milliliter of serum had no discernible effect on the distribution of PON AEase activity (**Fig. 4A**). Incubation of normal rabbit serum (n = 2) with increasing amounts of recombinant human SAA1.2 resulted in a small but dose-dependent decrease of HDL-associated PON AEase and an increase of PON AEase activity in the lipid-poor fraction, reaching 19% of total activity in the latter with 1,200 μg SAA per milliliter of serum (Fig. 4B). In contrast, incubation of normal mouse (C57) (n = 3) serum with only 600 μg of recombinant mouse SAA1 per milliliter of serum (the level commonly found during the acute phase response) (Fig. 4C) or human SAA 1.2 (data not shown) resulted in a decrease in the HDL-associated PON AEase with a significant increase in the lipid-poor fraction. Incubation with 1,200 μg SAA per milliliter of serum displaced most of the PON AEase to the lipid-poor fraction. We have previously shown (36) that the primary sequence of apoA-I

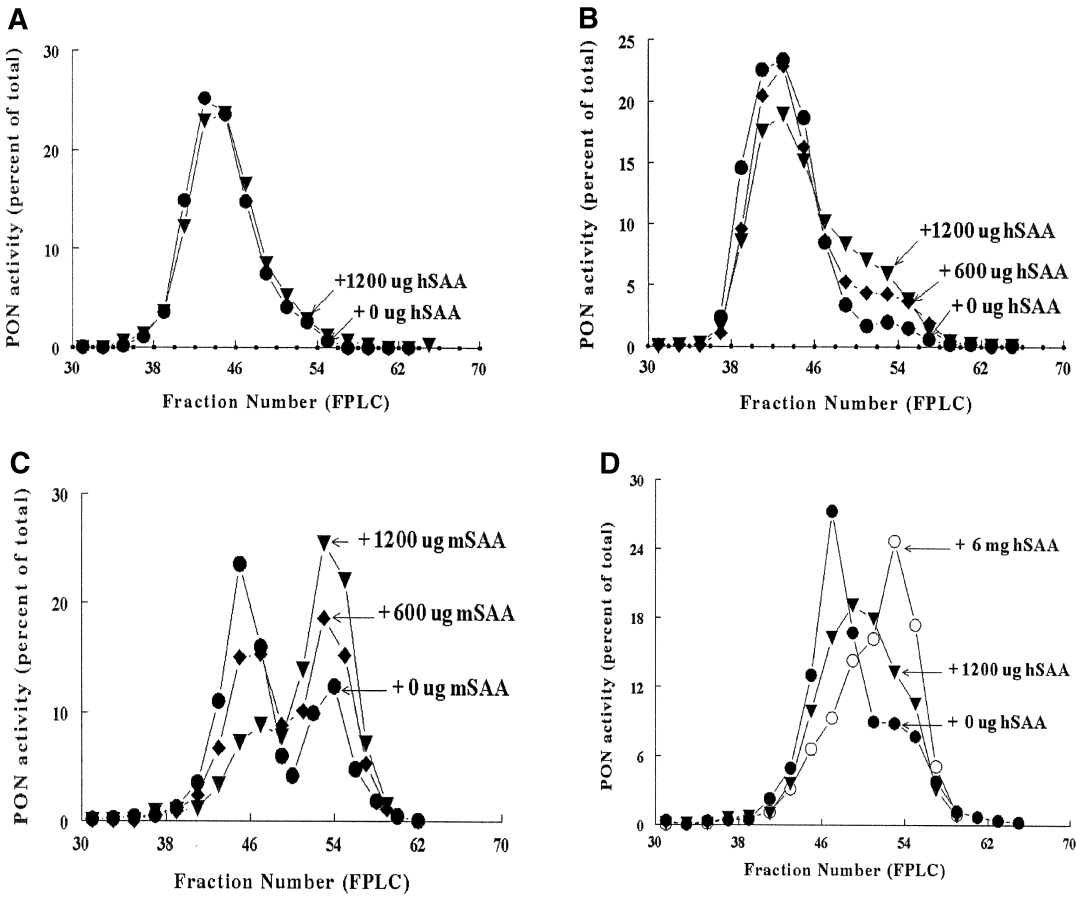

Fig. 4. Changes in PON AEase distribution following incubation with recombinant serum amyloid A (SAA) (expressed as per milliliters serum). A: Human serum (n = 3) incubated alone (circle) or with 1,200 μg of recombinant human SAA (triangle). B: Rabbit serum (n = 2) incubated alone (circle) or with 600 μg (diamond) or 1,200 μg (triangle) of recombinant human SAA. C: C57 serum (n = 3) incubated alone (circle) or with 600 μg (diamond) or 1,200 μg (triangle) recombinant mouse SAA. D: Human apoA-I transgenic mice incubated alone (circle), or with 1,200 μg (triangle) or 6 mg (open circle) recombinant human SAA.

influences its lipoprotein distribution. To test whether the distribution of mouse PON between HDL and the lipid-poor fraction may be sensitive to the nature of the apoA-I sequence in the HDL, sera from human AItgn mice were incubated with SAA. Incubation with 1,200 μg of recombinant human SAA1.2 per milliliter of serum, which almost completely displaced PON AEase from mouse HDL, resulted in only a partial displacement of PON AEase to the lipid-poor fraction in the AItgn mice (Fig. 4D). With the HDL of these latter animals, a higher amount of SAA (6 mg/ml serum) was needed to displace most of the PON AEase to the lipid-poor fraction. Thus, SAA shows a substantial displacement of PON AEase from murine but not from human HDL, and the presence of human apoA-I in the murine HDL attenuated the displacement of PON AEase by SAA.

In all the sera analyzed from the different species and the different strains of mice, PON consistently eluted in similar fractions whether in the presence or absence of apoA-I. In all of the mouse strains tested, the lipid-poor PON AEase eluted slightly ahead of albumin (Figs. 1, 2). Whether these are very small, protein-rich particles containing small amounts of lipids or represent aggregates of free PON associated with itself or with partner protein(s) has not been determined. Upon centrifugal flotation, >90% of the PON AEase activity was found in the lipid-poor bottom fraction of mouse serum, suggesting that PON was only loosely bound to HDL (**Fig. 5A**, **B**). Even in human serum where PON AEase was found exclusively in HDL, following high-salt centrifugation, most of the PON AEase activity was found in the lipid-poor fraction (Fig. 5C). ApoA-I does not redistribute to these fractions upon high-salt centrifugation.

DISCUSSION

This study reports a number of significant findings. First, PON AEase activity is uniquely associated with HDL in humans and rabbits but not in mice, where there is a significant but consistent amount of enzyme activity associated with a lipid-poor fraction as separated by FPLC. Second, apoA-I is not an absolute requirement for the PON AEase association with HDL. However, induced variations in serum apoA-I level are correlated with changes in HDL-associated PON AEase activity, while the level of lipid-poor

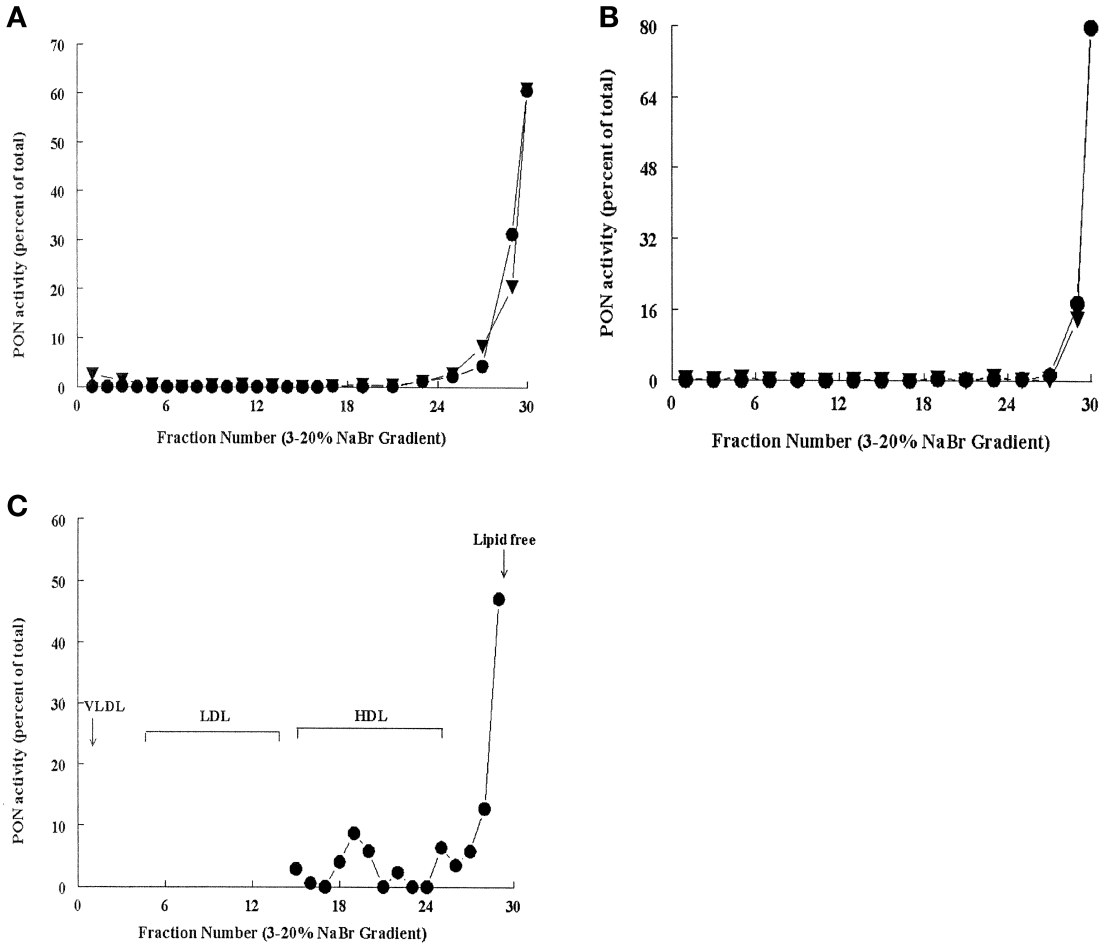

Fig. 5. Displacement of PON AEase by high salt during ultracentrifugal flotation. Two hundred microliters of serum was fractionated in the 3–20% NaBr gradient without EDTA. After dialysis in PBS, 40 μl of fraction was analyzed for PON activity. All results are expressed as percent of total. A: C57. B: ApoA-I$^{-/-}$ at baseline (circle) and 24 h post LPS injection (triangle). C: Human serum at baseline.

PON activity remains fairly constant. Third, PON AEase associates mostly with either apoA-I- or apoE-containing HDL-sized particles, and in the absence of both these apolipoproteins, PON AEase distributes almost exclusively to the lipid-poor fraction. Fourth, PON AEase activity does not associate with apoA-I when it is present in nonHDL lipoprotein fractions. Fifth, PON AEase activity can be displaced from HDL in vitro by incubation with recombinant human or murine SAA1. This occurs with rabbit HDL and more dramatically with murine HDL, but not with human HDL. In mouse serum containing human apoA-I as transgene, the PON AEase is less readily displaced from this HDL than that from HDL containing only mouse apoA-I.

One of the properties of HDL that makes it antiatherogenic is its capacity to serve as a reservoir of antioxidant enzymes capable of reducing the titer of oxidized phospholipids in the blood of hyperlipidemic animals and people. PON is one of the two major antioxidative enzymes present in the plasma that limit the accumulation of oxidized phospholipids in plasma lipoproteins. In mice lacking both apoE and PON, there is an increased accumulation of oxidized phospholipids in the plasma and an increase in atherosclerosis attributed to the lack of PON (37). The proposition that the antioxidant enzymes of HDL represent an important attribute of the antiatherogenic function of this lipoprotein suggests that all or most of the PON should be associated with HDL. Indeed, for both humans and rabbits, essentially all of the measurable PON AEase activity is associated with HDL separated by FPLC, a mild fractionation procedure that does not lead to either a dissociation of PON AEase from HDL or an impairment of its activity. On the other hand, a significant proportion (about 20–30%) of the PON AEase activity in wild-type mouse plasma is not associated with the major HDL peak, but is rather found in a lipid-poor fraction. Despite some variation in the absolute level of PON AEase activity in the total plasma under a variety of genetic conditions, there is little variation in the amount of active enzyme in the lipid-poor fraction. The finding of PON AEase activity in the lipid-poor fraction could be an artifact, but we do not believe that the mild preparative and fractionation methods employed are likely to induce such an artifact. The fact that the proportion of lipid-poor PON AEase activity varies as a function of genetic background argues that these distributions are unlikely to be artifactual. The PON AEase activity that eluted with albu-

min is designated lipid poor since this fraction is not completely devoid of phospholipid (Fig. 2 A–D) and cholesterol (data not shown).

Our results in this respect are somewhat different from those reported by Forte et al. (38), who did not find PON activity in the same lipid-poor fractions we report here. There are two differences between their studies and ours. They used a mouse strain of mixed genetic background (DBA × C57BL/6), while we used a more homogeneous C57BL/6 mouse. Second, we did our measurements on sera while they used heparinized plasma. We have compared the distributions of PON AEase activity in sera and heparinized plasma from wild-type C57 and AItgn mice. In both cases, sera and heparinized plasma yielded the same distribution. It is possible that PON does not exist free, but associates with a molecule(s) in the lipid-poor fraction that is limiting. ApoJ would be such a candidate molecule. ApoJ and PON have been found to coisolate (20).

The factors that control the plasma level of PON are poorly understood. It is striking that the rabbit has a so much higher steady-state level of plasma PON AEase activity than the other two species studied here, especially in view of the fact that of these three species, the rabbit is the most susceptible to cholesterol-induced atherosclerosis. It remains to be established whether this represents a higher antioxidant activity of the PON protein in rabbit, a higher specific activity of the rabbit PON protein, or whether the rabbit PON AEase activity is the net result of different proportions of PON1 and PON3 isoforms. The PON activity is much more labile to the acute phase reaction in the rabbit than in the mouse, and the substantial decrement we observed is consistent with the rabbit response to croton oil previously noted by Van Lenten et al. (23).

Steady-state levels in the plasma depend on the rates of synthesis and removal. Although we know relatively little of the rate of PON production by the liver, in the mouse, at least, it seems that the PON AEase in the HDL fraction and in the lipid-poor fraction may turn over at different rates. Our data in the mouse would be consistent with the removal of PON in association with intact HDL being the major mechanism for its disappearance from the plasma. There are several aspects of our observations that point in this direction. First, total PON activity is sustained at only modestly reduced levels, even with very low levels of the HDL, e.g., in the $E^{-/-}A^{-/-}$ mice (Table 2). Second, the level of PON AEase activity is lower in apoA-$I^{-/-}$ than in $apoE^{-/-}$ or wild-type mice. Forte et al. (39) also observed no change in PON activity in $apoE^{-/-}$ mice. In $LCAT^{-/-}$ mice, which have very low apoA-I plasma concentrations, there is also a substantial decline in PON activity (38), and this occurs without any change in hepatic PON mRNA level. This could be attributable to a more rapid disappearance of PON in association with apoE-HDL than with apoA-I-HDL. Third, there is a higher absolute level of lipid-poor PON in the absence of apoE than even in normal mouse plasma. This raises the possibility that apoE may play a significant role in the turnover of PON, especially when PON and apoE coexist on HDL particles. This possibility can only be established with reagents that can measure the mass of PON, and we are in the process of preparing a PON antibody for such measurements.

The results with mouse plasma raise three questions. First, the possibility that the PON AEase activity in the HDL fraction and the lipid-poor fraction are attributable to the same gene product must be considered. This will require antibodies to one or more of the murine PON gene products. The fact that SAA can displace PON AEase activity from the HDL to the lipid-poor fraction suggests that the two fractions may well represent different localizations of the same protein. Second is the question of the equivalence of the antioxidant activity of the PON in HDL and in the lipid-poor fraction. This cannot be assessed without a combined measurement of mass and enzyme activity. It is noteworthy that the arylesterase measurement used to trace PON activity in this and in most studies is not precisely the same as the antioxidant activity. For example, the two activities have different dependencies on calcium (6, 40) and on the state of PON glycosylation (6). There is clearly PON AEase activity in the lipid-poor fraction, though whether this fraction also has antioxidant activity remains to be established. The proximity to oxidized phospholipid substrate can be expected to be different in the phospholipid-rich HDL particle and the phospholipid-poor fraction.

The third question that is most relevant to this study relates to the determinants of PON association with HDL. PON is unusual among proteins secreted from hepatocytes in that it retains its hydrophobic "signal" peptide in the circulating form of the enzyme (41). This peptide is responsible for targeting the primary translation product to the phospholipid-rich endoplasmic reticulum. The same peptide is thought to play an important role in targeting PON to HDL particles (42). PON will target to phospholipid vesicles in the absence of apolipoprotein, though apoA-I is thought to stabilize this association. The colocalization of PON and apoA-I is probably a reflection of the fact that both proteins prefer a phospholipid-rich environment. This probably accounts for the presence of PON AEase in HDL fractions of mice lacking apoA-I. There are two possible interpretations of this finding. Either other HDL apolipoproteins, e.g., apoE, can substitute for apoA-I in stabilizing the HDL association, or the apolipoproteins simply play a role in forming phospholipid-rich particles. In mice lacking both apoA-I and apoE, all of the PON AEase activity was found in the lipid-poor fraction. In these mice, the HDL and lipid-poor fraction had no detectable phospholipid (Fig. 3C, D). $LCAT^{-/-}$ mice, which have markedly reduced HDL-C and apoA-I, also have reduced PON in their plasma, though the latter is not reduced (58%) to the same extent as are the former (90–94%) (39). However, the simple presence of phospholipid concentration on the surface of lipoproteins is not sufficient to furnish a "recipient" lipoprotein particle, as PON AEase does not associate with the phospholipids of LDL or VLDL, even when the latter contains apoA-I, as is the case in $apoE^{-/-}$ plasma lipoproteins. We are thus left with the hypothesis that PON associates with HDL ei-

ther because of its surface components, phospholipid and/or apolipoprotein, or because it prefers phospholipid surfaces of a particular narrow range of radii of curvature. We cannot clearly distinguish these possibilities. Mice expressing human apoA-I as a transgene have an HDL that is more retentive of mouse PON AEase than is HDL containing murine apoA-I. This is based upon the differences in displacement of PON AEase by SAA in vitro (Fig. 4). As human and mouse apoA-I generate HDL particles of different sizes (36), we cannot ascertain whether it is the lipoprotein size, or whether the HDL association of mouse PON AEase is sensitive to the sequence of apoA-I in the HDL with which it associates (36). In considering the relationship between PON and apoA-I, it is worth noting that there are ~40 times as many apoA-I molecules as PON molecules in the plasma [based on the mass results of Kujiraoka et al. (25), who reported the concentration of PON as 59.3 ± 1.3 μg/ml]. Thus, PON can only be associated with a small subset of HDL particles.

While apoA-I is not obligatorily required for the association of PON with HDL, its absence does influence the level and distribution of PON. The total PON AEase activity is lower in apoA-I$^{-/-}$ mice, and a greater proportion of the PON AEase activity is in the lipid-poor fraction. Even in human plasma deficient in apoA-I, an increased proportion of PON is found in a lipid-poor fraction (43). This distribution pattern could influence the risk of cardiovascular disease in these patients.

Our results of PON AEase distribution in mice lacking both apoA-I and apoE lead to speculation about the mechanism that accounts for PON secretion from the livers of these animals, which clearly secrete into the plasma almost normal amounts of the enzyme. A recent report on the secretion of PON from transfected CHO cells suggests that phospholipid and/or HDL may be necessary for the release of the enzyme from these cells (10). However, in our double knockout E$^{-/-}$A$^{-/-}$ animals, which have very little phospholipid in the HDL size range, there is significant PON in the lipid-poor fraction of the serum. A likely difference between CHO cells and livers in vivo could be that liver secretion of PON is in association with a "chaperone," which may be another lipoprotein or nonlipoprotein protein chaperone, e.g., apoJ.

Our results with serum PON AEase during the acute phase response in rabbits and mice are not easy to explain. In both species, the acute phase response results in a reduction of PON AEase activity much more profound in the rabbit than in the mouse (Table 1). Our in vitro displacement studies, on the other hand, suggest that the acute phase reactant, SAA, has a much greater capacity to displace PON AEase from murine than from rabbit or human HDL (Fig. 4). Thus, we suggest that the loss of PON AEase from rabbit HDL during the acute phase response is not due simply to the large increase of SAA in acute phase rabbit plasma and HDL. Van Lenten et al. (23) have shown that during the acute phase response, rabbit HDL becomes proinflammatory, and they relate this to the displacement of PON by SAA, among other changes. If this is the case, the PON must be rapidly cleared, as there is no evidence during the acute phase (Fig. 3B) of a new peak of PON activity in acute phase rabbit serum. Also, in mouse serum during the acute phase response (Fig. 3D), there is no major displacement of PON AEase activity. Taken together, these observations suggest that normal HDL and acute phase HDL are quite different particles. For example, acute phase HDL acquires ceruloplasmin, which could account for its loss of antioxidant activity (44).

One basis for the differences in PON AEase distribution among the three species studied here may rest upon the relative affinity of PON for HDL, whether lipid or apolipoprotein, of these three species. We used recombinant SAA incubated with HDL derived from each of the three species to test this proposition. In keeping with the different PON AEase distribution and not with the PON AEase response to the acute inflammation induced by croton oil and LPS, we found that recombinant SAA displaced PON AEase from HDL in the series of increasing displacement from human to rabbit to mouse, with substantial displacement evident in the mouse. This difference in retention on the HDL of these three species could depend upon any of several factors: on differences in PON primary sequences; on differences in apoA-I sequences; on differences in the interaction of PON with other molecules found on HDL, including their resident apolipoproteins; or on differences in HDL size and composition.

In conclusion, PON AEase selectively associates with HDL or HDL-sized particles, regardless of their specific apolipoprotein composition. In the mouse, at least a significant proportion of PON AEase is found in a lipid-poor fraction, and this partitioning of PON could influence its total antioxidant capability in the plasma. It is clear that much further work is required to resolve the issues emanating from these studies.

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