Separation and quantitative recovery of mouse serum arylesterase and carboxylesterase activity

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Abstract Paraoxonase-1 (PON1) is known to be associated with high density lipoproteins. We optimized buffer conditions to obtain quantitative recovery of PON1 (arylesterase) activity and analyzed the distribution of PON1 in mice using a combination of size-exclusion chromatography and ultracentrifugation. Size-exclusion chromatography of mouse serum separated the esterase activity into two peaks, one overlapping the high density lipoproteins and a second peak of lower molecular weight, consistent with serum carboxylesterase, which accounted for ~20% of the total esterase activity of normal mouse serum. Using conditions for the quantitative recovery of arylesterase activity, we fractionated serum by ultracentrifugation into d $<$ 1.21 g/ml, d $<$ 1.25 g/ml, d $>$ 1.21 g/ml, and d $>$ 1.25 g/ml fractions. We observed that PON1 arylesterase activity and mass were isolated in the d $<$ 1.21 g/ml fraction and that serum carboxylesterase was recovered in the d $>$ 1.25 g/ml fraction. The significance of the confounding of PON1 arylesterase activity by serum carboxylesterase was demonstrated by studying mice challenged with a high-fat, high-cholate diet for 14 days. It was shown that all of the decrease in arylesterase activity in response to this diet is attributable to the HDL-associated arylesterase activity (PON1). We conclude that mouse PON1 is quantitatively associated with high density lipoproteins. The contribution of serum carboxylesterase to the total esterase activity significantly confounds the interpretation of total arylesterase activity in mouse serum.—Connelly, P. W., G. F. Maguire, and D. I. Draganov. **Separation and quantitative recovery of mouse serum arylesterase and carboxylesterase activity.** ***J. Lipid Res.*** **2004.** 45: **561–566.**

Supplementary key words paraoxonase-1 • high density lipoproteins • diet

Paraoxonase-1 (PON1) has been identified as a component of HDL that may have a role in protecting LDL from copper-mediated oxidative modification (1–4). HDL is heterogeneous and can be defined by density, by size, or, using immunoaffinity chromatography, by apolipoprotein. The uncleaved N-terminal signal peptide of PON1 is necessary for the binding of PON1 to phospholipids (5) and the desorption from cells to HDL (6). As part of our studies of various transgenic and knockout mouse strains (7), we observed a quantitative association of PON1 arylesterase activity with HDL from C57Bl/6 mice when serum was separated by size-exclusion chromatography using Superose-6 but a poor recovery of PON1 arylesterase activity from serum after ultracentrifugation to isolate HDL. Furthermore, we observed a bimodal distribution of the arylesterase activity. This contrasts with the unimodal nature of normal mouse HDL (8). It also became apparent that serum carboxylesterase (CEase), an esterase that is present in relatively high concentrations in mouse serum (9), was a significant confounder when studying PON1 arylesterase with phenyl acetate as a substrate. This led us to review the conditions used in the isolation of lipoproteins for compatibility with maintaining serum arylesterase activity.

We and others typically isolate HDL from serum by ultracentrifugation between densities of 1.063 and 1.21 g/ml and remove excess salt by dialysis against phosphate-buffered saline without EDTA (1–4, 10, 11). We now report that the lipoprotein-associated serum arylesterase is inactivated during dialysis when time and buffer conditions typical for lipoprotein isolation are used. We focus on mouse serum arylesterase activity and show that it could be quantitatively preserved by decreasing the dialysis time and using buffers containing 2 mM calcium.

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Abbreviations: CEase, serum carboxylesterase; DTPA, diethylenetriamine penta-acetic acid; FPLC, fast-protein liquid chromatography; PON1, paraoxonase-1.

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MATERIALS AND METHODS

Animals

C57Bl/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and housed in the St. Michael's Hospital vivarium. Mice were fed a standard chow diet containing 4.5% fat (Labdiet 5001; PMI Nutrition International, Richmond, IN) or the atherogenic diet containing 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate (TD 90221; Harlan Teklad, Madison, WI) for 14 days. Animal protocols were approved by the St. Michael's Hospital Animal Care Committee.

Isolation of lipoproteins

Serum samples were obtained from C57Bl/6 mice after a 12 h fast. Two to 5 ml of serum was placed in a heat-sealable polyallomer tube (Quick-seal tubes; Beckman Coulter Canada, Inc.). Solid KBr was added to increase the density to 1.21 or 1.25 g/ml, and the solution was overlayered with d = 1.21 g/ml or d = 1.25 g/ml KBr, pH 7.5, containing 2 mM $CaCl_2$ and 100 μM diethylenetriamine penta-acetic acid (DTPA). The tube was centrifuged in a 50.4 rotor (Beckman Coulter Canada, Inc.) at 37,000 rpm (145,000 *g*) for 44 h at 10°C. The supernatant (1.006–1.21 or 1.006–1.25 g/ml) was removed and stored at 4°C. The 1.21–1.25 fraction was isolated by the addition of KBr to the infranatant from the 1.21 g/ml centrifugation to increase the density to 1.25 g/ml. This solution was overlayered with d 1.25 g/ml KBr, pH 7.5, containing 2 mM $CaCl_2$ and 100 μM DTPA and centrifuged as described above. All fractions were stored at 7–10°C before Superose 6HR chromatography.

Fast-protein liquid chromatography of lipoproteins

Samples were not dialyzed before separation on Superose 6HR. The chromatography itself serves as a desalting step; thus, the losses in PON1 activity associated with dialysis of samples (vide infra) can be avoided. Samples were applied to a Superose 6HR 10 mm × 30 cm column (Pharmacia Biotech). A 10 mM Tris-HCl, pH 7.4, buffer containing 2 mM $CaCl_2$, 100 μM DTPA, and 0.02% sodium azide was used to separate serum fractions. Typically, 100–200 μl of serum or ultracentrifuge fraction was applied using a Rheodyne 9725i polyether ether ketone (PEEK) injector with a 200 μl PEEK Upchurch sample loop. Samples were filtered through a 0.45 μm regenerated cellulose or polyvinylidene difluoride micro-spin filter (Alltech) before chromatography. Chromatography was done using a Beckman Coulter 126 solvent module at a flow rate of 0.5 ml/min with collection of fractions every minute. The system was fitted with an Upchurch PEEK prefilter holder equipped with a 2 μm PEEK frit.

To maintain the column and system in good working order, after every 20 samples, a 50 μl aliquot of 50% ethanol was injected and washed through with 15 ml of running buffer. This was then followed by an injection of 50 μl of 0.01% Tween 20 in water that was washed through with another 15 ml of running buffer. We have found that this combination of PEEK precolumn frit and column washing prevented increases in back pressure that occurred when a steel precolumn frit was used, particularly with mouse serum samples.

Dialysis

Dialysis membranes may have substantial metal content. Thus, the membranes (molecular weight cutoff 6,000–8,000; regenerated cellulose; Fisher Scientific, Mississauga, Ontario, Canada) were treated before use according to the manufacturer's protocol. Specifically, while stirring, membranes were heated at 80°C for 30 min in 1 liter of 10 mM sodium bicarbonate and then soaked for 30 min in 1 liter of 10 mM Na_2EDTA. Membranes were then stirred at 80°C for 30 min in 1 liter of distilled water, cooled, and stored in 0.05% sodium azide. Immediately before use, membranes were washed inside and out with distilled water.

Samples were transferred to pretreated dialysis bags and placed in 1 liter of dialysis buffer consisting of either 10 mM phosphate-buffered saline, pH 7.4, 2 mM $CaCl_2$, and 100 μM DTPA or 10 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$, and 100 μM DTPA in a high-density polypropylene container and gently stirred for 6 h at 7–10°C, at which time buffer was replaced and stirred for another 16 h. After dialysis, samples were transferred to polypropylene tubes and stored at 7–10°C.

Assay of arylesterase activity

Typically, 5 μl of serum was added to a total volume of 1 ml containing 10 mM phenyl acetate in 20 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$. In the case of ultracentrifugal fractions, the enzyme was assayed before dialysis using a 10–20 μl sample. An aliquot of each Superose fraction was assayed (25–200 μl depending on the PON-1 activity). The increase in OD at 270 nm was monitored every 3 s for 30 s using a Milton Roy Spectronic 1001 Plus spectrophotometer. Activities are reported as units per milliliter, where 1 U is defined as 1 μmol of phenyl acetate hydrolyzed per minute.

Assay of carboxylesterase activity

Sera were diluted 160 times with 0.15 M NaCl, pH 7.4. A 20 μl aliquot was added to 200 μl of 0.48 mM *p*-nitrophenyl valerate in 50 mM HEPES, pH 7.0. Aliquots of each Superose fraction were assayed (5–20 μl depending on activity). The increase in OD at 405 nm was followed between 5 and 20 min using a MCC 340 microtiter plate reader (Flow Laboratories). Activities are reported as units per milliliter, where 1 U is defined as 1 μmol of *p*-nitrophenyl valerate hydrolyzed per minute.

Measurement of lipids

All enzymatic lipid assays were performed using a Technicon RA1000 analyzer. The volume and proportion of each reagent were optimized for mouse samples. Total cholesterol was measured using enzymatic kit T01-1684-01, total triglyceride was measured using enzymatic kit T01-1868-02, and free glycerol was measured using enzymatic kit T01-2013-01 (Bayer, Mississauga, Ontario, Canada). Choline phospholipids were measured using enzymatic kit 990-54009, and free cholesterol was measured using enzymatic kit 274-47109 (Wako Chemicals, Richmond, VA).

Immunoblot for PON1

PON1 mouse monoclonal antibody 4C10.28 was raised against purified human serum PON1 at the University of Michigan Hybridoma Core and affinity-purified from ascites (9.9 mg/ml). The mouse d $<$ 1.21 g/ml serum fraction was separated by Superose 6HR chromatography. For analysis, 0.1 ml of each 0.5 ml fraction was lyophilized, redissolved in 100 μl of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 10 mM DTT, and 20 μl was applied to an 11% SDS-PAGE gel. Proteins were transferred to polyvinylidene difluoride membranes for Western blotting. Primary antibody was used at a 1:1,000 dilution. Secondary antibody was goat anti-mouse IgG/IgM alkaline phosphatase (Aurora ICN Biomedicals, Inc., Costa Mesa, CA) diluted 1:5,000. The immunoblot was visualized using Starlight chemiluminescent substrate and Optimembrane reagent (Aurora), and the signal was captured on Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

RESULTS

The peak of PON1 arylesterase activity precedes the peak of HDL cholesterol by size-exclusion chromatography

We profiled the distribution of arylesterase activity and serum cholesterol in C57Bl/6 mice by Superose-6 chro-

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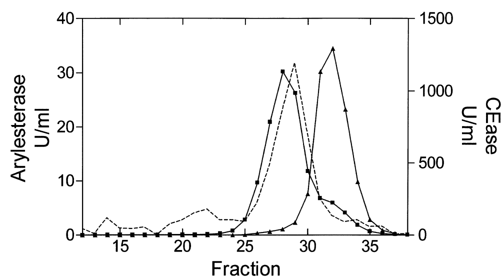

Fig. 1. Superose 6HR chromatography of pooled mouse serum. Arylesterase was determined using phenyl acetate as substrate (closed squares, left axis), and serum carboxylesterase (CEase) was determined using *p*-nitrophenyl valerate as substrate (closed triangles, right axis), as described in Materials and Methods. For reference, the distribution of cholesterol is superimposed as the dashed line.

matography (**Fig. 1**). We consistently observed that PON1 arylesterase activity preceded the peak of HDL cholesterol by one fraction. This relationship implied that PON1 was found predominantly in larger HDL particles. Mouse HDL is considered to be unimodal (12). However, larger, asymmetric, pre-β-HDL particles overlap spherical HDL particles when separated on the basis of size (13). Thus, the distribution of arylesterase activity was further investigated using ultracentrifugation.

Ultracentrifugation and the recovery of PON1 arylesterase activity

We used a common protocol for lipoprotein isolation by ultracentrifugation but included 2 mM $CaCl_2$ and 100 μM DTPA, instead of EDTA, to preserve the PON1 activity.

Dialysis of serum or serum fractions against either phosphate-buffered saline or Tris-buffered saline, without the inclusion of $CaCl_2$ and DTPA, resulted in the loss of 80% and 60% of the arylesterase activity, respectively (data not shown). Inclusion of 2 mM $CaCl_2$ in either buffer typically increased recoveries to 80%. Thus, avoiding buffers that bind Ca^{2+} (e.g., phosphate) and controlling the conditions of dialysis (duration, membrane pretreatment) are important for the reproducible recovery of PON1 arylesterase activity. We found that direct application of fractions to Superose 6, without dialysis, was an effective means to determine PON1 activity and lipoprotein distribution. We concluded that the arylesterase activity of PON1 survives ultracentrifugation in high-salt solutions but requires millimolar concentrations of Ca^{2+} in buffers used for salt removal by dialysis.

Mouse PON1 arylesterase is associated with HDL

The C57Bl/6 mouse is used in studies of atherosclerosis and as a background strain in many transgenic and knockout mouse models of lipoprotein metabolism. We collected serum from these mice after an overnight fast and separated the serum by ultracentrifugation into d < 1.21 g/ml, d = 1.21–1.25 g/ml, and d > 1.25 g/ml fractions. PON1 arylesterase activity was recovered to the same extent in both the d < 1.21 g/ml and d < 1.25 g/ml fractions (**Table 1**), suggesting that there was little or no PON1 arylesterase in the d > 1.21 g/ml fraction. The d = 1.21–1.25 g/ml fraction of mouse serum had minimal arylesterase activity (Table 1). The recovery of cholesterol was 94% and that of arylesterase was 84% when serum was separated with a single ultracentrifugation at d = 1.21 g/ml. The ultracentrifugal fractions were separated on a Superose 6HR column, and the resulting fractions were analyzed for cholesterol, arylesterase, and carboxylesterase distribution (**Fig. 2**). The bimodal distribution of arylesterase activity that was observed with whole serum (Fig. 1) was separated after ultracentrifugation into the peak from fractions 24–32 within the d < 1.21 g/ml fraction (Fig. 2, upper panel) and into the peak from fractions 30–35 within the d > 1.21 g/ml fraction (Fig. 2, lower panel). The results for the d > 1.25 g/ml fraction were essentially identical to those observed for the d > 1.21 g/ml fraction.

The distribution of PON1 protein was semiquantitatively estimated by Western blot analysis of Superose 6HR column fractions after separation of the proteins by SDS-polyacrylamide gel electrophoresis. We observed that mouse serum proteins interfered with the immunoblot analysis in the molecular weight region containing PON1; thus, whole serum and d > 1.25 g/ml fractions could not be analyzed (data not shown). The immunoblot of PON1 for the d < 1.21 g/ml fraction separated by Superose 6HR chromatography showed a unimodal distribution, with the peak of immunoreactivity coincident with the peak of arylesterase activity at fraction 28 (data not shown). The cross-reaction of this anti-human PON1 antibody with mouse PON1 was specific but less sensitive than enzymatic activity assays. Thus, the absence of detection of PON1 by Western blot could not be taken as evidence for the absence of PON1.

TABLE 1. Distribution and recovery of cholesterol, arylesterase, and serum carboxylesterase after ultracentrifugation of pooled mouse serum

Sample	Serum	d = 1.006–1.21	d > 1.21	Recovery	d = 1.006–1.25	d > 1.25	Recovery	d = 1.21–1.25
Cholesterol (mmol/l)	1.81, 1.57	1.66, 1.46	0.04, 0.08	91%, 99%	1.60, 1.45	0, 0.08	97%, 97%	0, 0.01
Arylesterase (U/ml)	162, 118	105, 72.5	31.5, 18.3	81%, 77%	104, 70.0	24.0, 19.3	79%, 76%	2.9, 0.2
Serum carboxylesterase (U/ml)	ND, 5023	ND, 34	ND, 4187	ND, 84%	ND, 36	ND, 4664	ND, 94%	ND, 1

The density of mouse serum was increased to 1.21 g/ml with solid KBr, overlayered with 1.21 g/ml KBr, 2 mM $CaCl_2$, and 100 μM diethylenetriamine penta-acetic acid (DTPA), and separated by ultracentrifugation into d = 1.006–1.21 g/ml supernatant and the d > 1.21 g/ml infranatant. The d > 1.21 g/ml infranatant was adjusted to a density of 1.25 g/ml with solid KBr and separated by ultracentrifugation into the d = 1.21–1.25 g/ml fraction and the d > 1.25 g/ml infranatant. The density of a separate aliquot of serum was increased to 1.25 g/ml as above and overlayered with 1.25 g/ml KBr, 2 mM $CaCl_2$, and 100 μM DTPA. The results shown are from two separate experiments. ND, not determined.

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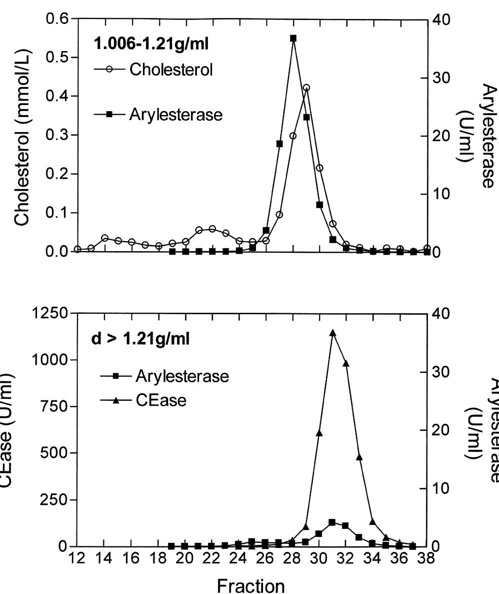

Fig. 2. Superose 6HR chromatography of ultracentrifugal fractions of pooled mouse serum. Upper panel: d < 1.21 g/ml fraction showing cholesterol (left axis) and arylesterase activity (right axis). Lower panel: d > 1.21 g/ml fraction showing arylesterase activity (right axis) and CEase activity (left axis). Cholesterol and esterase activities were analyzed as described in Materials and Methods.

CEase was recognized as a confounder in the use of phenyl acetate to assay PON1-specific arylesterase. We used *p*-nitrophenyl valerate to assay the CEase and observed a symmetrical, unimodal distribution that overlapped the tail of the HDL-associated arylesterase peak (PON1) and that was coincident with the second esterase peak. The CEase activity was quantitatively recovered in the d > 1.25 g/ml fraction (Fig. 2). The Superose 6HR profile of the CEase activity of the d > 1.25 g/ml fraction was completely coincident with that of whole serum, indicating that *p*-nitrophenyl valerate hydrolysis was an effective means of distinguishing between PON1 and CEase contributions to the esterase activity. From the ratio of the enzymatic activities with *p*-nitrophenyl valerate and phenyl acetate in the fractions lacking PON1, we were able to calculate the CEase contribution to the esterase activity in the fractions in which the two enzymes coelute. Using this ratio as a correction factor, the esterase activities of fractions 30 and 31 were adjusted to obtain a "corrected" PON1 arylesterase activity.

Differential response of lipoprotein and nonlipoprotein arylesterase to dietary cholate and cholesterol

The C57Bl/6 mouse is known to be responsive to a diet rich in saturated fat, cholate, and cholesterol (14). This diet has a combined effect of increasing VLDL plus LDL cholesterol and at the same time reducing HDL cholesterol. It has been reported that the diet also decreases the serum arylesterase activity (15). The changes in HDL reach a maximum response within 7 days after beginning the diet (15). C57Bl/6 mice were placed on the diet, and serum samples were collected at days 0, 3, 7, and 14. The serum was separated by fast-protein liquid chromatography (FPLC) and assayed for lipids and arylesterase activity using phenyl acetate. The results are shown in **Figs. 3** and **4**. Total serum arylesterase activity was reduced by 31% at day 3 and by 54% at day 7 (Figs. 3 and 4A). HDL cholesterol was reduced by 20% at day 3 and by 50% at day 7 (Figs. 3 and 4B). VLDL and LDL cholesterol increased from day 3 to day 7 (Figs. 3 and 4B). Total serum choline

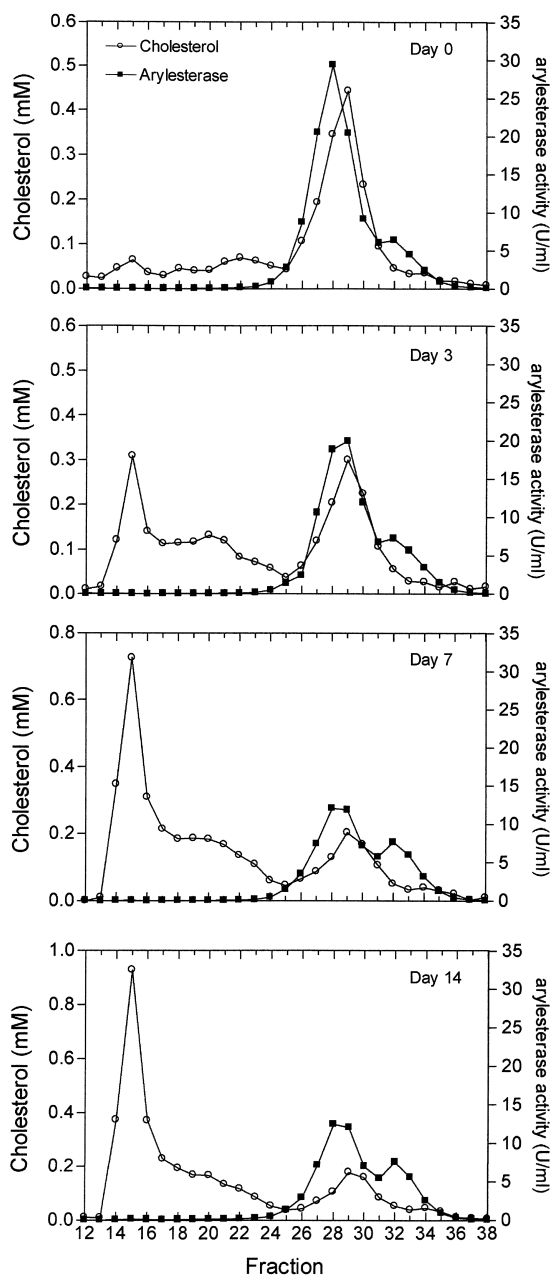

Fig. 3. The effect of a high-fat, high-cholate diet studied by Superose 6HR chromatography of mouse serum. Values shown are means of three pools (four mice in each pool). Day 0 represents sera from C57Bl/6 mice fed a standard chow diet containing 4.5% fat. These mice were then placed on the atherogenic diet, containing 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate, and serum was drawn on days 3, 7, and 14. Cholesterol and paraoxonase-1 arylesterase activities were analyzed as described in Materials and Methods.

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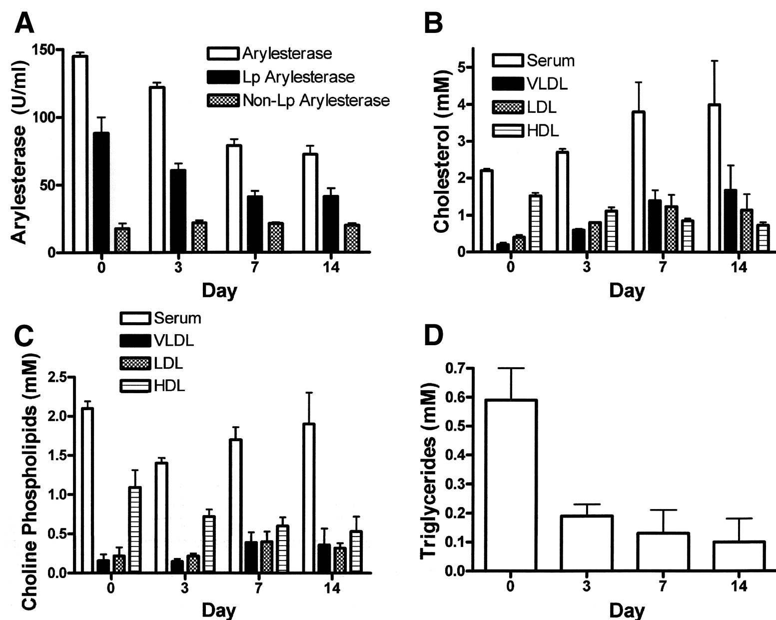

Fig. 4. Time course of arylesterase activity and lipids in serum of mice on the atherogenic diet at days 0, 3, 7, and 14. A: Total serum, lipoprotein (Lp)-associated, and nonlipoprotein (Non-Lp)-associated arylesterase activity. B: Total serum, VLDL, LDL, and HDL cholesterol. C: Total serum, VLDL, LDL, and HDL choline phospholipids. D: Total serum triglycerides. The means ± SD are shown.

phospholipids decreased at day 3 because of the decrease in HDL and increased on days 7 and 14, in parallel with the increase in VLDL and LDL (Fig. 4C). Total serum triglycerides (corrected for serum-free glycerol) decreased from day 3 to day 14 and were too low to reproducibly measure in the individual lipoprotein fractions (Fig. 4D).

Total serum esterase activity was defined by FPLC as lipoprotein-associated (fractions 12–30) and as nonlipoprotein-associated (fractions 31–39) (Figs. 3 and 4A). The lipoprotein-associated activity was reduced by the diet, whereas the nonlipoprotein-associated activity was unaffected by diet. The separation of esterase activity into two fractions had a major impact on the correlation with HDL cholesterol values (**Fig. 5**). Total esterase activity showed a sigmoidal relationship with HDL cholesterol. In contrast, lipoprotein-associated arylesterase activity showed a linear relationship with HDL cholesterol with a high correlation (r = 0.9). The effect of the diet was such that, at day 0, 80% of the serum esterase activity was lipoprotein-associated arylesterase, and at day 14, only 50% of the total serum esterase activity was lipoprotein-associated arylesterase.

DISCUSSION

We observed the quantitative recovery of PON1 arylesterase activity, the unimodal distribution of activity in whole serum and in the d < 1.21 g/ml fraction, and coincident size distribution for activity and mass of PON1. These results indicated that mouse serum contains two major esterases, PON1, quantitatively associated with HDL, and CEase, quantitatively recovered in the d > 1.25 g/ml serum proteins, with less than 1% of the arylesterase in the d = 1.21–1.25 g/ml fraction. The very low hydrolysis of *p*-nitrophenyl valerate by FPLC fractions 24–29 indicated that the activity in these fractions was attributable to PON1.

Erdos, Debay, and Westerman (16) reported that human serum arylesterase activity decreased during dialysis against Tris buffers and that the loss of activity could be prevented by the addition of 0.1 mM $CaCl_2$. We confirmed that the major reason for the loss of PON1 arylesterase activity during HDL isolation was dialysis against buffers without calcium. Excluding EDTA alone was not sufficient to preserve PON1's arylesterase activity. In fact, we found that avoiding dialysis resulted in the best recoveries of PON1 arylesterase activity.

The association of PON1, as characterized by enzymatic activity, with bovine high density lipoproteins was an early observation (17). However, there is limited information in the modern era about the distribution of PON1 activity and mass. Graham et al. (10) reported that human HDL_2 contained only 1% of the PON1 activity and that the majority of the activity was associated with human HDL_3, although recoveries after ultracentrifugation were low. We now attribute the low recoveries of activity to the use of suboptimal buffers during the dialysis step.

A major difficulty in the study of human HDL is the generation of pre-β-HDL particles as an artifact of re-

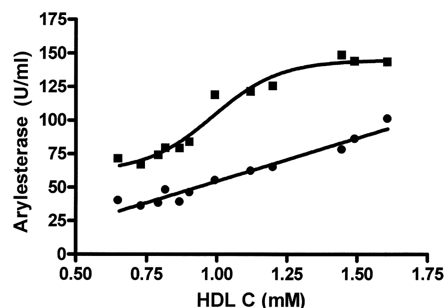

Fig. 5. The correlation of total (closed squares) and lipoprotein-associated (closed circles) arylesterase activity with HDL cholesterol (HDL C).

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peated ultracentrifugation. We have used size-exclusion chromatography as the benchmark for identifying the native distribution of PON1. Using this technique and a single ultracentrifugation step, rather than repeated sequential ultracentrifugation steps, to separate lipoproteins, we have identified HDL as the PON1-containing fraction in the mouse. In addition, we have determined that the majority of PON1 in this fraction is separated by size as particles larger than the peak for HDL cholesterol.

Blatter et al. (18) isolated human PON1 lipoprotein fractions by immunoaffinity chromatography. Their data are consistent with PON1 being in multiple subfractions of human HDL. All of these fractions have apolipoprotein A-I (apoA-I), whereas some of the fractions also contain apoA-IV or apoJ. Upon ultracentrifugation, they could not recover active PON1 but observed PON1 mass in the density interval greater than 1.21 g/ml.

Kelso et al. (19) observed that affinity-purified human apoJ was always associated with apoA-I and PON1. Although they showed that PON1 was a stoichiometric component of this complex, they also observed that quantitative removal of apoJ from human serum by immunoaffinity chromatography accounted for only a small fraction of the total PON1.

PON1 activity, mass, and genetic variation have been associated with vascular disease in humans (20). The knockout of PON1, when crossed into apoE knockout mice challenged with a high-fat diet (21), resulted in larger aortic plaques, whereas the overexpression of PON1 resulted in decreased plaque size (22). Thus, there is significant evidence that PON1 contributes to protection from the development of fatty streaks and atherosclerosis. PON1 appears to play a role within the cascade of changes that occur at the cellular and lipoprotein levels in the progression of atherosclerosis. The procedures reported here should facilitate the determination of the precise role of PON1 in physiology.

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