Purified human serum PON1 does not protect LDL against oxidation in the in vitro assays initiated with copper or AAPH[S]

John F. Teiber, Dragomir I. Draganov, and Bert N. La Du[1]

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI

Abstract **Purified serum paraoxonase (PON1) had been shown to attenuate the oxidation of LDL in vitro. We critically reevaluated the antioxidant properties of serum PON1 in the in vitro assays initiated with copper or the free radical generator 2,2′-azobis-2-amidinopropane hydrochloride (AAPH). The antioxidant activity of different purified PON1 preparations did not correlate with their arylesterase (AE), lactonase, or phospholipase A_2 activities or with the amounts of detergent or protein. Dialysis of three of these preparations resulted in a 30–40% loss of their AE activities but in a complete loss of their antioxidant activities. We also followed the distribution of the antioxidant activity during human serum PON1 purification by two purification methods. The antioxidant activity of the anion-exchange chromatography fractions did not copurify with PON1 using either method and could largely be accounted for by the "antioxidant" activity of the detergent present. In conclusion, using the copper or AAPH in vitro assays, no PON1-mediated antioxidant activity was detected, suggesting that the removal of PON1 from its natural environment may impair its antioxidative activity and that this assay with highly purified PON1 may be an inappropriate method with which to study the antioxidative properties of the enzyme.**—Teiber, J. F., D. I. Draganov, and B. N. La Du. **Purified human serum PON1 does not protect LDL against oxidation in the in vitro assays initiated with copper or AAPH.** ***J. Lipid Res.*** **2004.** 45: **2260–2268.**

Supplementary key words paraoxonase 1 • oxidized low density lipoprotein • antioxidants • atherosclerosis • 2,2′-azobis-2-amidinopropane hydrochloride

Paraoxonase (PON1; EC 3.1.8.1) is a calcium-dependent esterase that is associated with HDL in the serum. PON1 hydrolyzes aromatic carboxylic acid esters, toxic organophosphate compounds, and lactones, yet the natural substrates and physiological function(s) of PON1 remain to be established (1). Many studies have suggested a protective role for PON1 in the development of atherosclerosis (reviewed in Ref. 2). Most convincing are studies with PON1-knockout and transgenic mice. PON1-knockout mice are more prone to develop atherosclerotic plaques when fed a high-fat diet compared with controls, and mice overexpressing human PON1 are more resistant to atherosclerotic lesion development (3, 4).

Oxidation of LDL is a critical event in the genesis and progression of atherosclerosis (5). HDL can attenuate the oxidative modification of LDL, and the ability of PON1 to afford protection against atherosclerosis has been attributed to its ability to confer antioxidative properties to HDL (6). Accordingly, many in vitro assays have been developed or adapted to study the enzyme's antioxidative capabilities. A coculture artery wall model has been used to show that HDL from PON1-knockout mice could not protect LDL from oxidation (3), as measured by lipid peroxide formation or monocyte-endothelium interactions induced by oxidized LDL, but wild-type HDL could do so. Purified PON1 also exhibited antioxidative properties in this coculture system (3). HDL from mice overexpressing PON1 with a 5-fold increase in PON1 concentration was much more resistant to oxidation in vitro (7).

The mechanism(s) by which PON1 protects against oxidative stress and atherogenesis has not been definitively established. PON1 can inhibit the biological activities of oxidized intermediates of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (8, 9), which form during the oxidation of LDL. Studies have proposed different mechanisms by which PON1 could mediate its protective role, including a peroxidase activity, phospholipase A_2 (PLA_2) activity, and a hydrolytic activity toward cholesteryl linoleate hydroperoxides/hydroxides (10–13). Monitoring the rate and extent of LDL oxidation in vitro is a method commonly used to assess the mechanistic and inhibitory

Manuscript received 8 June 2004 and in revised form 23 August 2004.

Published, JLR Papers in Press, September 1, 2004.
DOI 10.1194/jlr.M400213-JLR200

Abbreviations: AAPH, 2,2′-azobis-2-amidinopropane hydrochloride; AE, arylesterase; DDM, *n*-dodecyl-β-D-maltoside; DLPC, L-α-dilauroylphosphatidylcholine; PLA_2, phospholipase A_2; PON1, paraoxonase; TBARS, thiobarbituric acid-reactive substances.

[1] To whom correspondence should be addressed.
e-mail: bladu@umich.edu

[S] The online version of this article (available at http://www.jlr.org) contains an additional figure.

capacity of antioxidants (14). Lipid peroxidation is initiated with copper, which forms free radicals at the LDL surface, or by water-soluble free radical generators such as 2,2′-azobis-2-amidinopropane hydrochloride (AAPH). Numerous studies have shown that purified human serum PON1 can inhibit LDL oxidation after initiation with copper or AAPH, suggesting that PON1 by itself can act as an antioxidant (15–17).

A method for purifying PON1 from serum that was developed in our laboratory has been commonly used to obtain PON1 for biochemical studies (18). Many of the proposed mechanisms by which PON1 protects against atherogenesis and oxidation are based on studies with the purified enzyme. It is now clear that minor contaminants are present in these preparations, which has resulted in erroneous conclusions about some of the properties of purified PON1. For example, purified PON1 was reported to have PLA_2 activity (13, 19). However, more recent evidence suggests that the reported activities are attributable to a small amount of PLA_2 contaminating the PON preparations (20, 21; our unpublished observations). Also, it was reported that crystals of PON1 had been obtained using enzyme purified by a similar method; however, upon analysis, it was found that the crystals were those of an unknown contaminating protein (22). We reported that human serum PON1 has statinase activity (23), but subsequently we found that this activity was the result of small amounts of PON3 in the purified PON1 preparation (1).

To obtain PONs devoid of serum protein contaminants, we have recently begun expressing, purifying, and characterizing the recombinant PONs from a baculovirus-mediated expression system (D. Draganov, J. Teiber, A. Speelman, R. Sunahara, and B. N. La Du, unpublished data). To study the antioxidative properties of the recombinant proteins, we adapted the in vitro LDL oxidation assay, described above, for a high-throughput, 96-well plate format. Surprisingly, no antioxidant activity by the recombinant enzymes was detected. We became concerned that PON1's ability to protect against oxidation in this in vitro assay may require the presence of additional serum components or might be attributable to minor contaminants copurifying with the enzyme isolated from mammalian serum. Therefore, we reanalyzed different purified PON1 preparations, which had antioxidant activity that was previously documented, and attempted to correlate it with the enzymatic activities of the samples. We also purified human serum PON1 by two different methods and followed the distribution of PON1 and the antioxidant activity throughout the purification.

MATERIALS AND METHODS

Materials

DEAE Bio-Gel A, Macro-Prep DEAE, and Extracti®-Gel D were from Bio-Rad (Hercules, CA). Cibacron Blue 3 GA-agarose type 3000, Tergitol NP-10, phenyl acetate, 1,1,3,3-tetramethoxypropane, and L-α-dilauroylphosphatidylcholine (DLPC) were from Sigma-Aldrich (St. Louis, MO). *n*-Dodecyl-β-D-maltoside (DDM) was from Dojindo (Gaithersburg, MD). Glycerol [United States Pharmacopeia/Food Chemicals Codex Grade(USP/FCC)] was from Fisher Scientific. AAPH was from Wako Chemicals (Richmond, VA). Lovastatin was from Merck (Rahway, NJ). 2-Thio platelet-activating factor was from Cayman Chemical (Ann Arbor, MI). Human plasma LDL was from Intracel (Frederick, MD) and was stored in 0.15 M NaCl and 0.01% EDTA at pH 7.2 and 4°C under argon and used before the expiration date. Human serum (type Q) was obtained from outdated citrated plasma (University of Michigan blood bank). All other chemicals were reagent grade or better from commercial sources.

PON1 purification

Our previous purification of PON1 was by the method of Gan et al. (18). Human serum (1 liter) was centrifuged for 20 min at 2,000 *g*, filtered through cheesecloth, and mixed with 800 ml of blue agarose in 50 mM Tris/HCl buffer (pH 8.0) containing 3 M NaCl, 1.5 mM $CaCl_2$, and 20 μM EDTA. Thirty minutes later, the agarose was washed batchwise six times with the high-salt buffer followed by two washes with 50 mM Tris/HCl (pH 8.0) containing 1 mM $CaCl_2$. The agarose was then poured into a column and developed with 25 mM Tris/HCl buffer (pH 8.0) containing 1 mM $CaCl_2$, 20% glycerol, and 0.1% sodium deoxycholate. The fractions containing the highest arylesterase (AE) activities were combined, and this pool (60 ml) was further purified using either the Gan method or the new DEAE chromatography method.

For the new method, DDM was added to the pool so its concentration was 7 mg/ml (2 mg DDM/mg protein) and the pool was gently mixed for 30 min at room temperature. Macro-Prep DEAE support [15 ml equilibrated in buffer A: 25 mM Tris/HCl buffer (pH 7.4), 1 mM $CaCl_2$, 10% glycerol, and 0.5 mg/ml DDM] was then added to the pool. Gentle mixing was continued until the AE activity of the supernatant was less than 3 U/ml. The supernatant was decanted and the support was poured into a column, washed with two column volumes of buffer A, and developed with a linear 30 ml of 0–0.6 M NaCl gradient in buffer A at 0.5 ml/min.

For the Gan method, the pool was mixed 1:1 with 25 mM Tris/HCl buffer (pH 8.0) containing 1 mM $CaCl_2$, 20% glycerol, and 2 mg/ml Tergitol. DEAE Bio-Gel support [15 ml equilibrated in buffer B: 25 mM Tris/HCl buffer (pH 8.0), 1 mM $CaCl_2$, 20% glycerol, and 1 mg/ml Tergitol] was added to the pool and then processed as described above. After washing with buffer B, the column was developed with a linear 90 ml of 0–0.15 M NaCl gradient in buffer B at 0.5 ml/min. To further purify one of the stored PON1 preparations (number 2), a 50 ml aliquot was diluted to 125 ml in buffer B, loaded onto the DEAE Bio-Gel column, and developed as described above for the Gan method.

Stored PON1 preparations purified by the Gan method

All of the preparations, except preparation 2, were purified through two DEAE chromatography steps and were A-type (192_{QQ}). Preparation 2 was AB-type (192_{QR}) and was purified on July 14, 2000, through one DEAE column. Preparations 4 and 9 were pooled fractions 42–47 and 48–53, respectively, from a second DEAE column, purified on December 4, 1998. Preparations 3, 10, and 6 were pooled second DEAE fractions 42–46, 47–53, and 53–56, respectively, purified on December 5, 1998. Preparation 5 was a pool of four different preparations purified in 1992 and 1993. Preparations 1 and 7 were purified on May 28, 1998, and February 26, 1999, respectively.

Enzymatic activities

AE activities were measured spectrophotometrically as described elsewhere using phenyl acetate as a substrate (18). One

unit of AE activity corresponds to 1 μmol/min. Statinase activity was determined by the hydrolysis of lovastatin. A 5 μl aliquot of each fraction and 1 μl of substrate (0.5 mg/ml in methanol) were added to a final volume of 50 μl of 25 mM Tris/HCl (pH 7.4) containing 1 mM $CaCl_2$. Samples were processed and analyzed by HPLC as described previously (24). One unit of statinase activity corresponds to 1 pmol/min. 2-Thio platelet-activating factor acetylhydrolase (PAF-AH) activity was measured on 96-well plates based on the protocol described in the Cayman PAF-AH assay kit (catalog number 760901). A 20 μl aliquot of each fraction was added to 145 μl of 100 mM Tris/HCl (pH 7.2) followed by 10 μl of a 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) solution [in 100 mM Tris/HCl (pH 7.0) and 18 mM sodium bicarbonate]. After shaking, 50 μl of a 450 μM solution of 2-thio PAF [in 100 mM Tris/HCl (pH 7.2)] was added to the samples. The plate was shaken and incubated at 30°C, and absorbance was continuously monitored at 414 nm. Enzymatic activities were calculated as described in the Cayman assay kit. One unit of PLA_2 activity corresponds to 1 nmol/min.

Tergitol quantification

Tergitol, which is the detergent used in the Gan purification method, was quantified by HPLC using a Beckman System Gold with a model 168 diode array detector set at 270 nm and a Supelco discovery column (C8; 15 cm × 4.6 mm, 5 μm). The samples (2.5 μl) were added to 97.5 μl of 87% acetonitrile containing 560 μM coumarin as an internal standard. Samples were briefly vortexed and then centrifuged for 1 min in a tabletop centrifuge at top speed. The supernatant (20 μl) was injected onto the HPLC column and eluted isocratically at 1 ml/min with a mobile phase of 78% acetonitrile containing 0.2% acetic acid. The retention times for coumarin and Tergitol were 2.1 and 2.9 min, respectively. Tergitol concentrations were calculated from a standard curve that was generated based on the peak height of standards of varying Tergitol concentrations.

LDL oxidation assays

LDL oxidation reactions were performed as previously described (10) with minor modifications. Reactions were performed on 96-well plates in PBS at a final volume of 0.2 ml and contained 100 μg LDL protein/ml. The plates were shaken immediately after addition of the samples, and reactions were initiated by adding either 20 μl of a copper sulfate (in double-distilled water) or AAPH (in PBS) solution and run at 37°C. Copper concentrations are reported as final and account for the chelation attributable to the EDTA present in the LDL. The absorbance was continuously monitored at 234 nm, and lag times were calculated from the kinetic profiles as previously described (8). Lipid peroxides were determined using 20 μl aliquots of the LDL oxidation reactions as described previously (25). Thiobarbituric acid-reactive substances (TBARS) were determined as previously described using 1,1,3,3-tetramethoxypropane for the standard curve (26).

Dialysis

Aliquots of 50 μl from the stored purified PON1 preparations, or buffer B as the control, were added to Slide-A-Lyzer® mini dialysis units (Pierce, Rockford, IL) and dialyzed overnight at 4°C against buffer B without Tergitol. Antioxidant activities were determined in the copper LDL system as described above.

Detergent removal and DLPC stimulation

Aliquots of 0.5 ml from the DEAE fractions were applied to a column containing 1.25 ml of the Extracti-gel. PON1 was eluted by gravity using buffer B without Tergitol. The Tergitol remaining in the fractions was too low to be quantitated.

DLPC was dispersed by sonication in 25 mM Tris/HCl (pH 7.4) containing 1 mM $CaCl_2$ at a concentration of 2 mg/ml. The Extracti-gel fraction, or buffer B without Tergitol (as the control), was mixed 1:1 with DLPC or buffer and incubated at room temperature for 1 h and then analyzed in the LDL oxidation assays as described above. Assays were performed in duplicate, and the error represents the range of the values.

RESULTS

Antioxidant activities of stored purified PON1 preparations

PON1 preparations that had been purified through two DEAE chromatography steps (except preparation 2) using the Gan method and stored from 4–12 years at 4°C were examined for antioxidant activity in the copper/LDL oxidation system. On a Coomassie blue-stained SDS-PAGE gel, at least 90% of the proteins from the preparations migrated as a band of ~45 kDa, which is the molecular mass of PON1. All of the preparations significantly delayed the lag time, with preparations 5 and 10 exhibiting very potent antioxidant activities (**Table 1**). However, the antioxidant activities were not proportional to the AE, statinase, or PLA_2 activities or with the concentration of protein or Tergitol in the preparations (Table 1). The ability of the stored preparations to protect LDL against oxidation initiated with AAPH was also determined. Peroxides were measured every hour from 2 to 6 h. Peroxides were decreased, relative to the control, by most of the preparations (Table 1, 4 h). The protection from AAPH-initiated oxidation did not parallel any of the enzymatic activities or concentrations of protein or Tergitol, nor did it correspond to the lag times in the copper-initiated assay. Results were similar at shorter and longer time points, with the extent of inhibition of peroxide formation diminishing as the time increased (data not shown). The lack of correlation between the antioxidant activities in the copper- and AAPH-initiated reactions indicates that the component(s) that protects against copper oxidation is different from the one that protects against AAPH oxidation.

Preparations 7, 4, and 2 were then dialyzed overnight using 3,500 molecular weight cutoff dialysis units. AE activities were decreased by 30, 37, and 30% in preparations 7, 4, and 2, respectively. However, they all lost their ability to prolong the lag time and to decrease the propagation rate in copper (5 μM)-induced LDL oxidation, suggesting the presence of a low molecular weight antioxidant component in these old preparations.

Antioxidant effects of the detergents

The amount of Tergitol in each preparation varied from 0.93 to 1.32 mg/ml, except for preparation 1, from which the detergent had been removed (Table 1). This prompted us to evaluate the effects of the detergents in the LDL oxidation assays. Both Tergitol and DDM had a pronounced ability to delay the lag time (**Fig. 1A**) and to decrease the propagation rate (data not shown). The delay in lag for both detergents appeared to exhibit a nonlinear concentration dependence that could be fit to

TABLE 1. Enzymatic activities, antioxidant properties, and concentrations of protein and Tergitol in the stored purified PON1 preparations from human plasma

PON1 Preparation	Arylesterase	PLA_2[a]	Statinase	Lag Time[a,b]	Peroxides[a,c]	Protein[a]	Tergitol[a]
		U/ml		*min*	*% of control*	*μg/ml*	*mg/ml*
1	189	133	58	133	67	162	0.13
2	240	236	31	181	69	465	1.15
3	268	234	34	132	88	273	0.94
4	342	38	29	141	84	288	1.03
5	379	165	39	>400	95	527	1.14
6	414	35	28	146	73	387	0.93
7	443	55	23	178	86	337	1.09
8	447	18	35	156	76	435	1.02
9	483	28	26	190	72	425	1.19
10	847	80	27	264	103	823	1.32

PLA_2, phospholipase A_2; PON1, paraoxonase.
[a] Assays were performed in duplicate, and the range of the duplicate values was less than 10%.
[b] Samples were diluted 1:10 for the reactions. Final copper concentration was 5 μM. The lag time of the control (buffer B alone) was 101 ± 4 min.
[c] Samples were diluted 1:10 for the reactions. Values are given as nmol lipid peroxides/mg LDL. Final 2,2′-azobis-2-amidinopropane hydrochloride concentration was 5 mM. The control is the peroxide level in reactions containing buffer B and was 340 ± 43 nmol/mg LDL.

second-order polynomial equations. This indicates that relatively minor differences in detergent concentrations could result in significantly delayed lag times. The detergents also had significant effects on the amount of peroxides formed in the copper/LDL oxidation system. Increasing the concentrations of either detergent resulted in lower peroxide accumulation at 2 h, whereas at 4 h the amount of peroxides was higher (Fig. 1B). These effects on peroxide accumulation are likely a reflection of the ability of the detergents to delay the lag time. For example, at lower detergent concentrations (i.e., short lag times), peroxide formation will occur earlier and by the later time points the peroxides will have begun to decompose. Increasing the amount of glycerol resulted in a shortening of the lag (Fig. 1A), with no effect on the maximum propagation rate (data not shown), and exhibited a linear concentration dependence. This prooxidant activity may be attributable to the presence of trace peroxides in the glycerol. Under the assay conditions, detergent concentrations up to 2 mg/ml had little effect on peroxide generation after initiation with AAPH.

Distribution of PON1 and the antioxidant activity during PON1 purification

The possibility that antioxidant components may be partially copurifying with PON1 prompted us to closely follow the antioxidant activity of fractions eluting during the DEAE chromatography step using the Gan method and an improved purification method. With the new method, PON1 was still not completely resolved from the PLA_2 or PON3, although it was superior to the Gan method in separating PON1 from these two proteins (**Figs. 2A**, **3A**). The specific activity of the peak AE fractions was also slightly higher using the new method (e.g., 537 U/mg for fraction 21 with the new method versus 454 U/mg for fraction 20 with the Gan method).

Fractions from the DEAE columns were screened for antioxidant activity in the copper/LDL oxidation system by determining their ability to delay the lag phase or decrease lipid peroxides. Purified PON1 was previously shown to exhibit substantial antioxidant activity in this assay at a copper concentration of 5 μM (15–17). Surprisingly, at this copper concentration, there was no peak in antioxidant activity that corresponded to the PON1 peak in fractions from either purification method, as measured by an increase in lag time or a decrease in lipid peroxide formation (Figs. 2B, C, 3B, C). At the 3 and 8 h time

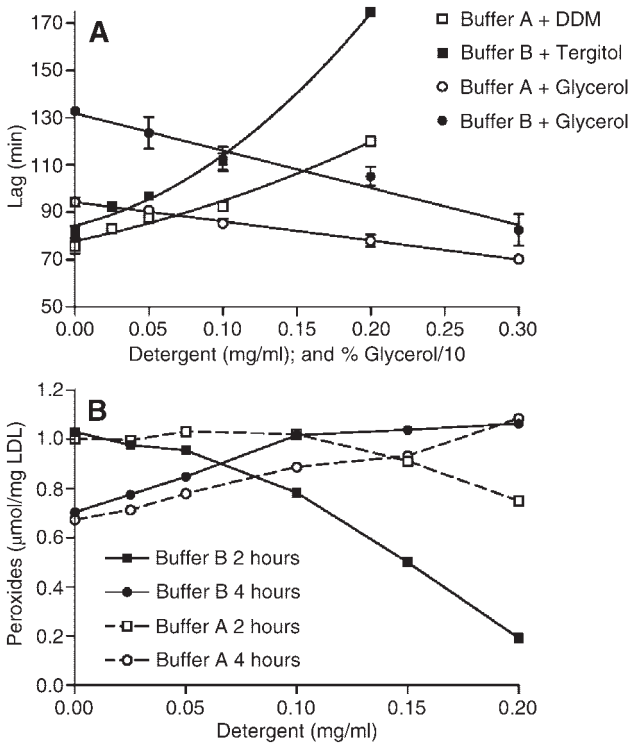

Fig. 1. Antioxidant activities of Tergitol, *n*-dodecyl-β-D-maltoside (DDM), and glycerol. Twenty microliter aliquots of buffer A or B containing increasing concentrations of detergents or glycerol were analyzed for their ability to increase the lag times (A) or decrease peroxide formation (B) in the in vitro LDL oxidation system initiated with 5 μM copper as described in Materials and Methods. Results are averages of two experiments; error bars represent the range.

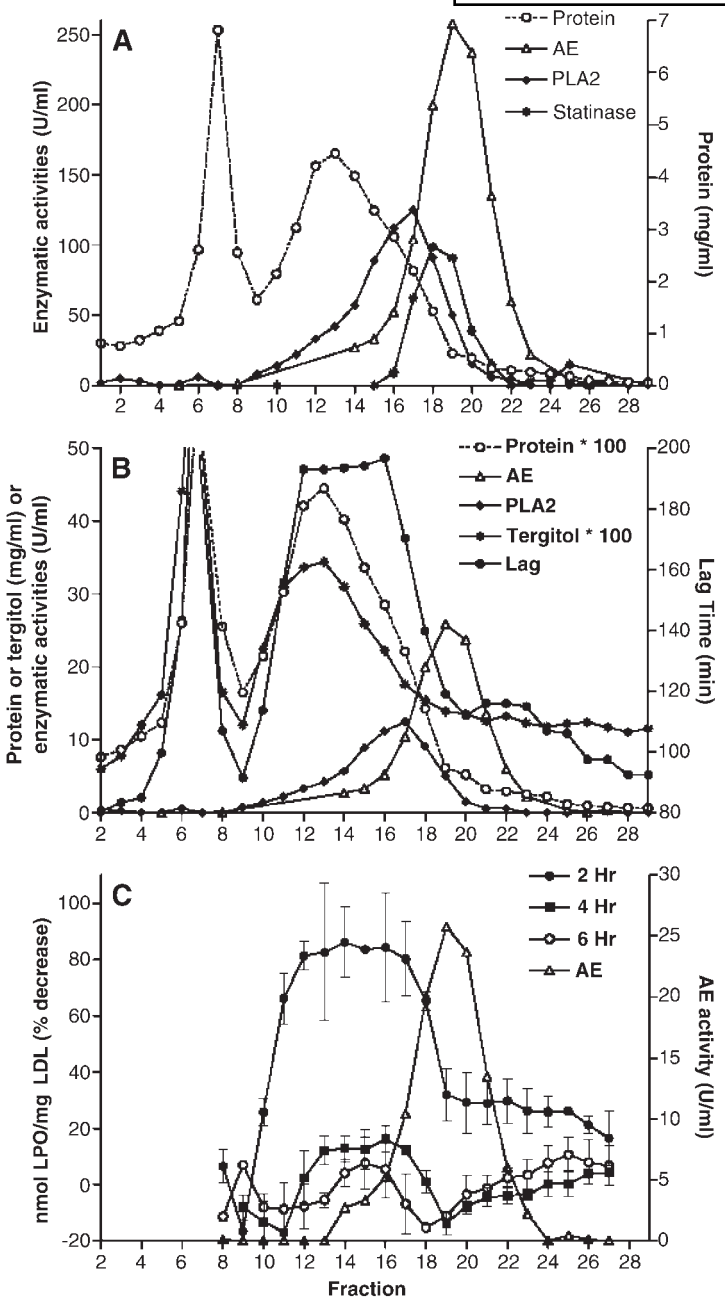

Fig. 2. Characterization of fractions from the DEAE chromatographic purification of the human serum paraoxonase (PON1) blue-agarose pool using the Gan method. A: PON1 arylesterase (AE), phospholipase A_2 (PLA_2), and PON3 (statinase) activities and protein concentrations of the DEAE fractions. B: AE and PLA_2 activities, protein and Tergitol concentrations, and lag times of the DEAE fractions in the copper (5 μM)/LDL oxidation assays. Lag times are from a single experiment that was representative of two separate experiments. C: Percentage decrease in lipid peroxide formation compared with buffer B and AE activities of the DEAE fractions. Oxidation was initiated with 5 μM copper. The values for the percentage decrease in lipid peroxides (LPO) are averages of two experiments; error bars represent the range.

points, there was also no decrease in lipid peroxides that corresponded to PON1 in the fractions (data not shown).

The increased lag times corresponded roughly to the protein concentration. Most probably this is attributable at least in part to the higher concentration of detergent coeluting with the protein. The concentration of Tergitol in the fractions purified by the Gan method did correspond to the protein concentrations and ranged from ~1 mg/ml, the concentration in buffer B, to 8 mg/ml (Fig. 2B). Because DDM does not have an appreciable extinction coefficient, its concentration in the samples could not readily be determined. There were minor peaks of antioxidant activity that coeluted with PLA_2, although further analysis of the fractions with high PLA_2 activity indicated that the antioxidant activity is not the result of this enzyme (see below). When the copper was decreased to 1 μM, to reduce the possibility of copper-mediated PON1 inactivation, the lag times increased substantially, but there was still no significant peak in antioxidant activity that coeluted with PON1 using fractions obtained by the new purification method (data not shown). Preliminary tests with fractions from the Gan purification method with 1 μM copper indicated that the lag times were too long to measure accurately and likely were attributable to the extensive inhibitory effects of Tergitol.

The generation of peroxides from the addition of 5 mM AAPH was determined each hour for 8 h using selected fractions from each purification method. The peroxide formation after 4 h, given as a percentage of the control, is shown in **Fig. 4**. Except for fraction 12, there was little inhibition of peroxide formation by the fractions, and their antioxidant activities did not correspond to their AE

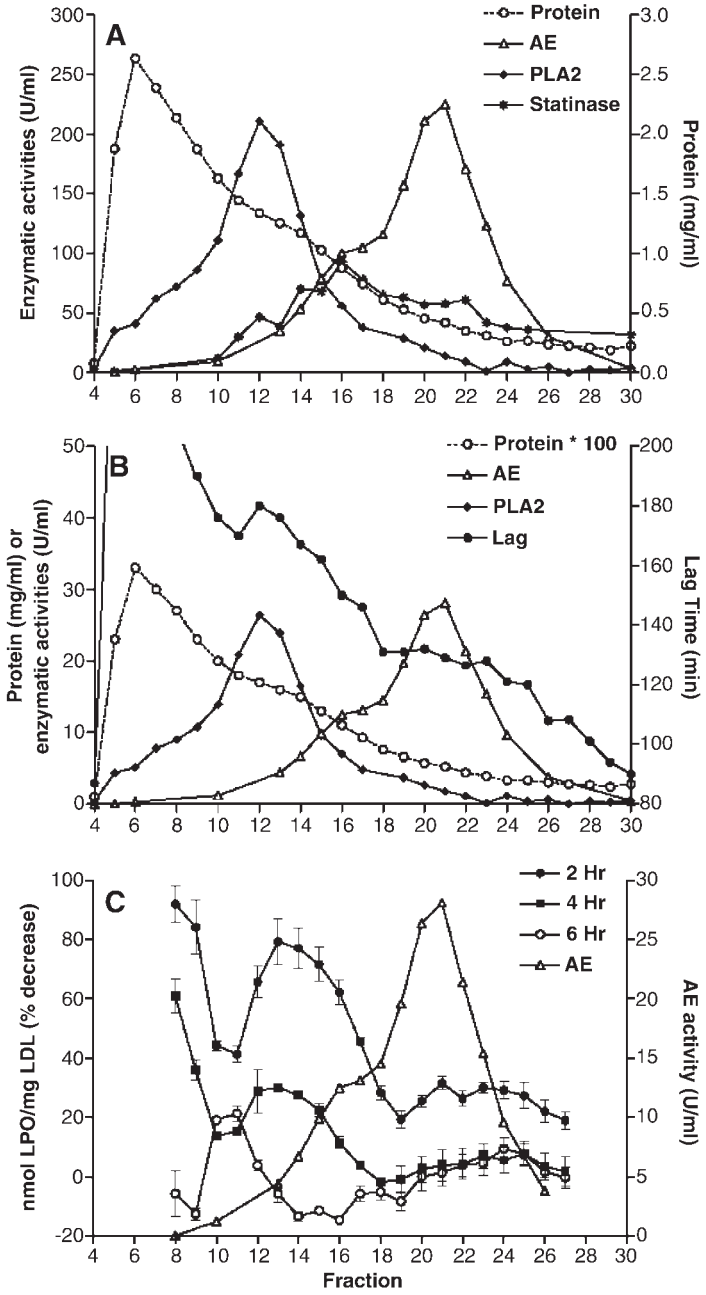

Fig. 3. Characterization of fractions from the DEAE chromatographic purification of the serum PON1 blue-agarose pool using the new method. A: PON1 AE, PLA_2, and PON3 (statinase) activities and protein concentrations of the DEAE fractions. B: AE and PLA_2 activities, protein concentrations, and lag times of the DEAE fractions in the copper (5 μM)/LDL oxidation assays. Lag times are from a single experiment that was representative of two separate experiments. C: Percentage decrease in lipid peroxide (LPO) formation compared with buffer A and AE activities of the DEAE fractions. Oxidation was initiated with 5 μM copper. The values for the percentage decrease in lipid peroxides are averages of two experiments; error bars represent the range.

activities. Results were similar at shorter and longer time points, with the extent of inhibition of peroxide formation diminishing slightly as the time was increased (data not shown).

The concentrations of LDL, PON1, and copper in the above assays were similar to those previously reported (10, 11). Because our final reaction volumes were lower than in the previous studies, it was possible that the inability to detect the antioxidant activity of PON1 was the result of the lower amount of total LDL and/or PON1 in our reactions. Therefore, the antioxidant activities in the copper-initiated system of several DEAE fractions purified by the new method were also examined in reaction volumes of 1 ml by measuring lipid peroxides and TBARS, as had been done in the previous studies. The antioxidant activity corresponded to the amount of protein, which probably reflects the amount of detergent in the fractions, but not PON1 (see supplementary figure).

Our inability to detect antioxidant activity of PON1 in our freshly purified preparations prompted us to characterize further the antioxidant activity present in one of the stored preparations. We selected preparation 2, which previously had only been purified through one DEAE chromatography step, and passed it over a second DEAE column using the Gan method. The antioxidant activity did not bind to the support and remained in the flow-through fraction (**Table 2**). PON1 eluted in two peaks, and the minor increases in lag times of 0–15 min (**Fig. 5A**) and decreases in peroxides of 0–40% (Fig. 5B) could be accounted for by the concentration of Tergitol in the fractions.

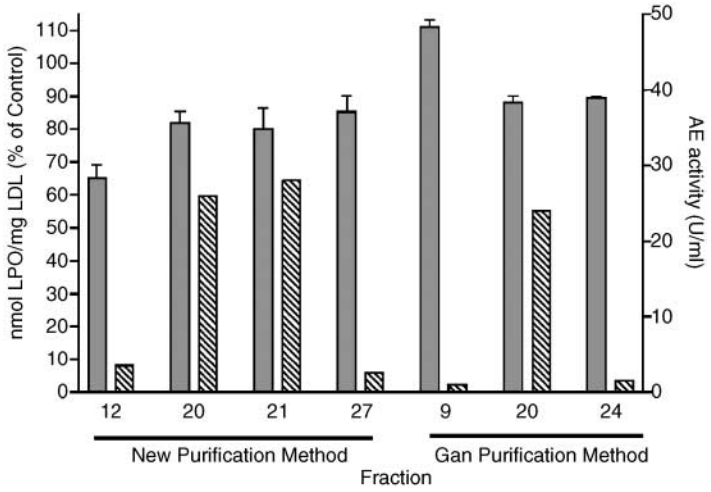

Fig. 4. Antioxidant activities of human serum DEAE fractions purified by the Gan and new methods. Fractions were analyzed for their ability to decrease peroxides in the in vitro LDL oxidation system initiated with 5 mM 2,2′-azobis-2-amidinopropane hydrochloride as describe in Materials and Methods. Gray bars, lipid peroxide (LPO); hatched bars, AE. Control values are the peroxide levels in reactions containing buffer A or B. Results are averages of two experiments; error bars represent the range.

Antioxidant activity of detergent-free and DLPC-stimulated DEAE fractions

To verify that any apparent antioxidant activity retained by PON1 was not diminished by interference with the detergent, fractions 7 and 14 from the second DEAE purification of PON1 preparation 2, and buffer B without Tergitol as a control, were each passed over an Extracti-gel column to remove the Tergitol and then analyzed in the LDL oxidation assays. At a copper concentration of 2 μM, the lag time for the control was 63 ± 5 min. Assays with fractions 7 and 14 contained AE activities of 0.9 and 37 U/ml, respectively, and had lag times of 67 ± 6 min and 70 ± 1 min. Similar assays in which oxidation was initiated by 2 mM AAPH had 148 ± 9, 150 ± 37, and 140 ± 8 nmol/mg LDL for the control, fraction 7, and fraction 14, respectively, after 5 h.

DLPC has been shown to stimulate the esterase activity of PON1 (27), and it was considered possible that DLPC may also be able to stabilize its putative antioxidant activity. Fraction 14 from the second DEAE step of preparation 2, which had been processed through the Extracti-gel column, was incubated in the presence or absence of DLPC and then analyzed in the LDL oxidation assay. For these assays, LDL was dialyzed overnight before use to remove the EDTA, and copper was used at a concentration of 1 μM to minimize any inactivation of PON1 by the metal. Preincubation with DLPC stimulated the AE activity by 61%, a similar extent of stimulation as has been reported previously (27). Lag times for the control (buffer B without Tergitol) and fraction 14, preincubated in the absence of DLPC, were 135 ± 0.5 min and 140 ± 0.5 min respectively. Preincubating with DLPC resulted in lag times of 167 ± 0.5 min and 166 ± 0.6 min for the control and fraction 14, respectively.

TABLE 2. Enzymatic and antioxidant activities of fractions from the second DEAE chromatography of purified PON1 preparation 2

Sample	Arylesterase	PLA_2[a]	Lag Time[a,b]
	U/ml	*U/ml*	*min*
Control (buffer B)	–	–	101 ± 4
Preparation 2	240	236 ± 4	181 ± 1
Flow-through	ND	ND	131 ± 1
Fraction 13	381	975 ± 49	106 ± 1
Fraction 23	452	766 ± 5	105 ± 4

ND, none detected.
[a] Values are averages of two determinations ± the range.
[b] Samples were diluted 1:10 for the LDL oxidation reactions. The final copper concentration was 5 μM.

DISCUSSION

It has been established that PON1 plays a protective role against atherosclerosis. Previous studies have shown that purified PON1 appears to act as an antioxidant, and this property is presumed to at least partially account for this protection. In light of recent studies showing that minor contaminants remaining in purified PON1 preparations have led to erroneous conclusions about its catalytic properties, it was important to reevaluate PON1's antioxidative functions. In this study, purified PON1 failed to protect LDL from copper- or AAPH-initiated oxidation in vitro. These results lead to the conclusion that either this assay is inappropriate for the study of purified PON1's antioxidant properties or that for PON1 to elicit its protective properties in vitro some additional component(s) is required with the purified enzyme.

The lack of correlation between PON1, PON3, PLA_2, protein concentrations, or Tergitol concentrations with the protection of LDL from oxidation in the stored purified PON1 preparations suggested the presence of a minor unknown antioxidant component. Except for preparation 2, all of the stored preparations are at least 90% pure, as determined by SDS-PAGE Coomassie blue-stained gels. However, many additional proteins are present as minor contaminants (data not shown). Therefore, starting with human plasma, PON1 was purified through the DEAE chromatography step using both the Gan method and an improved method. Each elution fraction was analyzed to identify antioxidant components that partially copurify with PON1 or any PON1-mediated antioxidant activity that might be obscured by the "contaminant(s)."

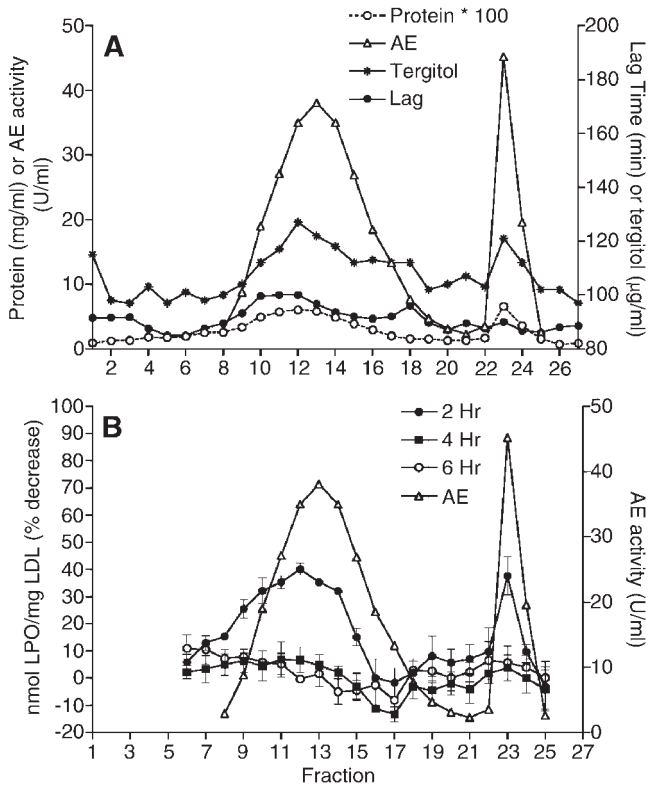

Fig. 5. Characterization of fractions from the DEAE chromatographic purification of serum PON1 preparation 2 using the Gan method. The stored purified PON1 preparation 2 was passed through a second DEAE chromatographic column using the Gan purification method. A: Lag times, PON1 AE activities, and protein and Tergitol concentrations of the DEAE fractions. Oxidation was initiated with 5 μM copper. Lag times are from a single experiment that was representative of two separate experiments. B: Percentage decrease in lipid peroxide (LPO) formation compared with buffer B and AE activities of the DEAE fractions. Oxidation was initiated with 5 μM copper. The values for the percentage decrease in lipid peroxides are averages of two experiments; error bars represent the range.

The majority of the antioxidant activity in the DEAE fractions corresponded to the total protein concentration and could be attributed largely to the detergent that coeluted with the protein (Figs. 2, 3). There were some minor antioxidant peaks that appeared to correspond to the PLA_2 fractions with both purification methods. However, when the stored PON1 preparation (number 2) was purified through an additional DEAE step, the fractions that had high PLA_2 activities did not have any significant antioxidant activities (Table 2). This suggests that the aforementioned peaks of antioxidant activity are not attributable to contaminating PLA_2. This agrees with results from the stored preparations that show a lack of correspondence between antioxidant activities and PLA_2 activity. Our results also concur with a previous study in another laboratory that reported an inability of LDL-associated phospholipase A to protect LDL for oxidation in vitro (28).

The antioxidant activity in preparation 2 did not bind to the support during an additional DEAE purification step, suggesting that the antioxidant may not have originated in the plasma, or if so, that it had lost its affinity for the DEAE support. Also, the antioxidant activity of the stored preparations was lost after dialyses overnight in 3,500 molecular weight cutoff dialysis units, indicating that it might be a low molecular weight component. The source of the antioxidant(s) is not known but may even be attributable to agents leeching from the storage tubes or be a by-product of microbial growth. This is currently under investigation.

DEAE fractions were initially analyzed at PON1 and copper or AAPH concentrations that had previously been shown to be adequate for demonstrating that PON1 has significant antioxidant activity. However, we were unable to detect PON1-mediated antioxidant activity with purified PON1. The esterase activity of PON1 is known to be sensitive to inactivation by oxidized lipids and oxidized LDL (17), and the antioxidant activity might also be vulnerable to such oxidants. Previous studies using this LDL oxidation system have shown that HDL can lose its ability to prevent LDL oxidation at higher concentrations of copper (5 μM) and AAPH (10 mM) (29, 30). When we reduced the copper or AAPH concentrations, removed the detergent, or preincubated with DLPC, antioxidant activity associated with PON1 was still not detected. Typical HDL concentrations that have been used to show inhibition of LDL oxidation in this system are in the range of 0.1–0.2 mg HDL protein/ml: this would correspond to ~5–10 U/ml AE activity (29, 30). In our assays, the PON1 concentrations ranged from 20 to 40 U/ml, indicating that the lack of observable antioxidant activity should not be the result of too low a PON1 concentration.

These findings suggest that purified human serum PON1 cannot protect LDL from copper- and AAPH-mediated in vitro oxidation. However, more sensitive techniques, such as mass spectroscopy, may reveal minor reductions in specific oxidized products. If purified PON1 does protect LDL from oxidation in this system, it may only be detectable over extended times and in mildly oxidative conditions. Confounding factors such as trace metal-catalyzed reactions, nonspecific degradation of oxidized products, and enzyme inactivation would be much more pronounced over long incubation times.

Many studies have demonstrated that the antioxidant activity of HDL is at least partially attributable to the presence of PON (3, 7, 10, 30, 31). Our inability to detect antioxidant activity of the purified enzyme suggests that removal of PON1 from its natural environment could destabilize the enzyme or impair its ability to protect against oxidation. Also, important PON1-protein interactions or the presence of essential cofactors may be disrupted or lost during purification. Preliminary assays in which we preincubated purified enzyme with PON1-deficient HDL isolated from PON1-knockout mice did not result in increased antioxidant capacity of the HDL (data not shown), but simply adding PON1 to the HDL may not be sufficient to restore its antioxidant activity. It has been shown that the subfractions of HDL containing PON1 are the most potent inhibitors of copper- and AAPH-initiated LDL oxidation in the in vitro system (30); however, there

is no direct evidence that the protection observed is mediated by PON1. The increased antioxidant capacity may result from other components that localize to the same subfractions containing PON1.

Our results suggest that this in vitro LDL oxidation assay may be inappropriate to study the antioxidant properties of purified PON1. Systems in which other components are also present may be necessary for PON1 to exhibit its protective properties, and these may be required to elucidate the antiatherogenic mechanisms of PON. Additionally, we found that the detergents showed significant "antioxidant" activities in this assay, and detergent concentrations vary in the purified preparations and in the fractions eluting from the DEAE column. Therefore, it is critically important to control for the amount of detergent in the purified preparations before evaluating the antioxidant activity of PON1 in this assay. Our improved purification protocol will help provide a useful preparation of PON1 for these future in vitro investigations.

This work was funded by Michigan Life Sciences Corridor Fund Grant 001796.

REFERENCES

1. Draganov, D. I., and B. N. La Du. 2004. Pharmacogenetics of paraoxonases: a brief review. *Naunyn Schmiedebergs Arch Pharmacol.* **369:** 78–88.
2. Mackness, M. I., B. Mackness, and P. N. Durrington. 2002. Paraoxonase and coronary heart disease. *Atheroscler. Suppl.* **3:** 49–55.
3. Shih, D. M., L. Gu, Y. R. Xia, M. Navab, W. F. Li, S. Hama, L. W. Castellani, C. E. Furlong, L. G. Costa, A. M. Fogelman, and A. J. Lusis. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature.* **394:** 284–287.
4. Tward, A., Y. R. Xia, X. P. Wang, Y. S. Shi, C. Park, L. W. Castellani, A. J. Lusis, and D. M. Shih. 2002. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. *Circulation.* **106:** 484–490.
5. Chisolm, G. M., and D. Steinberg. 2000. The oxidative modification hypothesis of atherogenesis: an overview. *Free Radic. Biol. Med.* **28:** 1815–1826.
6. Durrington, P. N., B. Mackness, and M. I. Mackness. 2001. Paraoxonase and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **21:** 473–480.
7. Oda, M. N., J. K. Bielicki, T. T. Ho, T. Berger, E. M. Rubin, and T. M. Forte. 2002. Paraoxonase 1 overexpression in mice and its effect on high-density lipoproteins. *Biochem. Biophys. Res. Commun.* **290:** 921–927.
8. Watson, A. D., J. A. Berliner, S. Y. Hama, B. N. La Du, K. F. Faull, A. M. Fogelman, and M. Navab. 1995. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **96:** 2882–2891.
9. Navab, M., S. Y. Hama, G. M. Anantharamaiah, K. Hassan, G. P. Hough, A. D. Watson, S. T. Reddy, A. Sevanian, G. C. Fonarow, and A. M. Fogelman. 2000. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. *J. Lipid Res.* **41:** 1495–1508.
10. Aviram, M., M. Rosenblat, C. L. Bisgaier, R. S. Newton, S. L. Primo-Parmo, and B. N. La Du. 1998. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J. Clin. Invest.* **101:** 1581–1590.
11. Aviram, M., E. Hardak, J. Vaya, S. Mahmood, S. Milo, A. Hoffman, S. Billicke, D. Draganov, and M. Rosenblat. 2000. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation.* **101:** 2510–2517.
12. Rozenberg, O., D. M. Shih, and M. Aviram. 2003. Human serum paraoxonase 1 decreases macrophage cholesterol biosynthesis: possible role for its phospholipase-A2-like activity and lysophosphatidylcholine formation. *Arterioscler. Thromb. Vasc. Biol.* **23:** 461–467.
13. Rodrigo, L., B. Mackness, P. N. Durrington, A. Hernandez, and M. I. Mackness. 2001. Hydrolysis of platelet-activating factor by human serum paraoxonase. *Biochem. J.* **354:** 1–7.
14. Pinchuck, I., and D. Lichtenberg. 2002. The mechanism of action of antioxidants against lipoprotein peroxidation: evaluation based on kinetic experiments. *Prog. Lipid Res.* **41:** 279–314.
15. Mackness, M. I., S. Arrol, and P. N. Durrington. 1991. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett.* **286:** 152–154.
16. Aviram, M., S. Billecke, R. Sorenson, C. Bisgaier, R. Newton, M. Rosenblat, J. Erogul, C. Hsu, C. Dunlop, and B. La Du. 1998. Paraoxonase active site required for protection against LDL oxidation involves its free sulfhydryl group and is different from that required for its arylesterase/paraoxonase activities: selective action of human paraoxonase allozymes Q and R. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1617–1624.
17. Aviram, M., M. Rosenblat, S. Billecke, J. Erogul, R. Sorenson, C. L. Bisgaier, R. S. Newton, and B. La Du. 1999. Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. *Free Radic. Biol. Med.* **26:** 892–904.
18. Gan, K. N., A. Smolen, H. W. Eckerson, and B. N. La Du. 1991. Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. *Drug Metab. Dispos.* **19:** 100–106.
19. Ahmed, Z., A. Ravandi, G. F. Maguire, A. Emili, D. Draganov, B. N. La Du, A. Kuksis, and P. W. Connelly. 2002. Multiple substrates for paraoxonase-1 during oxidation of phosphatidylcholine by peroxynitrite. *Biochem. Biophys. Res. Commun.* **290:** 391–396.
20. Marathe, G. K., G. A. Zimmerman, and T. M. McIntyre. 2003. Platelet-activating factor acetylhydrolase, and not paraoxonase-1, is the oxidized phospholipid hydrolase of high density lipoprotein particles. *J. Biol. Chem.* **278:** 3937–3947.
21. Connelly, P. 2004. The role of apolipoprotein AI and paraoxonase 1 in the formation of bioactive phospholipids oxidation products (Abstract in First International Conference: Paraoxonases Basic and Clinical Directions of Current Research, Ann Arbor, MI, April 22–24, 2004).
22. Fokine, A., R. Morales, C. Contreras-Martel, P. Carpentier, F. Renault, D. Rochu, and E. Chabriere. 2003. Direct phasing at low resolution of a protein copurified with human paraoxonase (PON1). *Acta Crystallogr. D Biol. Crystallogr.* **59:** 2083–2087.
23. Billecke, S., D. Draganov, R. Counsell, P. Stetson, C. Watson, C. Hsu, and B. N. La Du. 2000. Human serum paraoxonase (PON1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. *Drug Metab. Dispos.* **28:** 1335–1342.
24. Draganov, D. I., P. L. Stetson, C. E. Watson, S. S. Billecke, and B. N. La Du. 2000. Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. *J. Biol. Chem.* **275:** 33435–33442.
25. el-Saadani, M., H. Esterbauer, M. el-Sayed, M. Goher, A. Y. Nassar, and G. Jurgens. 1989. A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J. Lipid Res.* **30:** 627–630.
26. Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. *Methods Enzymol.* **52:** 302–310.
27. Kuo, C. L., and B. N. La Du. 1995. Comparison of purified human and rabbit serum paraoxonases. *Drug Metab. Dispos.* **23:** 935–944.
28. Schnitzer, E., I. Pinchuk, M. Fainaru, D. Lichtenberg, and S. Yedgar. 1998. LDL-associated phospholipase A does not protect LDL against lipid peroxidation in vitro. *Free Radic. Biol. Med.* **24:** 1294–1303.
29. Raveh, O., I. Pinchuk, M. Fainaru, and D. Lichtenberg. 2001. Kinetics of lipid peroxidation in mixtures of HDL and LDL: mutual effects. *Free Radic. Biol. Med.* **31:** 1486–1497.
30. Kontush, A., S. Chantepie, and M. J. Chapman. 2003. Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress. *Arterioscler. Thromb. Vasc. Biol.* **23:** 1881–1888.
31. Cao, H., A. Girard-Globa, F. Berthezene, and P. Moulin. 1999. Paraoxonase protection of LDL against peroxidation is independent of its esterase activity towards paraoxon and is unaffected by the Q→R genetic polymorphism. *J. Lipid Res.* **40:** 133–139.