Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities[S]

Dragomir I. Draganov,[1] John F. Teiber, Audrey Speelman, Yoichi Osawa, Roger Sunahara, and Bert N. La Du[2]

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109

Abstract **The paraoxonase (PON) gene family in humans has three members, *PON1*, *PON2*, and *PON3*. Their physiological role(s) and natural substrates are uncertain. We developed a baculovirus-mediated expression system, suitable for all three human PONs, and optimized procedures for their purification. The recombinant PONs are glycosylated with high-mannose-type sugars, which are important for protein stability but are not essential for their enzymatic activities. Enzymatic characterization of the purified PONs has revealed them to be lactonases/lactonizing enzymes, with some overlapping substrates (e.g., aromatic lactones), but also to have distinctive substrate specificities. All three PONs metabolized very efficiently 5-hydroxy-eicosatetraenoic acid 1,5-lactone and 4-hydroxy-docosahexaenoic acid, which are products of both enzymatic and nonenzymatic oxidation of arachidonic acid and docosahexaenoic acid, respectively, and may represent the PONs' endogenous substrates. Organophosphates are hydrolyzed almost exclusively by PON1, whereas bulky drug substrates such as lovastatin and spironolactone are hydrolyzed only by PON3. Of special interest is the ability of the human PONs, especially PON2, to hydrolyze and thereby inactivate *N*-acyl-homoserine lactones, which are quorum-sensing signals of pathogenic bacteria.[jlr] None of the recombinant PONs protected low density lipoprotein against copper-induced oxidation in vitro.**—Draganov, D. I., J. F. Teiber, A. Speelman, Y. Osawa, R. Sunahara, and B. N. La Du. **Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J. Lipid Res.* 2005.** 46: **1239–1247.**

Supplementary key words *N*-acyl-homoserine lactones • protein expression and purification • arylesterase • low density lipoprotein oxidation

The paraoxonase (PON) gene family in humans has three members, *PON1*, *PON2*, and *PON3*, aligned next to each other on the long arm of chromosome 7q21.3-22.1 (1). They show high similarity in their structural characteristics and have ~65% identity at the amino acid level (1). The three genes are well conserved in mammals, sharing 79–95% identity at the amino acid level and 81–95% identity at the nucleotide level between different species (1–3). Phylogenetic analysis reveals PON2 to be the oldest member of the family (3). All PON2 and PON3 cDNAs sequenced to date lack the three nucleotides of codon 106, which are present in PON1 (1, 3). The latter is by far the best studied member of the family. PON1 hydrolyzes the toxic oxon metabolites of a number of organophosphorous insecticides such as parathion, diazinon, and chlorpyrifos (4, 5) and even nerve agents such as sarin and soman (5, 6). PON1 also hydrolyzes aromatic esters, preferably those of acetic acid (4). Phenyl acetate is one of the most commonly used substrates for following PON1's enzymatic activity in serum. More recently, PON1 has been shown to catalyze the hydrolysis of a variety of aromatic and aliphatic lactones (7, 8) as well as the reverse reaction, lactonization, of γ- and δ-hydroxycarboxylic acids (9). PON3s purified from rabbit serum, rat liver microsomes, and stably transfected HEK 293 cells all have low arylesterase and almost no PON activities, but they share some lactone substrates with PON1 (e.g., dihydrocoumarin) (10–12). Dihydrocoumarin is the only substrate reported to date for PON2 (12).

Human PON1 is synthesized in the liver and secreted into the blood, where it is associated exclusively with HDLs (13, 14). The secreted protein retains its hydropho-

Manuscript received 23 December 2004 and in revised form 23 February 2005.

Published, JLR Papers in Press, March 16, 2005.
DOI 10.1194/jlr.M400511-JLR200

Abbreviations: ACN, acetonitrile; AHL, acylhomoserine lactone; DDM, *n*-dodecyl-β-D-maltoside; 5,6-DHTL, 5,6-dihydroxytrienoic acid 1,5-lactone; 5,6-EET, 5,6-epoxyeicosatrienoic acid; EndoH, endoglycosidase H; 4-HDoHE, (±)4-hydroxy-5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid; 5-HETEL, (±)5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid 1,5-lactone; PLA_2, phospholipase A_2; PON, paraoxonase; thio-PAF, 2-thio platelet-activating factor.

[1] To whom correspondence should be addressed.
e-mail: draganov@umich.edu
[2] Deceased 30 January, 2005.

[S] The online version of this article (available at http://www.jlr.org) contains additional text, figures, and references.

bic leader sequence, which is a structural requirement for PON1's association with HDL (14, 15). Similar to PON1, PON3 is expressed mostly in the liver and at low levels in the kidney (16). Rabbit and human PON3 are found in serum associated with HDL (10, 16) but are ~2 orders of magnitude less abundant than PON1 (10). PON3 mRNA and protein have been identified in murine but not in human macrophages (12). PON2 is not detectable in serum, although it is expressed in many tissues, including brain, liver, kidney, and testis (17), and may have multiple mRNA forms (18).

The physiological roles of the PONs are not known. PON1's association with HDL in serum led to the suggestion by Mackness (13) that the enzyme might have a role in lipid metabolism and protect against the development of atherosclerosis. HDL's ability to prevent oxidative modifications of LDL has been attributed to PON1 (19–22), and serum PON1 levels are inversely proportional to the risk of coronary heart disease (23, 24). The antiatherogenic role of PON1 is further supported by studies in transgenic mice lacking or overexpressing the enzyme (25, 26). PON1-knockout mice are more susceptible to developing atherosclerosis than are wild-type mice, and their HDL, in contrast to wild-type HDL, fails to prevent LDL oxidation in cultured artery wall cells (25). Like PON1, both human PON2 and PON3 have been shown to prevent cell-mediated oxidative modification of LDL (16, 17). However, the exact endogenous substrates and mechanism of the PONs' protective activities remain to be elucidated.

Recently, human PON1 was expressed in insect cells and the purified recombinant protein was shown to exhibit similar kinetic properties to PON1 purified from serum (27). Here, we describe a similar expression system for all three human PONs in insect cells and optimized procedures for their purification. We characterized the enzymatic activities of the recombinant PONs with a wide range of organophosphate, aromatic ester, lactone, and hydroxycarboxylic acid substrates and tested their ability to protect LDL against copper-induced oxidation.

MATERIALS AND METHODS

Materials

Enzymes for molecular biological procedures were purchased from Promega and New England Biolabs. pcDNA3.1(+) and pFastBac1(+) vectors, DH10Bac™ *Escherichia coli* cells, penicillin-streptomycin (10,000 U/ml penicillin, 10 mg/ml streptomycin), Geneticin® (G-418 sulfate), Cellfectin® reagent, Pluronic® F-68, Sf-900 II, and Grace's insect media were obtained from Invitrogen. Insect Xpress™ medium and fetal bovine serum were purchased from BioWhittaker, Inc. (Walkersville, MD). DEAE-Microprep and low molecular weight prestained Precision Plus protein standards were obtained from Bio-Rad. Concanavalin A-Sepharose was purchased from Amersham. Chlorpyrifos oxon and diazoxon were purchased from Chem Service, Inc. (±)4-hydroxy-5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid (4-HDoHE), (±)5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid 1,5-lactone (5-HETEL), and 2-thio platelet-activating factor (thio-PAF) were obtained from Cayman Chemical (Ann Arbor, MI). Lovastatin was provided by Merck. All other chemicals were of analytical grade or better and purchased from Sigma-Aldrich or Fisher.

Expression and purification of recombinant human PONs

The generation of recombinant baculoviruses and the purification procedure for the individual PONs are described in detail in the supplementary data online. Briefly, the recombinant PONs were expressed in *Trichoplusia ni* High Five™ insect cells (Invitrogen) and extracted from the crude membrane fraction with a detergent [*n*-dodecyl-β-D-maltoside (DDM)]. PON1 and PON2 were purified to homogeneity using Microprep-DEAE and Concanavalin A-Sepharose chromatography, but for PON3 purification an additional third step (Superdex 200) was necessary. Representative purifications of the recombinant human PONs are shown in supplementary Figs. I–III and are summarized in **Table 1**.

Native and denaturing PAGE, zymograms, and Western blotting

Nondenaturing and SDS-PAGE were performed according to Laemmli (28). The nondenaturing gels contained 0.05% DDM and no SDS. The proteins were visualized by Coomassie Blue or silver staining. For activity staining (zymogram), gels were immersed in substrate solution, prepared by dissolving 80 mg of β-naphthyl acetate and 40 mg of Fast Blue RR salt in 20 ml of ethylene glycol monomethyl ether and then diluted to 50 ml with 25 mM Tris-HCl, pH 8.0, and 5 mM $CaCl_2$. Red bands representing catalytically active protein developed within seconds. The gels were fixed (20% isopropyl alcohol and 10% acetic acid), scanned, and stained with Coomassie Blue. For Western blotting, gels run under denaturing or nondenaturing conditions were transferred to polyvinylidene difluoride membranes. The primary antibodies used were as follows: for PON1, mouse monoclonal antibody 3C6.33 (3.3 mg/ml, affinity purified from ascites; University of Michigan Hybridoma Core); for PON2, affinity-purified polyclonal rabbit antibody raised against human PON2-specific peptide (HLKEEKPRARELRISRGFDLA); and for PON3, rabbit anti-human PON3 serum (provided by Dr. S. T. Reddy, University of California Los Angeles). Secondary antibodies coupled with alkaline phosphatase (Sigma) were used at 1:5,000 dilutions.

The molecular masses of the native PONs were determined by a Ferguson plot (29, 30) using BSA (monomer, 66 kDa; dimer, 132 kDa), alcohol dehydrogenase (150 kDa), and ferritin (443 kDa) as standards.

Enzymatic deglycosylation of the recombinant PONs

Purified recombinant PONs were enzymatically deglycosylated with endoglycosidase H (EndoH) or peptide:*N*-glycosidase F according to the manufacturer's protocols (New England Biolabs, Inc.). EndoH deglycosylation of the recombinant PONs under nondenaturing conditions was carried out overnight at room temperature in 50 mM sodium citrate buffer, pH 5.5, supplemented with 50 mM $CaCl_2$.

Enzymatic assays

Ultraviolet/visible spectrophotometric assays were performed on a Cary 3 instrument (Varian) equipped with a temperature block adjusted to 25°C. The initial rates were calculated from the curve slopes using the Cary WinUV Bio software package. Substrate stock solutions (100 mM) were prepared in methanol except phenyl acetate and paraoxon (water suspensions of 20 and 4 mM, respectively) and unless specified otherwise were used at 1 mM final concentration (1% methanol final concentration in the reaction mixture). Arylesterase activity with the substrates phenyl acetate, *p*-NO_2-phenyl acetate, *p*-NO_2-phenyl propionate, and *p*-NO_2-phenyl butyrate was measured in 50 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$ (31, 32). Organophosphatase activity with

TABLE 1. Summary of the purification of recombinant human PONs from HiFive insect cells

PON	Volume	Protein	Activity	Specific Activity	Yield	Fold Purification
	ml	*mg*	*units*	*U/mg*	%	*n*
Human PON1			Phenyl acetate hydrolysis			
Cell homogenate	100	234.2	9,000	38.4		1.0
Solubilized membranes	20	54.6	11,600	212.3	100	5.6
100,000 *g* supernatant	19	38.0	10,925	287.5	94	7.5
DEAE pool	12	5.9	6,070	1021.0	52	26.6
Concanavalin A pool	7	1.8	2,230	1225.0	19	31.9
Human PON2			4-HDoHE lactonization			
Cell homogenate	80	148.0	1.043	0.007		1.0
Solubilized membranes	20	100.0	1.316	0.013		1.9
100,000 *g* supernatant	18	31.7	1.437	0.045	100	6.5
DEAE pool	6	0.9	0.411	0.466	29	65.6
Concanavalin A pool	4	0.4	0.212	0.530	13	75.7
Human PON3			Lovastatin hydrolysis			
Cell homogenate	100	123.0	0.418	0.003		1.0
Solubilized membranes	20	80.2	0.618	0.008		2.3
100,000 *g* supernatant	19	36.2	0.724	0.020	100	5.9
DEAE pool	10	4.5	0.219	0.049	30	14.4
Concanavalin A pool	6	1.0	0.065	0.065	9	19.0
Superdex 200 pool	3.5	0.2	0.054	0.270	7.5	79.4

4-HDoHE, (±)4-hydroxy-5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid; PON, paraoxonase. Results are given for typical purifications. The enzymatic activities were measured as described in Materials and Methods. Yield = (activity of the fractions combined for the next step)/(activity of the solubilized membranes or the 100,000 *g* supernatant) × 100 and does not include all of the activity actually recovered. One unit of enzymatic activity is defined as 1 μmol of substrate metabolized per minute.

the substrates paraoxon, chlorpyrifos oxon (0.32 mM final concentration), and diazoxon was measured in 100 mM Tris-HCl buffer, pH 8.5, 2 mM $CaCl_2$, and 2 M NaCl (5, 33). Lactonase activity with the substrates dihydrocoumarin, 2-coumaronone, and homogentisic acid lactone was assayed in 50 mM Tris-HCl buffer, pH 7.4, and 1 mM $CaCl_2$ (10).

Lactonase activity with aliphatic lactones was determined by a continuous pH-sensitive colorimetric assay modified from Billecke et al. (7) using a SPECTRAmax® PLUS microplate reader (Molecular Devices, Sunnyvale, CA). The reactions (200 μl final volume) containing 2 mM HEPES, pH 8.0, 1 mM $CaCl_2$, 0.004% (w/v) Phenol Red, and 2–10 μl of purified enzyme were initiated with 2 μl of 100 mM substrate solution in methanol and were carried out at 37°C for 3–10 min. The rates were calculated from the slopes of the absorbance decrease at 558 nm with correction at 475 nm (isosbestic point) using a rate factor (mOD/μmol H^+) estimated from a standard curve generated with known amounts of HCl. The spontaneous hydrolysis of the lactones and acidification by atmospheric CO_2 were corrected for by carrying out parallel reactions with the same volume of storage buffer instead of enzyme.

The lactonization of 4-HDoHE (10 μM) and coumaric acid (100 μM) as well as the lactone hydrolysis of 5-HETEL (10 μM), lovastatin, spironolactone, and canrenone (25 μM) at the final substrate concentrations indicated in parentheses were analyzed by HPLC using a Supelco Discovery C-18 column (250 × 4.6 mm, 5 μm particles) and a Beckman System Gold 126 equipped with a Beckman 128 diode array detector as described (7, 9, 10).

The hydrolysis of acylhomoserine lactones (AHLs) was analyzed by HPLC on a Waters 2695 system equipped with Waters 2996 photodiode array detector set at 197 nm using a Supelco Discovery C-18 column (250 × 4.6 mm, 5 μm particles). Enzymatic reactions were carried out at room temperature in 50 μl volume of 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 25 μM AHL (from 2 mM stock solution in methanol, except for 3-oxo-hexanoyl homoserine lactone, which was 25 mM stock and 250 μM final), and 2–5 μl of purified enzyme. Reactions were stopped with 50 μl of acetonitrile (ACN) and centrifuged to remove the protein. Supernatants (40 μl) were loaded onto the HPLC system and eluted isocratically with 85% ACN/0.2% acetic acid (tetradeca-homoserine lactone), 75% ACN/0.2% acetic acid (dodeca-homoserine lactone), 50% ACN/0.2% acetic acid (hepta-homoserine lactone), or 20% ACN/0.2% acetic acid (3-oxo-hexanoyl homoserine lactone). The retention times for the open acid forms and the lactones under these conditions were as follows: 5.8/7.5 min for tetradeca-homoserine lactone, 4.9/6.9 min for dodeca-homoserine lactone, 3.6/4.8 min for hepta-homoserine lactone, and 4.1/5.6 min for 3-oxo-hexanoyl homoserine lactone.

The hydrolysis of thio-PAF was measured on 96-well plates according to the manufacturer's protocol (Cayman PAF-AH assay kit, catalog number 760901). All enzymatic activities are expressed in units per milligram of purified protein where 1 U is defined as 1 μmol of substrate metabolized per minute.

Transient expression of PONs in HEK 293T cells

HEK 293T cells were grown on six-well plates and transfected at ~70% confluence by the calcium phosphate precipitation method (34) with empty pcDNA3.1 vector (mock) or pcDNA3.1 vector containing human PON1, PON2, or PON3 cDNA (2 μg DNA/well). Two days after transfection, cells were collected and sonicated in 0.5 ml of 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM $CaCl_2$. The membrane fraction was isolated by centrifugation at 16,000 *g* for 15 min and resuspended in the same buffer.

LDL oxidation assays

LDL oxidation reactions were performed on 96-well plates in PBS at a final volume of 0.2 ml and contained 100 μg LDL protein/ml (Intracel, Frederick, MD) as described previously (35). The reactions were initiated by adding 20 μl of copper sulfate in distilled water (5 μM final concentration) and run at 37°C. The absorbance was monitored continuously at 234 nm, and lag times were calculated from the kinetic profiles. Lipid peroxides and thiobarbituric acid-reactive substances were determined as described (35).

RESULTS

Characterization of the purified PONs

The purified PON1 appeared as a double band of 39 and 42 kDa on SDS-PAGE, whereas PON2 and PON3 appeared as single 39 kDa bands (**Fig. 1A**). Purified recombinant PONs were electrophoresed on 7.5% nondenaturing PAGE and stained for enzymatic activity with β-naphthyl acetate/RR Fast Blue (Fig. 1B). The enzymatically active form of the proteins accounted for more than 90–95% of the total PON mass in these preparations, as judged from Western blot analysis (data not shown). We examined the migration of the recombinant human PONs on a set of gels of various polyacrylamide concentrations and analyzed the data graphically using Ferguson plots (see supplementary Fig. IV). The estimated molecular masses for the native PONs were 91.9, 95.6, 127.5, and 94.9 kDa for serum PON1, recombinant PON1, PON2, and PON3, respectively, with ~10% error in the estimation for the individual PONs. Given that the PON monomers have a molecular mass of ~40 kDa, the expected size of the PON dimers would be ~80 kDa, and 120 kDa for trimeric complexes. Thus, it was concluded that in the presence of DDM above its critical micelle mass, the native enzymatically active forms of the purified PON1 and PON3 are probably dimers, but PON2 forms a trimer.

Next, we examined whether the differences in the PONs are attributable to different extents and types of glycosylation of the recombinant proteins. All three human PONs expressed in HiFive cells were deglycosylated by EndoH, suggesting glycosylation with high-mannose-type sugars (**Fig. 2**). In contrast, PON1 and PON3 from human serum were deglycosylated only by peptide:*N*-glycosidase F, indicating a complex type of sugar in the secreted serum PONs. The extent of glycosylation seems to account for the differences in the migration on SDS-PAGE of the recombinant PON1 and PON3 and the PONs purified from human serum.

The difference in glycosylation between the recombinant and serum PON1 and PON3 does not seem to alter their substrate specificity or to be required for their enzymatic activities. In contrast to Brushia et al. (27), we did not find a significant loss of PON1's hydrolytic activity after deglycosylation with EndoH under nondenaturing conditions (data not shown). Under acidic conditions, PON1 has lower affinity for Ca^{2+} (9, 36); therefore, we supplemented the reaction buffer with 50 mM $CaCl_2$ to preserve PON1's activity. Similarly, EndoH deglycosylation of the recombinant PON2 and PON3 did not abolish their enzymatic activities. However, the type and extent of glycosylation of the recombinant PONs may be responsible for their reduced stability, compared with the PONs purified from serum or transfected HEK 293 cells. Purified recombinant PON1 preparations typically lost ~20% of their arylesterase activity within the first month after purification when stored at 4°C, compared with less than 10% loss for the enzyme purified from serum. There was no further significant loss of the PON1 activity afterward, suggesting the existence of two pools in the purified PON1, a labile one and a stable one. Recombinant PON2 and PON3 seem to be more stable than PON1. There was less than 15% loss in their activities over 6 months of storage at 4°C.

Enzymatic activities of the purified recombinant human PONs

The enzymatic activities of the purified recombinant PONs were studied over a range of organophosphate and aromatic esters, lactones, and hydroxycarboxylic acids (**Ta-**

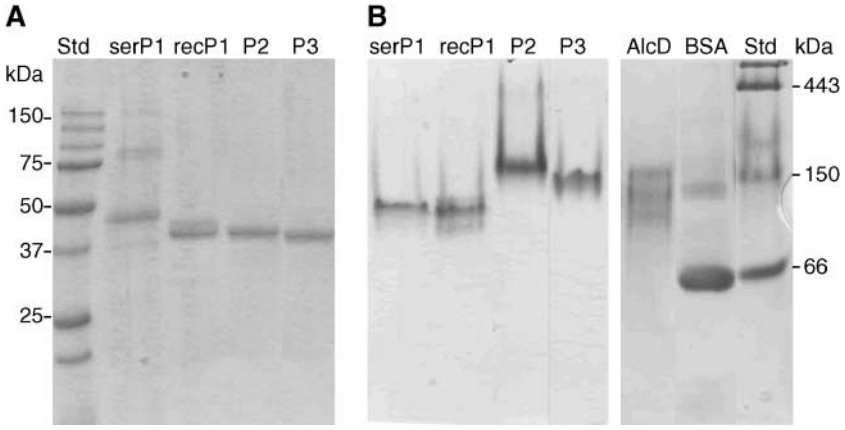

Fig. 1. Denaturing and nondenaturing electrophoresis of the purified recombinant paraoxonases (PONs). A: Purified PONs were electrophoresed on 12% SDS-PAGE and stained with Coomassie Blue as described in Materials and Methods. B: Nondenaturing electrophoresis was performed on a 7.5% polyacrylamide gel containing 0.05% *n*-dodecyl-β-D-maltoside and no SDS. Staining for enzymatic activity with β-naphthyl acetate/Fast Blue RR salt (left panel) and for protein with Coomassie Blue (right panel) was performed as described in Materials and Methods. Four micrograms (A) or 10 μg (B) of protein was loaded per lane: purified serum PON1 (serP1), recombinant PON1 (recP1), PON2 (P2), PON3 (P3), alcohol dehydrogenase (AlcD; the upper band is 150 kDa), BSA (monomer, 66 kDa; dimer, 132 kDa), and nondenaturing PAGE protein standards mix (Std) containing 2.5 μg of carbonic anhydrase (29 kDa), BSA, alcohol dehydrogenase, apoferritin (443 kDa), and thyroglobulin (669 kDa).

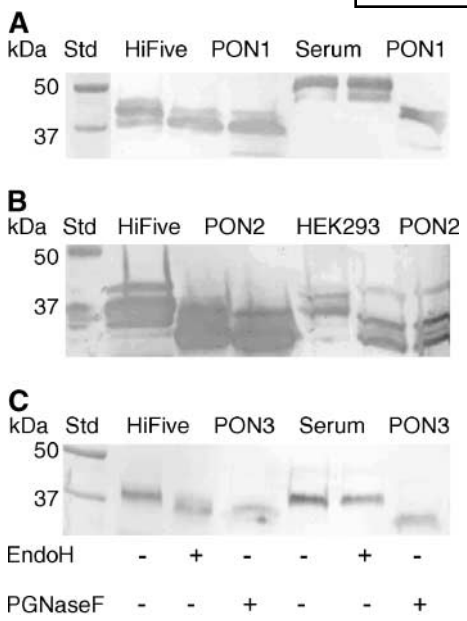

Fig. 2. Enzymatic deglycosylation of the recombinant human PONs. Purified recombinant PON1 (A), PON2 (B), and PON3 (C) were deglycosylated with endoglycosidase H (EndoH) or peptide:*N*-glycosidase F (PNGaseF) as described in Materials and Methods. The digests were run on 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and Western blotted as described in Materials and Methods. For comparison with mammalian processing, purified PON1 and PON3 from human serum (A, C) or PON2 (B) from HEK 293 membranes were used.

ble 2). Organophosphatase activity was limited to PON1; PON3 had very low PON activity and did not hydrolyze diazoxon and chlorpyrifos oxon. All three PONs hydrolyzed aromatic esters, but with some noticeable differences. Phenyl acetate is one of the best substrates for PON1 but was hydrolyzed at a modest rate by PON3 and very slowly by PON2. Introduction of a nitro group in the *para* position dramatically decreased PON1 activity while increasing PON2 and PON3 activities. The rate of *p*-NO_2-phenol ester hydrolysis decreased from acetate > butyrate > propionate for PON1 and PON3 but increased in that order for PON2. Dihydrocoumarin and 2-coumaronone were very good substrates for PON1 and PON3 and were hydrolyzed by PON2 also, although less efficiently. Interestingly, the hydroxyl group in homogentisic acid lactone seemed to promote PON1's lactonase activity but abolished it for PON2 and PON3. We studied PON lactonase activity over a series of aliphatic five- and six-member ring lactones. In general, PON1 and PON3 activities increased as the length of the substituent in the γ or δ position increased. The rate of δ-valerolactone hydrolysis by PON1 was an exception. The presence of a double bond within the lactone ring (α-angelica lactone) increased the rate of hydrolysis by PON1 and PON3. γ-Phenyl-γ-butyrolactone was hydrolyzed by all three PONs. Six-member ring lactones were hydrolyzed more efficiently than their five-member ring analogs. None of the aliphatic lactones was hydrolyzed by PON2 under these assay conditions (up to 10 min at 37°C).

The limited solubility of the more hydrophobic substrates required analysis at low micromolar concentrations and HPLC quantitation of the reaction products. At 10 μM substrate concentration, 5-HETEL is probably the best substrate we have identified for each of the PONs, but the limitations of its solubility precluded further kinetic experiments. Bulky drug substrates such as lovastatin, spironolactone, and canrenone were hydrolyzed only by PON3. All three human PONs catalyzed the lactonization of 4-HDoHE, but only PON1 and PON3 were able to lactonize coumaric acid.

The acyl-homoserine lactones, except 3-oxo-hexanoyl homoserine lactone, were assayed at a concentration of 25 μM, which is in the range produced by laboratory bacterial cultures (37). Because of the lower sensitivity of the HPLC assay for 3-oxo-hexanoyl homoserine lactone, we used a higher substrate concentration (250 μM) to accurately quantitate its hydrolysis. Interestingly, all of the AHLs were preferably hydrolyzed by PON2. The hydrolysis was stereoselective; after allowing the reaction to proceed until completion, only half of the DL-AHLs were hydrolyzed. The only L-form we had available was L-3-oxo-hexanoyl homoserine lactone, and it was hydrolyzed completely by PON2. No AHL hydrolysis was observed in the presence of 5 mM EDTA (data not shown).

Highly purified serum PON1 was reported to possess phospholipase A_2 (PLA_2) activity (38, 39). We used thio-PAF as a surrogate substrate to test the recombinant PONs for PLA_2 activity. None of the PONs hydrolyzed thio-PAF (detection limit, 10 nmol/min). Serum PON1 purified by the method used to purify the recombinant PONs also did not have thio-PAF hydrolyzing activity (35) and did not reduce or hydrolyze phospholipids oxidized with peroxynitrite (40). Marathe, Zimmerman, and McIntyre (41) were also able to separate a PAF-acetyl hydrolase-like PLA_2 activity from PON1.

To test whether the substrate specificity of the PONs described above for the recombinant proteins is maintained under cellular conditions, we overexpressed each of the human PONs in HEK 293T cells by transient transfections and measured the enzymatic activities of the membrane fractions with representative substrates (**Table 3**). The substrate specificity of the PON-transfected cells was in accordance with the data obtained with the purified proteins overexpressed in HiFive insect cells. Approximately 60% of the total arylesterase and lactonase activity of PON1-transfected cells was secreted into the medium, whereas only ~10% of the total lactonase activity of PON3-transfected cells was secreted. The lactonase activity of PON2-transfected cells remained cell-associated (data not shown). Data from the transient transfections indicate that organophosphates and phenyl acetate can be used as specific substrates for PON1, acyl-homoserine lactones can be used as relatively specific substrates for PON2, and lovastatin and spironolactone can be used for PON3.

Recombinant human PONs do not protect LDL against copper ion-induced oxidation

Monitoring the rate and extent of LDL oxidation induced in vitro by metal ions or free radical generators is a method commonly used to assess the mechanistics and in-

TABLE 2. Specific enzymatic activities of the purified recombinant human PONs

Substrate	PON1	PON2	PON3
Organophosphatase activity (U/mg)			
Paraoxon	1.94 ± 0.11	ND	0.205 ± 0.05
Chlorpyrifos oxon (0.32 mM)	40.9 ± 0.9	ND	ND
Diazoxon	113 ± 5	ND	ND
Arylesterase activity (U/mg)			
Phenyl acetate	1,120 ± 50	0.086 ± 0.0013	4.1 ± 0.3
p-NO_2-acetate	15.0 ± 0.03	0.7 ± 0.07	39.0 ± 4.1
p-NO_2-propionate	13.6 ± 0.04	0.96 ± 0.06	20.7 ± 3.2
p-NO_2-butyrate	1.3 ± 0.015	1.4 ± 0.03	11.4 ± 0.7
Lactonase activity (U/mg)			
Dihydrocoumarin	129.9 ± 8.30	3.1 ± 0.2	126.1 ± 12
2-Coumaronone	135.7 ± 10.3	10.9 ± 0.4	40.7 ± 3.8
Homogentisic acid lactone	329.5 ± 13.1	ND	ND
γ-Butyrolactone	32.1 ± 2.73	ND	0.81 ± 0.1
γ-Valerolactone	45.0 ± 3.7	ND	6.2 ± 0.4
γ-Hexalactone	51.7 ± 4.2	ND	23.9 ± 3.2
γ-Heptalactone	57.2 ± 2.3	ND	27.7 ± 2.7
γ-Octalactone	69.2 ± 4.3	ND	25.6 ± 3.2
γ-Nonalactone	144.7 ± 11.3	ND	30.9 ± 2.7
γ-Decanolactone	173.8 ± 14.7	ND	45.6 ± 3.6
γ-Undecanolactone	127.6 ± 10.5	ND	71.4 ± 3.1
α-Angelica lactone	183.0 ± 16	ND	20.7 ± 3.2
γ-Phenyl-γ-butyrolactone (0.5 mM)	63.0 ± 3.1	0.68 ± 0.08	11.4 ± 0.7
δ-Valerolactone	671 ± 14	ND	14.5 ± 0.7
δ-Hexalactone	72 ± 2.3	ND	11.7 ± 1.2
δ-Nonalactone	150 ± 12.3	ND	11.1 ± 0.9
δ-Decanolactone	251 ± 13	ND	44.3 ± 3.2
δ-Undecanolactone	287 ± 17	ND	84.4 ± 2.7
δ-Tetradecanolactone (0.5 mM)	154 ± 24	ND	22.7 ± 2.2
5-HETEL (10 μM)	75.4 ± 8.36	1.83 ± 0.08	27.5 ± 3.6
DL-3-Oxo-hexanoyl-HSL (250 μM)	0.0334 ± 0.0031	0.2683 ± 0.0384	ND
L-3-Oxo-hexanoyl-HSL (250 μM)		0.5080 ± 0.0661	
DL-Heptanoyl-HSL (25 μM)	0.0036 ± 0.0004	0.0311 ± 0.0026	0.0049 ± 0.0023
DL-Dodecanoyl-HSL (25 μM)	0.0167 ± 0.0005	0.4588 ± 0.0371	0.0877 ± 0.0014
DL-Tetradecanoyl-HSL (25 μM)	0.0035 ± 0.0013	0.4239 ± 0.0204	0.0255 ± 0.0003
Lovastatin (25 μM)	ND	ND	0.266 ± 0.022
Spironolactone (25 μM)	ND	ND	0.011 ± 0.001
Canrenone (25 μM)	ND	ND	0.013 ± 0.001
Lactonizing activity (U/mg)			
Coumaric acid (100 μM)	0.047 ± 0.004	ND	0.013 ± 0.0007
4-HDoHE (10 μM)	1.51 ± 0.16	0.52 ± 0.03	13.7 ± 2.0

5-HETEL, (±)5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid 1,5-lactone; HSL, homoserine lactone; ND, not detectable under these assay conditions. Data are averages from two to four measurements ± SD or range. One unit = 1 μmol of substrate metabolized per minute. All substrates were at 1 mM final concentration unless indicated otherwise.

hibitory capacity of antioxidants. Numerous studies have shown that purified serum PON1 can inhibit LDL oxidation after initiation with copper or 2,2′-azobis-2-amidinopropane hydrochloride (19–21). Therefore, we tested the purified recombinant PONs but found that they failed to protect human LDL against copper-induced oxidation assayed by monitoring of the conjugated diene formation (**Fig. 3**). Also, there were no significant differences in the amount of lipid peroxides and thiobarbituric acid-reactive substances generated up to 6.5 h after initiation of LDL oxidation. No antioxidant activity copurified with any of the PONs (data for recombinant PON1 are shown in supplementary Fig. V). This concurs with our recent study (35), which demonstrated that purified human serum PON1 does not have antioxidant activity in this in vitro assay. We conclude that copper-induced in vitro LDL oxidation is an inappropriate method to study the antioxidant properties of highly purified PONs.

TABLE 3. Enzymatic activities of the membrane fraction isolated from HEK 293T cells transiently transfected with human PONs

Substrate	Mock	PON1	PON2	PON3
Phenyl acetate	0.07 ± 0.05	3,266 ± 666	0.23 ± 0.11	48.2 ± 10.5
5-HETEL	2.38 ± 0.57	249 ± 60	20.7 ± 0.9	161 ± 5.9
DL-Tetradecanoyl-HSL	ND	ND	0.46 ± 0.05	ND
Lovastatin	ND	ND	ND	0.27 ± 0.06

HSL, homoserine lactone; ND, not detectable under these assay conditions. Activities are expressed in nanomoles per minute per milligram of protein and are averages ± SD from three samples assayed in duplicate.

DISCUSSION

The three PON genes, *PON1*, *PON2*, and *PON3*, are highly conserved in mammals, suggesting an important physiological role(s) for them. PON-like proteins can be found

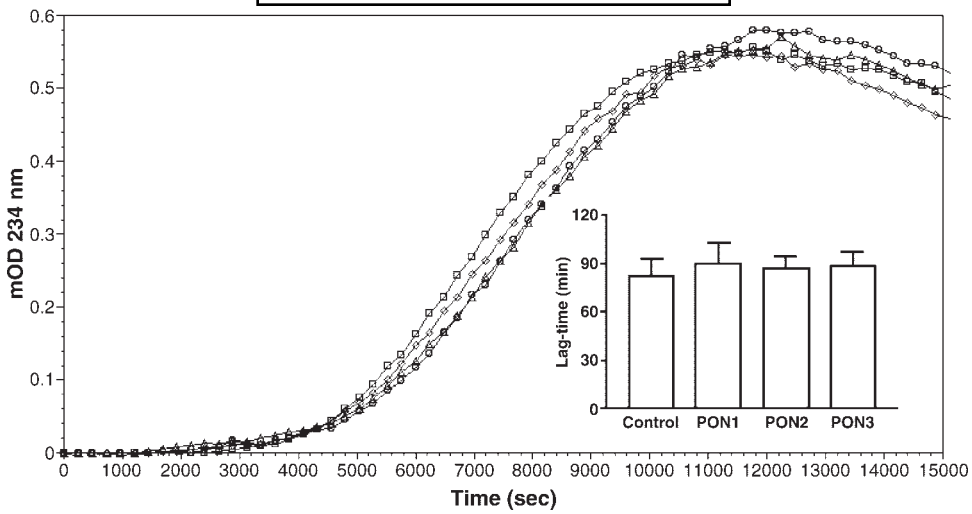

Fig. 3. Purified recombinant human PONs do not protect LDL against copper ion-induced oxidation. LDL oxidation reactions were performed on 96-well plates in PBS at a final volume of 0.2 ml and contained 100 μg LDL protein/ml and 10 μg of recombinant protein purified through a concanavalin A column (PON1, circles; PON2, diamonds; PON3, triangles). The reactions were initiated with 5 μM copper sulfate and run at 37°C. The figure shows a typical profile of conjugated diene formation monitored at 234 nm; control (squares) was a flow-through fraction from the concanavalin A chromatography with similar protein concentration and no PON activity. Inset: Average and standard deviations of the lag times from three LDL oxidation experiments, each performed in duplicate. mOD, miliOD units.

in all animal species (1–3), and even in fungi and bacteria. Lactone hydrolase from *Fusarium oxysporum* shares aromatic lactone substrates with the human PONs, but it does not have PON activity (42). Based on the evolutionary relationships (3, 42) and the substrate specificity of the human PONs described here, we proposed that the PON proteins are primarily lactonases/lactonizing enzymes. Noteworthy, 5-HETEL and 4-HDoHE, which are products of both enzymatic and nonenzymatic oxidation of arachidonic acid and docosahexaenoic acid, respectively, are metabolized by all three PONs with very high efficiency. 5-HETEL formation has been described in cell systems from human intrauterine tissue (43) and B-lymphocytes stimulated with a calcium ionophore (44). 5-HETEL was more effective than the open acid, (±)5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, in inhibiting 5-lipoxygenase (45, 46) and in thromboxanes and prostaglandin E_2 synthesis in peritoneal macrophages (47), which was attributed to the more favorable binding conformation of the lactone. (±)5-Hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, but not 5-HETEL, was shown to be a specific PLA_2 inhibitor (48). Therefore, the presence of 5-HETEL in vivo may have an effect on cells that use PLA_2, 5-lipoxygenase, and/or cyclooxygenases, and its hydrolysis by the PONs may modulate this effect. Of the three human PONs, only PON2 is expressed in the above-mentioned cells and tissues, but its role in in vivo 5-HETEL metabolism remains to be demonstrated. There are many oxidized metabolites of polyunsaturated fatty acids with distinct biological activities that are structurally similar to 5-HETEL and 4-DHoHA, including iodolactones (49), resolvins (50), and lipoxins (51). Based on our findings, it is likely that at least some of these are PON substrates. One example is 5,6-dihydroxytrienoic acid 1,5-lactone (5,6-DHTL), which is a product of the spontaneous intramolecular rearrangement of 5,6-epoxyeicosatrienoic acid (5,6-EET), a cytochrome P450-derived arachidonic acid metabolite (52). 5,6-DHTL produced extremely potent vasodilation in canine coronary arterioles via activation of K_{Ca} channels with a mean effective dose of −13.1 log[M] (53). We have studied the conversion of 5,6-EET to the dihydroxy acid (5,6-dihydroxytrienoic acid) in the presence of purified PON enzymes from human and rabbit plasma (PON1 and PON3), a reaction otherwise catalyzed by epoxide hydrolases. We have found that PONs did not influence the rate of 5,6-EET conversion to the lactone but rapidly hydrolyzed the latter to 5,6-dihydroxytrienoic acid (P. Stetson, D. Draganov, and B.N. La Du, unpublished results).

Only a few substrates have been found for human PON2, and its enzymatic activities, except for the AHLs, are much lower than those of PON1 and PON3. PON2 is considered the oldest member in the PON family (2, 3) and thus may have only the core enzymatic activities for the PON family. Degradation AHLs could represent an important physiological role for the PONs, and especially for human PON2. AHLs are now recognized as important quorum-sensing mediators in bacteria with major roles in the virulence of a number of pathogens; thus, AHL-degrading enzymes could be important for host defense (54). Apart from regulating the expression of virulence factors, homoserine lactones themselves may function as virulence determinants and modulate immune responses and other eukaryotic cell functions (55, 56). An enzyme-mediated inactivation of *Pseudomonas aeruginosa* quorum-sensing signal was described recently in human airway epithelia and some cultured mammalian cell lines (57). AHL degradation could proceed through

opening of the lactone ring or by cleavage of the acyl side chain from the lactone ring, and it is not clear which of the two (or both) activities is responsible for the AHL inactivation described by Chun et al. (57). However, the authors provide evidence for the cell membrane association of the AHL inactivator. The enzymatic activities of PON2 described here and its membrane localization (17) make us suggest a role for PON2 in the inactivation of long-chain homoserine lactones and thus in the disruption of quorum sensing in pathogenic bacteria. Moreover, PON2 expression is upregulated under oxidative stress, accompanying the infectious process, and during the monocyte maturation to macrophages (12), which may represent an important innate immune response.

PON1 has been shown to protect mice against atherosclerosis (25, 26), and this protection has been attributed to its ability to protect LDL against oxidation and/or to attenuate the biological activity of oxidized LDL (19–22). Recently, we demonstrated that PON1 purified from human serum does not protect LDL against oxidation in vitro (35). In that report, we showed also that the antioxidant activity copurifies with, but can be separated from, serum PON1 and is actually attributable to a low molecular mass contaminant (<3 kDa) as well as to the detergent present in the preparations. None of the recombinant PONs was able to protect LDL against copper-induced oxidation in vitro, suggesting either that PONs do not have antioxidant activity (e.g., scavenging or destroying lipid peroxides) or they may need a specific phospholipid and/or protein environment to exert such activity. A directly evolved form of PON1 was crystallized very recently, and a model for PON1 association with HDL has been proposed (58). According to this model, the N-terminal domain of PON1 forms a unique active site lid that is also involved in HDL binding and orients the active site toward the HDL hydrophobic interior. We would expect similar orientation of PON2 and PON3 toward the membrane phospholipids. This is supported by the hydrophobic nature of most PON substrates. Thus, to study the "true" substrate specificity and enzymatic kinetics of the PONs, they may need to be reconstituted in an appropriate lipid/lipoprotein environment, similar to other membrane enzymes, such as cytochrome P450 family members.

In conclusion, we demonstrate that PON enzymes are lactonases and that certain lactones/hydroxy acids may represent their endogenous substrates. We propose that the physiological role of the PONs is the metabolism of lipid mediators arising from oxidation of polyunsaturated fatty acids, resulting in modulation of the local anti-inflammatory response.

NOTE IN PROOF

In a recent paper, O. Khersonsky and D. S. Tawfik have examined PON1 activity with more than 50 substrates belonging to three different classes: esters, phosphotriesters, and lactones (Khersonsky, O., and D. S. Tawfik. 2005. Structure-reactivity studies of serum paraoxonase PON1 suggest that its native activity is lactonase. *Biochemistry.* Epub ahead of print. March 26, 2005; doi:10.1021/bi047440d). Their results suggest that PON1 is not an esterase or a phosphotriesterase, but rather a lactonase.

This work was supported by Michigan Life Sciences Corridor Fund Grant 001796 and in part by UM#01-0013 subcontract of Grant DAMD17-01-1-0741 to Dr. Robert Haley (University of Texas Southwestern Medical Center, Dallas, TX) and National Institutes of Health Grant ES-08365 (to Y.O.).

REFERENCES

1. Primo-Parmo, S. L., R. C. Sorenson, J. Teiber, and B. N. La Du. 1996. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. *Genomics.* **33:** 498–507.
2. La Du, B. N., M. Aviram, S. Billecke, M. Navab, S. Primo-Parmo, R. C. Sorenson, and T. J. Standiford. 1999. On the physiological role(s) of the paraoxonases. *Chem. Biol. Interact.* **119–120:** 379–388.
3. Draganov, D. I., and B. N. La Du. 2004. Pharmacogenetics of paraoxonases: a brief review. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **369:** 78–88.
4. La Du, B. N. 1992. Human serum paraoxonase/arylesterase. *In* Genetic Factors Influencing the Metabolism of Foreign Compounds: International Encyclopedia of Pharmacology and Therapeutics. W. Kalow, editor. Pergamon Press, New York. 51–91.
5. Davies, H. G., R. J. Richter, M. Keifer, C. A. Broomfield, J. Sowalla, and C. E. Furlong. 1996. The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. *Nat. Genet.* **14:** 334–336.
6. Broomfield, C. A., and K. W. Ford. 1991. Hydrolysis of nerve gases by plasma enzymes. *In* Proceedings of the 3rd International Meeting on Cholinesterases, La Grande-Motte, France. 161.
7. 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.
8. Biggadike, K., R. M. Angell, C. M. Burgess, R. M. Farrell, A. P. Hancock, A. J. Harker, W. R. Irving, C. Ioannou, P. A. Procopiou, R. E. Shaw, et al. 2000. Selective plasma hydrolysis of glucocorticoid gamma-lactones and cyclic carbonates by the enzyme paraoxonase: an ideal plasma inactivation mechanism. *J. Med. Chem.* **43:** 19–21.
9. Teiber, J. F., D. I. Draganov, and B. N. La Du. 2003. Lactonase and lactonizing activities of human paraoxonase (PON1) and rabbit serum PON3. *Biochem. Pharmacol.* **66:** 887–896.
10. 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.
11. Rodrigo, L., F. Gil, A. F. Hernandez, O. Lopez, and A. Pla. 2003. Identification of paraoxonase 3 in rat liver microsomes. Purification and biochemical properties. *Biochem. J.* **376:** 261–268.
12. Rosenblat, M., D. Draganov, C. E. Watson, C. L. Bisgaier, B. N. La Du, and M. Aviram. 2003. Mouse macrophage paraoxonase 2 activity is increased whereas cellular paraoxonase 3 activity is decreased under oxidative stress. *Arterioscler. Thromb. Vasc. Biol.* **23:** 468–474.
13. Mackness, M. I. 1989. Possible medical significance of human serum 'A-' esterases. *In* Enzymes Hydrolysing Organophosphorus Compounds. E. Rainer, W. N. Aldridge, and F. C. G. Hoskin, editors. Ellis Horwood, Chichester, UK. 202–213.
14. Hassett, C., R. J. Richter, R. Humbert, C. Chapline, J. W. Crabb, C. J. Omiecinski, and C. E. Furlong. 1991. Characterization of cDNA clones encoding rabbit and human serum paraoxonase: the mature protein retains its signal sequence. *Biochemistry.* **30:** 10141–10149.
15. Sorenson, R. C., C. L. Bisgaier, M. Aviram, C. Hsu, S. Billecke, and B. N. La Du. 1999. Human serum paraoxonase/arylesterase's retained hydrophobic N-terminal leader sequence associates with high density lipoproteins by binding phospholipids: apolipoprotein A-1 stabilizes activity. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2214–2225.
16. Reddy, S. T., D. J. Wadleigh, V. Grijalva, C. Ng, S. Hama, A. Gangopadhyay, D. M. Shih, A. J. Lusis, M. Navab, and A. M. Fogelman. 2001. Human paraoxonase-3 is an HDL-associated enzyme with bi-

ological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids. *Arterioscler. Thromb. Vasc. Biol.* **21:** 542–547.
17. Ng, C. J., D. J. Wadleigh, A. Gangopadhyay, S. Hama, V. R. Grijalva, M. Navab, A. M. Fogelman, and S. T. Reddy. 2001. Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *J. Biol. Chem.* **276:** 44444–44449.
18. Mochizuki, H., S. W. Scherer, T. Xi, D. C. Nickle, M. Majer, J. J. Huizenga, L. C. Tsui, and M. Prochazka. 1998. Human PON2 gene at 7q21.3: cloning, multiple mRNA forms, and missense polymorphisms in the coding sequence. *Gene.* **213:** 149–157.
19. Mackness, M. I., S. Arrol, and P. N. Durrington. 1991. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett.* **286:** 152–154.
20. 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.
21. 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.
22. 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.
23. Durrington, P. N., B. Mackness, and M. I. Mackness. 2001. Paraoxonase and atherosclerosis. *Arteriosler. Thromb. Vasc. Biol.* **21:** 473–480.
24. Costa, L. G., T. B. Cole, G. P. Jarvik, and C. E. Furlong. 2003. Functional genomic of the paraoxonase (PON1) polymorphisms: effects on pesticide sensitivity, cardiovascular disease, and drug metabolism. *Annu. Rev. Med.* **54:** 371–392.
25. Shih, D. M., L. Gu, Y. R. Xia, M. Navab, W. F. Li, S. Hama, L. W. Castellani, C. E. Furlong, L. G. Costa, and A. M. Fogelman. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature.* **394:** 284–287.
26. Shih, D. M., S. T. Reddy, and A. J. Lusis. 2002. CHD and atherosclerosis: human epidemiological studies and transgenic mouse models. *In* Paraoxonase (PON1) in Health and Disease. L. G. Costa and C. E. Furlong, editors. Kluwer Academic Publishers, Norwell, MA. 93–124.
27. Brushia, R. J., T. M. Forte, M. N. Oda, B. N. La Du, and J. K. Bielicki. 2001. Baculovirus-mediated expression and purification of human serum paraoxonase 1A. *J. Lipid Res.* **42:** 951–958.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
29. Ferguson, K. A. 1964. Starch-gel electrophoresis—application to the classification of pituitary proteins and polypeptides. *Metabolism.* **13:** 985–1002.
30. Bryan, J. K. 1977. Molecular weights of protein multimers from polyacrylamide gel electrophoresis. *Anal. Biochem.* **78:** 513–519.
31. Kuo, C. L. and B. N. La Du. 1995. Comparison of purified human and rabbit serum paraoxonases. *Drug Metab. Dispos.* **23:** 935–944.
32. Eckerson, H. W., C. M. Wyte, and B. N. La Du. 1983. The human serum paraoxonase/arylesterase polymorphism. *Am. J. Hum. Genet.* **35:** 1126–1138.
33. Furlong, C. E., R. J. Richter, S. L. Seidel, L. G. Costa, and A. G. Motulsky. 1989. Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. *Anal. Biochem.* **180:** 242–247.
34. Kingston, R. E. 1993. Introduction of DNA into mammalian cells. *In* Current Protocols in Molecular Biology. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, editors. John Wiley & Sons, New York. 9.1.1–9.1.7.
35. Teiber, J. F., D. I. Draganov, and B. N. La Du. 2004. Serum PON1 does not protect LDL against oxidation induced by copper and AAPH in vitro. *J. Lipid Res.* **45:** 2260–2268.
36. Kuo, C. L., and B. N. La Du. 1998. Calcium binding by human and rabbit serum paraoxonases. Structural stability and enzymatic activity. *Drug Metab. Dispos.* **26:** 653–660.
37. Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg. 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA.* **92:** 1490–1494.
38. 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.
39. Ahmed, Z., A. Ravandi, G. F. Maguire, A. Emili, D. Draganov, B. N. La Du, A. Kukis, and P. W. Connelly. 2002. Multiple substrates for paraoxonase-1 during oxidation of phosphatidylcholine by peroxynitrite. *Biochem. Biophys. Res. Commun.* **290:** 3937–3947.
40. Connelly, P. W., D. Draganov, and G. F. Maguire. 2005. Paraoxonase-1 does not reduce or modify oxidation of phospholipids by peroxynitrite. *Free Radic. Biol. Med.* **38:** 164–174.
41. 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.
42. Kobayashi, M., M. Shinohara, C. Sakoh, M. Kataoka, and S. Shimizu. 1999. Lactone-ring-cleaving enzyme: genetic analysis, novel RNA editing, and evolutionary implications. *Proc. Natl. Acad. Sci. USA.* **95:** 2787–2792.
43. Saeed, S. A., and M. D. Mitchell. 1983. Lipoxygenase activity in human uterine and intrauterine tissues: new prospects for control of prostacyclin production in pre-eclampsia. *Clin. Exp. Hypertens. B.* **2:** 103–111.
44. Schulam, P. G., and W. T. Shearer. 1990. Evidence for 5-lipoxygenase activity in human B cell lines. A possible role for arachidonic acid metabolites during B cell signal transduction. *J. Immunol.* **144:** 2696–2701.
45. Haviv, F., J. D. Ratajczyk, R. W. DeNet, Y. C. Martin, R. D. Dyer, and G. W. Carter. 1987. Structural requirements for the inhibition of 5-lipoxygenase by 15-hydroxyeicosa-5,8,11,13-tetraenoic acid analogues. *J. Med. Chem.* **30:** 254–263.
46. Kerdesky, F. A. J., S. P. Schmidt, J. H. Holms, R. D. Dyer, G. W. Carter, and D. W. Brooks. 1987. Synthesis and 5-lipoxygenase inhibitory activity of 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid analogs. *J. Med. Chem.* **30:** 1177–1186.
47. Chang, J., B. Lamb, L. Marinari, A. F. Kreft, and A. J. Lewis. 1985. Modulation by hydroxyeicosatetraenoic acids (HETEs) of arachidonic acid metabolism in mouse resident peritoneal macrophages. *Eur. J. Pharmacol.* **107:** 215–222.
48. Chang, J., E. Blazek, A. F. Kreft, and A. J. Lewis. 1985. Inhibition of platelet and neutrophil phospholipase A2 by hydroxyeicosatetraenoic acids (HETES). A novel pharmacological mechanism for regulating free fatty acid release. *Biochem. Pharmacol.* **34:** 1571–1575.
49. Dugrillon, A. 1996. Iodolactones and iodoaldehydes—mediators of iodine in thyroid autoregulation. *Exp. Clin. Endocrinol. Diabetes.* **104 (Suppl. 4):** 41–45.
50. Serhan, C. N., K. Gotlinger, S. Hong, and M. Arita. 2004. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis. *Prostaglandins Other Lipid Mediat.* **73:** 155–172.
51. Kieran, N. E., P. Maderna, and C. Godson. 2004. Lipoxins: potential anti-inflammatory, proresolution, and antifibrotic mediators in renal disease. *Kidney Int.* **65:** 1145–1154.
52. Fulton, D., J. R. Falck, J. C. McGiff, M. A. Carroll, and J. Quilley. 1998. A method for the determination of 5,6-EET using the lactone as an intermediate in the formation of the diol. *J. Lipid Res.* **39:** 1713–1721.
53. Oltman, C. L., N. L. Weintraub, M. VanRollins, and K. C. Dellsperger. 1998. Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation. *Circ. Res.* **83:** 932–939.
54. Roche, D. M., J. T. Byers, D. S. Smith, F. G. Glansdorp, D. R. Spring, and M. Welch. 2004. Communication blackout? Do *N*-acylhomoserine-lactone-degrading enzymes have any role in quorum sensing? *Microbiology.* **150:** 2023–2028.
55. Tateda, K., Y. Ishii, M. Horikawa, T. Matsumoto, S. Miyairi, J. C. Pechere, T. J. Standiford, M. Ishiguro, and K. Yamaguchi. 2003. The *Pseudomonas aeruginosa* autoinducer *N*-3-oxododecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils. *Infect. Immun.* **71:** 5785–5793.
56. Hooi, D. S. W., B. W. Bycroft, S. R. Chhabra, P. Williams, and D. I. Pritchard. 2004. Differential immune modulatory activity of *Pseudomonas aeruginosa* quorum-sensing signal molecules. *Infect. Immun.* **72:** 6463–6470.
57. Chun, C. K., E. A. Ozer, M. J. Welsh, J. Zabner, and E. P. Greenberg. 2004. Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. *Proc. Natl. Acad. Sci. USA.* **101:** 3587–3590.
58. Harel, M., A. Aharoni, L. Gaidukov, B. Brumshtein, O. Khersonsky, R. Meged, H. Dvir, R. B. G. Ravelly, A. McCarthy, L. Toker, et al. 2004. Structure and evolution of the serum paraoxonase family of detoxifying and antiatherosclerotic enzymes. *Nat. Struct. Mol. Biol.* **11:** 412–419.