Paraoxonase Inhibits High-density Lipoprotein Oxidation and Preserves its Functions

A Possible Peroxidative Role for Paraoxonase

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Abstract

HDL levels are inversely related to the risk of developing atherosclerosis. In serum, paraoxonase (PON) is associated with HDL, and was shown to inhibit LDL oxidation. Whether PON also protects HDL from oxidation is unknown, and was determined in the present study. In humans, we found serum HDL PON activity and HDL susceptibility to oxidation to be inversely correlated ($r^2 = 0.77$, $n = 15$). Supplementing human HDL with purified PON inhibited copper-induced HDL oxidation in a concentration-dependent manner. Adding PON to HDL prolonged the oxidation lag phase and reduced HDL peroxide and aldehyde formation by up to 95%. This inhibitory effect was most pronounced when PON was added before oxidation initiation. When purified PON was added to whole serum, essentially all of it became HDL-associated. The PON-enriched HDL was more resistant to copper ion–induced oxidation than was control HDL. Compared with control HDL, HDL from PON-treated serum showed a 66% prolongation in the lag phase of its oxidation, and up to a 40% reduction in peroxide and aldehyde content. In contrast, in the presence of various PON inhibitors, HDL oxidation induced by either copper ions or by a free radical generating system was markedly enhanced. As PON inhibited HDL oxidation, two major functions of HDL were assessed: macrophage cholesterol efflux, and LDL protection from oxidation. Compared with oxidized untreated HDL, oxidized PON-treated HDL caused a 45% increase in cellular cholesterol efflux from J-774 A.1 macrophages. Both HDL-associated PON and purified PON were potent inhibitors of LDL oxidation.

Searching for a possible mechanism for PON-induced inhibition of HDL oxidation revealed PON (2 paraoxonase U/ml)-mediated hydrolysis of lipid peroxides (by 19%) and of cholesteryl linoleate hydroperoxides (by 90%) in oxidized HDL. HDL-associated PON, as well as purified PON, were also able to substantially hydrolyze (up to 25%) hydrogen peroxide (H_2O_2), a major reactive oxygen species produced under oxidative stress during atherogenesis. Finally, we analyzed serum PON activity in the atherosclerotic apolipoprotein E–deficient mice during aging and development of atherosclerotic lesions. With age, serum lipid peroxidation and lesion size increased, whereas serum PON activity decreased.

We thus conclude that HDL-associated PON possesses peroxidase-like activity that can contribute to the protective effect of PON against lipoprotein oxidation. The presence of PON in HDL may thus be a major contributor to the antiatherogenicity of this lipoprotein. (*J. Clin. Invest.* 1998. 101: 1581–1590.) Key words: paraoxonase • HDL • LDL • lipid peroxidation • apolipoprotein E deficient mice

Introduction

Serum paraoxonase (PON)[1] is a calcium-dependent esterase that is known to catalyze hydrolysis of organophosphates, and is widely distributed among tissues such as liver, kidney, intestine, and also serum, where it is associated with HDL (1–4). PON specificity towards endogenous serum and tissue substrates is not well-characterized, and therefore synthetic substrates are used to monitor the enzyme's activity. Serum PON activity was shown to be reduced in patients after myocardial infarction (5), in patients with familial hypercholesterolemia (6), and in patients with diabetes mellitus (6, 7) in comparison to healthy subjects. Although PON can offer protection against the toxicity of some organophosphates, its physiological role is still not known; however, evidence exists for a protective effect of PON against oxidative damage (3, 8–15). PON was suggested to contribute to the antioxidant protection conferred by HDL on LDL oxidation (8–15). The effect of HDL-associated PON or of purified PON on the LDL oxidation process, including its initiation (conjugated dienes formation), propagation (peroxides formation), and decomposition (aldehydes formation) phases could be analyzed by using PON inhibitors. The inhibitory effect of HDL on LDL oxidation was suggested to be related to metal ion chelation, or to a peroxidase-like activity. HDL-PON was able to hydrolyze long-chain oxidized phospholipids that were isolated from oxidized LDL (14, 15). HDL-associated enzymes other than PON, such as lecithin/cholesterol acyltransferase (LCAT) and platelet-acti-

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1. *Abbreviations used in this paper:* AAPH, 2,2 azobis-2-amidinopropane hydrochloride; LCAT, lecithin/cholesterol acyltransferase; PAF-AH, platelet-activating factor acyl hydrolase; PON, paraoxonase; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

vating factor acyl hydrolase (PAF-AH) were also implicated in the antioxidative properties of HDL (9, 16).

Under oxidative stress, not only is LDL susceptible to lipid peroxidation, but all other serum lipids, including those present in HDL, are also prone to oxidation. In fact, HDL has been shown to be the major carrier of lipid hydroperoxides in human serum (17, 18). In this context it is of interest that HDL-associated cholesteryl ester hydroperoxides are more rapidly reduced to their less reactive hydroxides than are those associated with LDL (19). Oxidative modification of HDL has also been shown to impair the ability of the lipoprotein to promote cholesterol efflux (20, 21). Thus, inhibition of HDL oxidation by PON may preserve the antiatherogenic functions of HDL in reverse cholesterol transport, as well as its protection of LDL from oxidation.

The present study demostrates for the first time the relationship between PON activity and susceptibility of HDL to oxidation. We have also directly analyzed the effect of HDL-associated PON (by using appropriate PON inhibitors), and of purified PON (isolated from human serum) on HDL oxidation, and on the consumption of the lipoprotein-associated peroxides.

Methods

Materials. 2,2 azobis-2-amidinopropane hydrochloride (AAPH) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The paraoxonase inhibitors were discovered by screening a diverse subset of the Parke-Davis/Warner-Lambert chemical library using purified human PON. Compounds selected (Fig. 1) were then subjected to kinetic studies to determine their mode of inhibition. PON inhibitors were used at concentrations up to 100 μM, and were dissolved in DMSO at a final concentration of 1.4%. Enzyme activities were unaffected at this concentration of DMSO.

The mode of inhibition of the various compounds on PON Q activity was studied using increasing paraoxon concentrations (0.10, 0.25, 0.50, 0.75, and 1.00 mM) and a fixed concentration of the compounds (100 μM). Kinetic analysis of the inhibition curves demonstrated noncompetitive inhibition for all compounds.

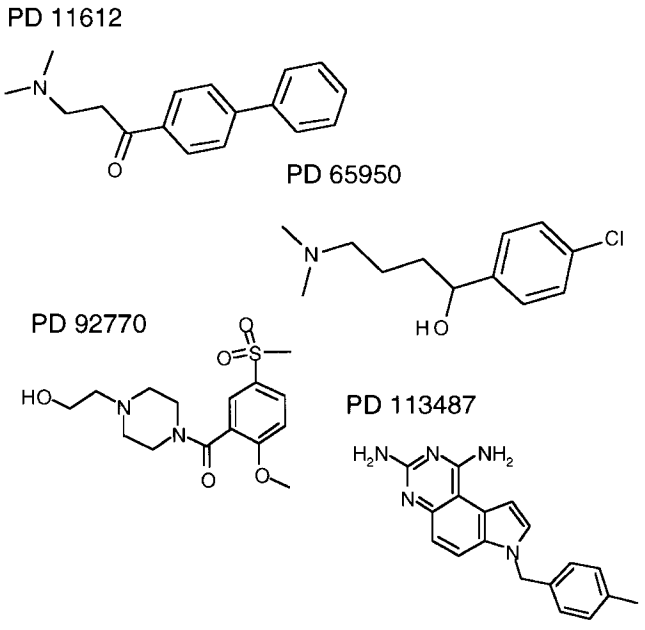

Figure 1. Structures of paraoxonase inhibitors.

Lipoproteins. Serum LDL and HDL were derived from fasted normolipidemic human volunteers from the Rambam Medical Center (Haifa, Israel) as well as from the atherosclerotic, hypercholesterolemic apolipoprotein E–deficient (E°) mice. Lipoproteins were prepared by discontinuous density gradient ultracentrifugation (22). The LDL and HDL were washed at their appropriate densities (1.063 and 1.210 g/ml, respectively) and dialyzed against 150 mM NaCl (pH 7.4) at 4°C. The lipoproteins were then sterilized by filtration (0.45 μm) and stored at a concentration of 3–6 mg protein/ml under nitrogen in the dark at 4°C and used within 2 wk. The lipoproteins were found to be free of LPS contamination as analyzed by the Limulus Amebocyte Lysate assay (Assoc. of Cape Cod, Inc., Woods Hole, MA).

Serum lipid peroxidation was induced by incubating serum with 100 mM AAPH for 2 h at 37°C. Before oxidating the lipoproteins, HDL and LDL were dialyzed against PBS, EDTA-free solution, pH 7.4 under nitrogen at 4°C. Then, the lipoproteins (100 μg of protein/ml) were incubated with 5 μM $CuSO_4$, or with 5 mM AAPH for 6 h at 37°C. The kinetic of lipoprotein oxidation was analyzed (conjugated dienes formation) by monitoring the absorbance change at 234 nm (23). Lipoprotein oxidation was terminated by adding 10 μM (final concentration) of butylated hydroxytoluene, 1 mM (final concentration) Na_2 EDTA, and refrigerating at 4°C. The extent of lipoprotein oxidation was measured directly in the medium by the thiobarbituric acid reactive substances (TBARS) assay, using malondialdehyde (MDA) for the preparation of a standard curve (24). Lipoprotein oxidation was also determined by the lipid peroxidation test, which analyzes lipid peroxides by their capacity to convert iodide to iodine, as measured photometrically at 365 nm (25). The effects of both PON and PON inhibitors on lipoprotein oxidation were substantial and very significant ($P < 0.01$), and therefore the assays used to determine lipoprotein oxidation were sufficiently sensitive. Lipoprotein cholesteryl linoleate hydroperoxides were measured by HPLC (26).

Macrophage cholesterol efflux. The J-774 A.1 murine macrophage-like cell line was purchased from American Type Culture Collection (Rockville, MD). Cells were plated at 1×10^6 cells/16-mm dish in DMEM supplemented with 10% FCS. For the cholesterol efflux studies (27), J-774 A.1 macrophages were incubated for 1 h at 37°C with [^{3}H]cholesterol (0.5 μCi/ml, 55 Ci/nmol, added in ethanol; Amersham Corp., Arlington Heights, IL). With this procedure, [^{3}H]cholesterol is selectively incoporated into the plasma membrane. At the end of the incubation, the cells were washed 3× with cold PBS and further incubated for 3 h at 37°C in the absence or presence of HDL (100 μg protein/ml). Radioactivity was measured in the medium and in the cells, and cellular cholesterol efflux was expressed as the percentage of radioactivity in the medium from the radioactivity in the cells + medium.

Serum paraoxonase purification. PON was purified from human subjects previously identified as being homozygous for PON Q. In brief, to purify serum PON, 1 M $CaCl_2$ (50 mL) were added per liter of serum and centrifuged at 8,000 *g* for 30 min at 4°C to remove the fibrin clot. The supernatant was mixed with Blue Agarose (Cibacron Blue 3 GA, Agarose, Type 3,000; Sigma Chemical Co., St. Louis, MO) in a solution containing 3 M NaCl, 50 mM Tris/HCl, pH 8.0, with 1 mM $CaCl_2$ and 5 μM EDTA as previously described (28–30). PON was eluted with 0.1% deoxycholate as previously described (28–30). The Blue Agarose–eluted PON was further purified by DEAE Bio gel (Bio-Rad Laboratories, Richmond, CA) anion exchange chromatography using a NaCl linear gradient, except that the nonionic detergent tergitol (NP-10; Sigma Chemical Co.) replaced Emulgen 911 (Kao Corp., Tokyo, Japan). To remove residual contamination of albumin, LCAT, and PAF-AH, a Con-A column was used with a 0–0.15 M linear gradient of methyl-α-D-mannopyranoside. Protein con-A protein fragments were removed by Centricon 100 microconcentrators (Amicon Inc., Beverly, MA). The purity of the enzyme was verified by sodium dodecyl sulfate-PAGE (28).

Paraoxonase activity measurements. The rate of hydrolysis of

paraoxon was assessed by measuring liberation of *p*-nitrophenol at 412 nm at 25°C. The basal assay mixture included 1.0 mM paraoxon and 1.0 mM $CaCl_2$ in 50 mM glycine/NaOH buffer, pH 10.5. Nonenzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. The E_{412} for the reaction was 18,290 M^{-1} cm^{-1}. 1 U of paraoxonase activity produced 1 nmol of *p*-nitrophenol per min (29, 30). 1 U of the PON activity equals 1 nmol of paraoxon hydrolyzed/min/ml, and is correlated to 0.5 μg of PON protein/ml. Arylestrase activity was measured using phenylacetate as the substrate (31). Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 1.0 mM phenylacetate and 0.9 mM $CaCl_2$ in 20 mM Tris HCl, pH 8.0. Nonenzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. The E_{270} for the reaction was 1,310 M^{-1} cm^{-1}. 1 U of arylesterase activity is equal to 1 μmol of phenylacetate hydrolyzed/min/ml (28, 29, 31). Purified enzyme has nearly 2,000 U of arylesterase activity per mg protein.

LCAT and PAF-AH determinations. Purified PON Q was assessed for LCAT activity by the proteoliposome assay described by Chen and Albers (32). PAF-AH activity was determined by using 2-acetyl [^{3}H]PAF as substrate. The [^{3}H]acetate generated after *sn*-2-hydrolysis was separated from the radiolabeled substrate by solid phase extraction chromatography and quantitated using liquid scintillation counting (16). The purified PON Q used in these experiments had no detectable LCAT or PAF-AH activity.

Analysis of apolipoprotein E–deficient mice aortic atherosclerotic lesions. Apolipoprotein E–deficient mice were kindly provided to us by Dr. Jan Breslow (The Rockefeller University, New York, NY). Mice were maintained on a regular chow diet containing 4% fat (Purina Chow, Rehovot, Israel) and were killed at intervals up to 12 mo. The hearts and their entire aortas were rapidly removed and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) with 0.01% calcium chloride at room temperature. The aortic arch was dissected free from the surrounding fatty tissue, and the first 4 mm of the ascending aorta (beginning with the aortic valve) was removed and processed as previously described in detail (33–35). Lesional areas were determined using a computerized quantitative image-analysis system (Cue-2; Olympus Corp., Lake Success, NY) with appropriate morphometric software (33–35).

Statistical analyses. The Student's *t* test was used to analyze the significance of the results. Results are given as mean±SD.

Results

Association between PON activity and lipid peroxidation in serum and in HDL. The effect of ex vivo oxidative stress on serum lipid peroxidation and on HDL oxidation in relation to serum PON activity, was studied in 15 normal volunteers. Lipid peroxides formed in serum or in HDL were analyzed after 3 h of incubation at 37°C with the free radical generator AAPH (100 mM or 5 mM, respectively). As shown in Fig. 2, PON activity was inversely related to the ability of AAPH to induce lipid peroxidation in serum ($r^2 = 0.70$, $P < 0.01$) or in HDL ($r^2 = 0.77$, $P < 0.01$). All subjects used in this study were of the PON Q phenotype, as assessed by the failure of 1 M of NaCl to stimulate their serum PON activity. Human serum PON also contains arylesterase activity. Therefore, we also analyzed this enzymatic activity under the above experimental conditions. Serum arylesterase activity was also inversely related to AAPH-induced lipid peroxidation (not shown) in serum ($r^2 = 0.45$) or in HDL ($r^2 = 0.47$); however, these correlations were much weaker than the ones observed for PON activity (Fig. 2).

Inhibitory effect of PON on HDL oxidation. The effect of exogenously added purified PON on copper ion (5 μM $CuSO_4$)-induced HDL oxidation was studied. PON (0–2 U/ml) substantially and in a concentration-dependent manner inhibited HDL oxidation as shown by a prolongation of the lag time required for initiation of HDL oxidation (Fig. 3 *A*), as well as by the reduction in malondialdehyde (up to 95%; Fig. 3 *B*) and in peroxides (up to 85%; Fig. 3 *C*) formed after 4 h of incubation. At 2 U/ml of PON, inhibition of HDL oxidation was only observed when it was added before the oxidation initiation. When added to HDL 2 h after $CuSO_4$ addition, PON was unable to prevent the formation of, or decrease the amount of, oxidation products (data not shown).

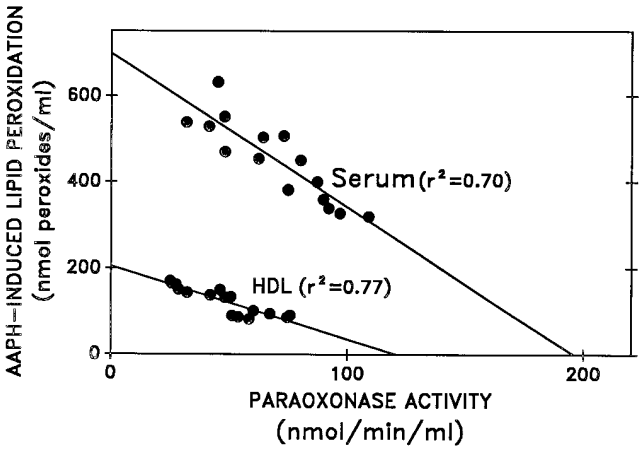

Figure 2. The relationships between AAPH-induced lipid peroxidation in whole human serum or in HDL, and serum paraoxonase activity. Serum and HDL samples were separately prepared from the blood of 15 healthy normolipidemic volunteers. Paraoxonase activity was measured in all serum and HDL samples. For the lipid peroxidation analyses, serum or HDL (500 μg of protein/ml) was incubated at 37°C for 3 h with 100 mM or with 5 mM of AAPH, respectively. At the end of the incubation period, the extent of lipid peroxidation was determined by the peroxides assay.

In human serum, essentially all endogenous serum PON activity is HDL-associated (2). Therefore, we next analyzed the distribution of exogenously added purified PON to serum (100 U/ml) among serum lipoproteins after a 2-h incubation at 37°C. No significant increment in PON activity could be found in the VLDL or LDL fractions (data not shown). Most of the added enzyme was HDL-associated, resulting in a significant increment (by 54%) in HDL-associated PON activity (Table I). Copper ion (5 μM $CuSO_4$)-induced oxidation was substantially inhibited in PON-enriched HDL, as evident by a 66% increment in the lag time required for initiation of HDL oxidation. Furthermore, a significant ($P < 0.01$) reduction in malondialdehyde equivalents (39%) and peroxides (42%) was observed in the PON-enriched HDL (Table I). To better understand the role of HDL-associated PON in its protective effect against oxidation, specific serum PON inhibitors were used. Table II demonstrates the effects of the paraoxonase inhibitors on serum PON and on serum arylesterase activity. All four compounds substantially (56–76%) inhibited serum PON activity, with only a minor inhibitory effect (~ 15%) on serum arylesterase activity, suggesting that these agents are potent and specific inhibitors of the esterase PON.

PON inhibitors were added to HDL, and copper ion (5 μM $CuSO_4$)-induced oxidation was then initiated. Without inhibitor, copper-induced formation of conjugated dienes (the

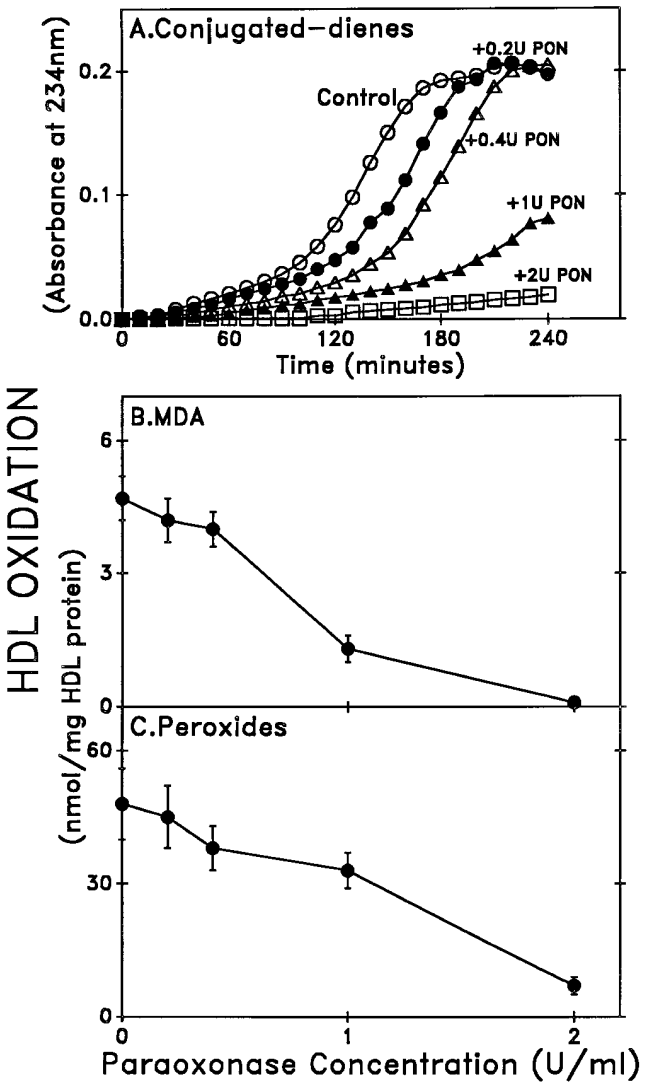

Figure 3. Paraoxonase time and concentration effect on HDL oxidation. HDL (100 μg of protein/ml) was incubated with 5 μM $CuSO_4$ at 25°C in the absence (*Control*) or presence of increasing PON concentrations. Lipoprotein oxidation was determined by kinetic analyses of conjugated dienes formation (*A*), as well as by determination of malondialdehyde (*MDA*; *B*) and peroxide (*C*) formation after 4 h of incubation. Results are given as mean±SD ($n = 3$).

changes in the absorbance at 234 nm) in HDL (100 μg of protein/ml) required 120 min of lag time before oxidation was initiated. Adding the PON inhibitor PD-92770 (100 μM) shortened the lag time to only 25 min, and elevated conjugated dienes formation twofold (Fig. 4). A similar, however less pronounced effect, was observed with another PON inhibitor, PD-113487 (Fig. 4). Paraoxonase inhibitor effects on HDL oxidation were also analyzed by determining the amounts of TBARS formed, measured as MDA equivalents, and of lipid peroxides. For this purpose, HDL (100 μg of protein/ml) was preincubated with 100 μM of each PON inhibitor, and was then oxidized by incubation with 5 μM of $CuSO_4$ for 6 h at 37°C. As shown in Table III, HDL treated with the PON inhibitors showed an increased susceptibility to oxidation in comparison to the control nontreated HDL. Similar results were

Table I. The Effect of HDL Enrichment with Paraoxonase on its Subsequent Oxidation by Copper Ions

HDL paraoxonase		HDL oxidation		
	Specific activity	Lag time	MDA	Peroxides
	nmol/mg HDL protein/min	*min*	*nmol/mg HDL protein*	
Control	26±3	80±8	9.6±0.8	48±2
+ Paraoxonase	40±2*	133±10*	5.9±0.6*	28±3*

HDL was obtained from serum after 2 h of incubation at 37°C in the absence (control) or presence of 100 U/ml of purified serum paraoxonase. The HDL that was separated from the serum (100 μg of protein/ml) was then incubated with 5 μM $CuSO_4$, and the kinetic changes in the absorbance at 234 nm (conjugated dienes formation) were measured. The lag time required for initiation of HDL oxidation was obtained from this analysis. After conjugated diene formation reached its maximal level (3 h), the extent of HDL oxidation was also measured by the TBARS (MDA equivalents) and by the lipid peroxides assays, as described in Methods. Results are given as mean±SD ($n = 3$). *$P < 0.01$ (vs. control).

obtained upon 5 mM AAPH–induced HDL oxidation (data not shown). The extent of HDL oxidation was found to be inversely related to the extent of PON inhibition (see Tables II and III). Upon using the most potent PON inhibitor, PD-65950, a concentration-dependent reduction in PON activity and an increase in HDL susceptibility to copper-induced oxidation was shown (Table IV). At 100 μM of PD-65950 (the highest concentration used), paraoxonase activity was inhibited by 76%, while HDL peroxides (+50%) and aldehydes (+ 81%) were substantially elevated in comparison to the values obtained for control, nontreated HDL (Table IV).

Inhibition of HDL oxidation by paraoxonase preserves the lipoprotein biological functions. We next studied the effect of PON-induced inhibition of HDL oxidation on two major functions of HDL: its ability to induce cellular cholesterol efflux, and its inhibitory effect on LDL oxidation. As HDL oxidation has been shown to impair the ability of the lipoprotein to induce cellular cholesterol efflux (20, 21), we questioned the possible involvement of PON in this phenomenon. J-774 A.1 macrophages were prelabeled by incubation with ^{3}[H]cholesterol for 1 h at 37°C. Next, the cells were challenged with 100 μg of

Table II. The Effect of PON Inhibitors on Serum Paraoxonase and Arylesterase Activities

Inhibitor added	Paraoxonase activity	Arylesterase activity
	nmol/ml/min	*μmol/ml/min*
Control (none)	98±9	94±12
+ PD-113487	42±5*	84±12
+ PD-92770	31±5*	79±12
+ PD-11612	26±4*	82±13
+ PD-65950	24±4*	81±12

Human serum was incubated for 30 min with 100 μM of each of the PON inhibitors. Paraoxonase and arylesterase activities were measured as described in Methods. Results are given as mean±SD ($n = 3$). *$P <$ 0.01 (vs. control).

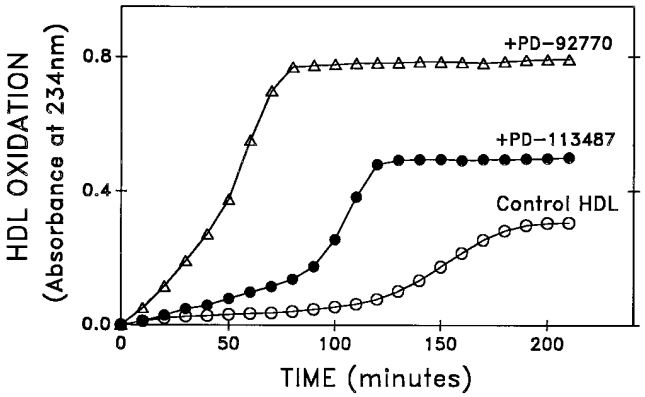

Figure 4. The effect of HDL-associated PON on HDL oxidation: kinetic analysis of conjugated dienes formation in the absence or presence of PON inhibitors. HDL (100 μg of protein/ml) was incubated with 5 μM $CuSO_4$ at 25°C for 3 h in the absence (*Control HDL*), or with 100 μM of the PON inhibitors PD-92770 or PD-113487. Conjugated diene formation was kinetically monitored at 234 nm. A representative experiment (one out of three) is shown.

Table IV. Dose Effect of the Paraoxonase Inhibitor PD-65950 on $CuSO_4$-induced HDL Oxidation

Inhibitor concentration	HDL paraoxonase	HDL oxidation	
	Specific activity	MDA	Peroxides
μM	*nmol/mg HDL protein/min*	*nmol/mg HDL protein*	
0	27±4	11.7±0.2	60±4
10	24±2	12.5±0.3	69±6
25	21±3*	12.9±0.1*	79±5*
50	19±2*	13.3±0.2*	84±5*
100	17±2*	21.2±0.2*	90±14*

HDL (100 μg of protein/ml) was preincubated for 30 min at 37°C with increasing concentrations (0–100 μM) of the PON inhibitor PD-65950. At the end of the incubation period, PON activity in the HDL was measured. The HDL samples were further incubated with 5 μM $CuSO_4$ for 6 h at 37°C, and the extent of HDL oxidation was measured by the TBARS and by the lipid peroxides assays. Results represent mean±SD ($n = 3$). *$P < 0.01$ (vs. 0 concentration).

protein/ml of native HDL or oxidized HDL (prepared by incubation with 5 μM $CuSO_4$ for 4 h at 37°C, in the absence or presence of PON). After 3 h of incubation with native or oxidized HDL, cellular cholesterol efflux was determined. Oxidation of HDL resulted in a 53% reduction in its ability to induce cellular cholesterol efflux, in comparison to native HDL (Fig. 5). However, HDL that was oxidized in the presence of 2 U/ml of purified PON induced a 40% enhanced cholesterol efflux in comparison to the HDL that was oxidized in the absence of PON (Fig. 5). PON alone had no effect on cellular cholesterol efflux from the cells (data not shown).

HDL has also been shown to inhibit LDL oxidation. In the present study using purified PON as well as PON inhibitors (Fig. 6), we assessed whether HDL-associated PON is involved in this protective effect of HDL on LDL oxidation. Kinetic monitoring of conjugated diene formation upon LDL (100 μg of protein/ml) incubation with 5 μM $CuSO_4$ at 37°C revealed a lag time of 60 min. Adding HDL (100 μg of protein/ml) or purified PON (20 U per 1 mg of LDL protein) to LDL substantially inhibited conjugated diene formation (Fig. 6). Even after 180 min of incubation, the propagation phase of LDL oxidation was still retarded. In contrast, when HDL was added together with 100 μM of the PON inhibitor PD-65950 to the LDL, the lag time required for initiating LDL oxidation was reduced to only 40 min. Furthermore, maximal conjugated diene formation in this experiment was increased in the presence of PD-65950 by about threefold, in comparison to the value obtained after incubating LDL + HDL in the absence of the PON inhibitor (Fig. 6). The PON inhibitor itself had no effect on LDL oxidation (data not shown).

Table III. The Effect of Paraoxonase Inhibitors on HDL Oxidation

Inhibitor added	HDL oxidation	
	MDA	Peroxides
	nmol/mg HDL protein	*nmol/mg HDL protein*
Control (none)	11.8±0.4	61±4
+ PD-113487	14.0±0.1*	70±3*
+ PD-92770	14.3±1.1*	76±5*
+ PD-11612	15.7±0.5*	79±9*
+ PD-65950	21.6±1.0*	89±7*

HDL (100 μg of protein/ml) was incubated with 5 μM $CuSO_4$ in the absence (control) or presence of 100 μM of each of the PON inhibitors for 6 h at 37°C. At the end of the incubation, HDL oxidation was measured by the TBARS, and by the lipid peroxide assays. Results represent mean±SD ($n = 3$). *$P < 0.01$ (vs. control).

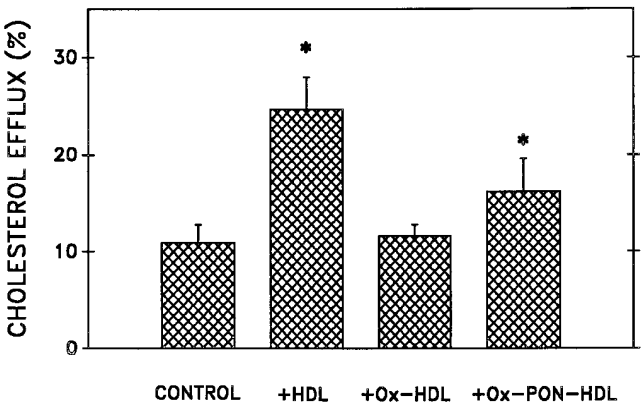

Figure 5. The effect of HDL oxidation in the presence of paraoxonase on lipoprotein-induced cellular cholesterol efflux. J-774 A.1 macrophages (1×10^6 cells/well) were incubated with 0.5 μCi/ml of [^{3}H]cholesterol for 1 h at 37°C, followed by cell wash (3×) and a further incubation for 3 h at 37°C, without (*Control*) or with 100 μg of protein/ml of HDL, or oxidized HDL or HDL that was oxidized in the presence of 2 U/ml of purified PON. Cholesterol efflux from the cells was than determined as described under Methods. Results are given as mean±SD of three different experiments.

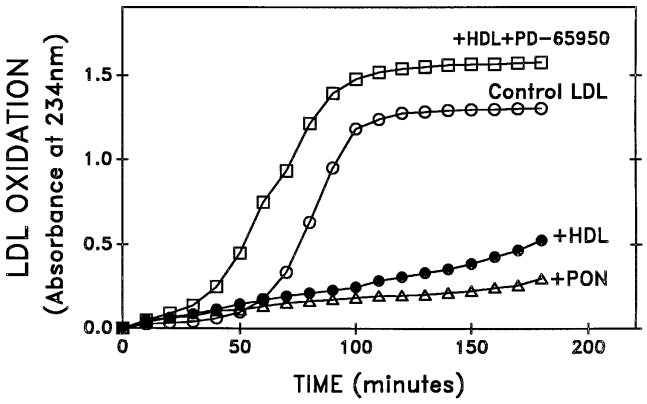

Figure 6. The effect of HDL-associated PON and of purified PON on LDL oxidation. LDL (100 μg of protein/ml) was incubated with 5 μM $CuSO_4$ at 25°C for 3 h alone (*Control LDL*), in the presence of HDL (100 μg of protein/ml), or with HDL + 100 μM of the PON inhibitor PD-65950, or with purified PON (20 U per mg LDL protein). Conjugated diene formation was kinetically monitored. A representative experiment (one out of three) is shown.

Peroxidase-like activity of paraoxonase. PON's inhibitory effect on HDL oxidation may be related to a possible hydrolytic action of PON on lipoproteins peroxides. Therefore, the effect of purified PON on the hydrolysis of peroxides in oxidized HDL was studied (Fig. 7 *A*). Incubation of oxidized HDL (100 μg of protein/ml) with 2 U/ml of purified PON for 2 h at 37°C reduced lipoprotein peroxides by 19% (Fig 7 *A*), and reduced HDL cholesterol linoleate hydroperoxides by 90% (Fig. 7 *B*). Analysis of cholesteryl linoeate hydroperoxides (CL-OOH) by HPLC revealed that reduction in the levels of HDL-associated CL-OOH was paralleled by the formation of cholesteryl lineoleate hydroxides, and not by the formation of fatty acid hydroperoxides. These results suggest that the action of paraoxonase on the cholesteryl ester hydroperoxides is a peroxidase-like activity.

The effect of HDL-associated PON, as well as that of purified PON, on hydrolysis of hydrogen peroxide (H_2O_2) was also studied. This peroxide is probably involved in atherogenesis, and is produced during lipid peroxidation from several reactive oxygen species (ROS). Incubation of hydrogen peroxide (15 μg/ml) with HDL (100 μg of protein/ml) produced a time-dependent reduction (up to 25%) in the hydrogen peroxide concentration (Fig. 8). No hydrogen peroxide consumption could be shown when the PON inhibitor, PD-65950 (100 μM) was included in the incubation (Fig. 8). In contrast, purified PON (2 U/ml) rapidly hydrolyzed H_2O_2 (by up to 23%) with a biphasic kinetic shape (Fig. 8). These results suggest that paraoxonase may act not only on lipid peroxides, but also on hydrogen peroxide.

Paraoxonase activity and atherosclerosis. Oxidative stress and the consequent serum lipid peroxidation were shown to be associated with accelerated atherosclerosis (36). Thus, we have studied serum PON activity in the E° mice as a function of age. We analyzed the extent of atherosclerotic lesions, the serum lipid peroxidative state (not induced oxidation), and the changes in serum PON activity. Fig. 9 clearly demonstrates an inverse relationship between the atherosclerotic lesion progression, (Fig. 9 *A*), and the increment in serum lipid peroxidation (Fig. 9 *B*) on one hand, and reduction in serum PON activity on the other (Fig. 9 *C*).

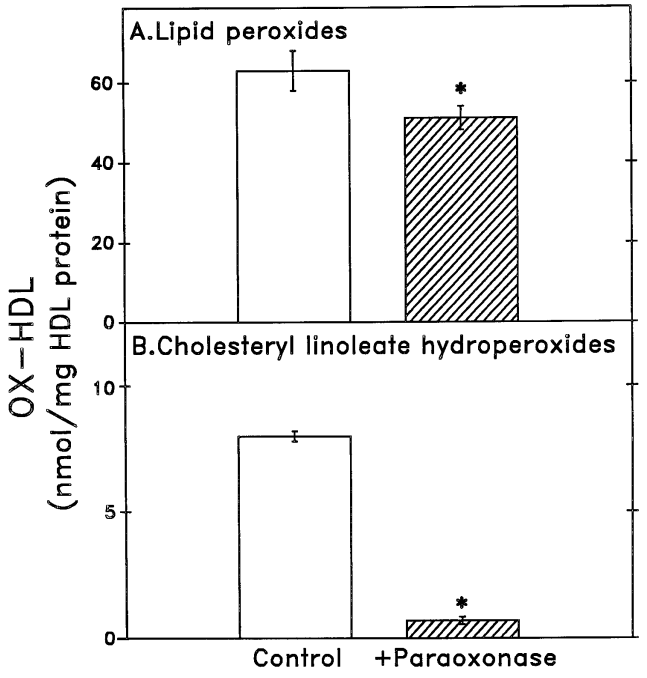

Figure 7. The effect of purified paraoxonase on total peroxides and on cholesteryl linoleate hydroperoxides hydrolysis in oxidized HDL. Oxidized HDL (*Ox-HDL*) was prepared by incubating HDL (1 mg protein/ml) with 5 μM $CuSO_4$ for 6 h at 37°C. HDL oxidation was terminated by adding 1mM Na_2 EDTA and 25 μM butylaled hydroxytoluene. Then, 100 μg of protein/ml of the oxidized lipoprotein were further incubated for 2 h at 37°C without (*Control*) or with 2 U/ml of purified serum paraoxonase. The lipoprotein total lipid peroxides as well as the cholesteryl linoleate hydroperoxides content was measured at the end of the incubation period. Results are given as the mean±SD (n = 3).

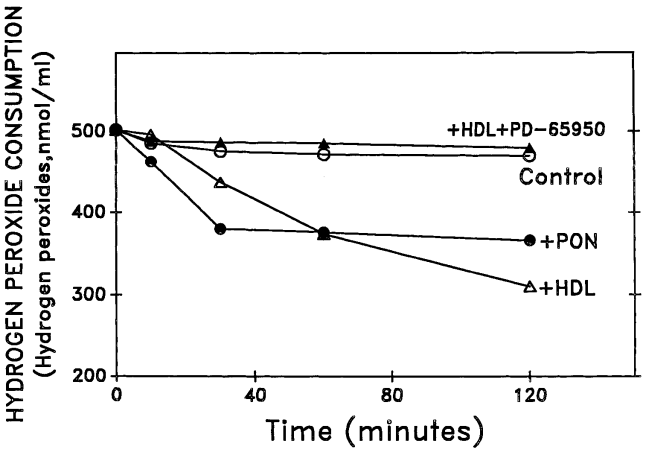

Figure 8. The effect of HDL-associated PON and of purified PON on the hydrolysis of hydroperoxide. Hydrogen peroxide (15 μg of H_2O_2/ml) was incubated at 37°C without (*Control*) or with HDL (200 μg protein/ml, +HDL), or with HDL plus the PON inhibitor PD-65950. Hydrogen peroxide was also incubated with 2 U/ml of purified serum PON (+PON). The amount of hydroperoxides was kinetically monitored by using the peroxides assay, as described in the Methods section. A representative experiment (one out of three) is shown.

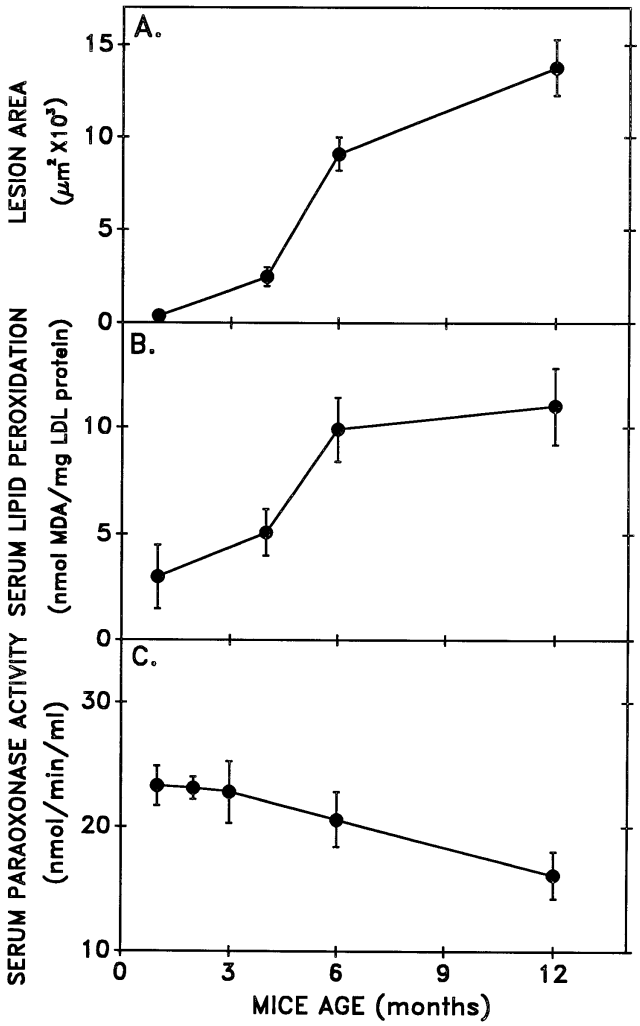

Figure 9. Age-related changes in the atherosclerotic lesion area (*A*), in the LDL oxidative state (*B*), and in serum PON activity (*C*) in the apolipoprotein E–deficient mice. Blood samples were drawn from the apolipoprotein E deficient mice at 1, 4, 6, and 12 mo (10 animals in each group). The atherosclerotic lesion area in the aortas from these mice (*A*) was determined as described under the Methods section. Serum lipid peroxidation state was measured by the TBARS assay (*B*). Serum paraoxonase activity (*C*) was measured as described under the Methods section. Results are the mean±SD of 10 different mice in each group.

Discussion

The present study demonstrates that HDL-associated PON efficiently protects HDL from oxidative damage. This property of PON may be of physiological significance. HDL and its associated PON quite possibly interrupt a process that would otherwise lead to oxidative damage. Our studies also demonstrate that under oxidative stress, PON protects HDL so that HDL can still act as a potent acceptor for cellular cholesterol efflux.

The protective role of PON against HDL oxidation is supported by the following seven observations. First, we observed an inverse correlation between serum HDL-PON activity and HDL oxidizability in normal human volunteers with the PON Q phenotype. This observation may explain the significant inverse relationships between serum PON activity and HDL oxidation in this group. In contrast, recent epidemiological evidence suggests that the PON R allele may be a risk factor for coronary heart disease (37). The PON R alloenzyme in HDL, unlike the PON Q, was recently shown to be a weaker inhibitor of LDL oxidation (38), a phenomenon that can be related to its atherogenicity. Second, we observed a concentration-dependent inhibitory effect of purified PON on HDL oxidation. Third, PON enrichment of serum resulted in an increased resistance of HDL to oxidation. Most of the added PON was found to be HDL-associated. Fourth, increased susceptibility to oxidation of HDL was observed when this lipoprotein was pretreated with PON inhibitors. Fifth, we observed a direct relationship between the abilities of the various PON inhibitors to reduce serum PON activity (Table II) and to increase HDL oxidizability (Fig. 4 and Table III). Sixth, the concentration-dependent effect of PD-65950 on reducing HDL PON activity inversely paralleled its effect on increasing HDL oxidizability (Table IV). Lastly, we observed an inverse correlation between serum PON activity and serum lipid oxidative state in the atherosclerotic apolipoprotein E–deficient mice. The apoE-deficient mice are characterized by accelerated atherosclerosis and enhanced oxidative stress (39–42). Similarly, the wild-type C57BL/6J mouse is susceptible to diet-induced aortic fatty streak when challenged with an atherogenic diet (43). Under these conditions, the wild-type mice have suppressed levels of serum PON, and lose the ability to protect their LDL from oxidation. Injection of mildly oxidized LDL to these mice also resulted in a significant reduction in serum PON activity (44).

The ability of PON to inhibit lipoprotein oxidation, and also to reduce lipoprotein-associated peroxides, may represent two separate properties of PON. First, PON prevents accumulation of oxidized lipid during induced oxidation, and second, PON uses and therefore eliminates preformed oxidized lipoproteins. However, both of these effects may result from PON's ability to hydrolyze specific lipoprotein peroxides during lipoprotein oxidation (14, 15). Our finding that prior addition of purified PON to HDL provided the maximal inhibitory effect on the oxidation process suggests that PON may act on the small amount of preexisting HDL-associated peroxides. The presence of even such a small amount of peroxides in the lipoprotein can significantly contribute to its subsequent oxidation in the presence of transition metal ions (45). Thus, removal of these lipid peroxides can substantially delay lipoprotein oxidation. The ineffectiveness of PON to inhibit HDL oxidation when added at later stages of lipoprotein oxidation may be related to inactivation of PON by reactive oxygen species (35). LDL oxidation is considered to be of major importance in the pathogenesis of atherosclerosis (46, 47), and therefore many studies have aimed to discover the means, as well as to understand the endogenous mechanism, of inhibiting LDL oxidation (48–53). Mechanisms to remove atherogenic oxidized lipoproteins from the circulation (and from extracellular spaces) by enhanced cellular uptake (mainly in the liver), as well as by their conversion to a less atherogenic lipoprotein, were suggested (36, 54). PON appears to play a role in both of these processes. Only small increases in HDL concentrations greatly influence atherogenicity, and this effect may be related to increased PON activity as well as to other HDL-associated proteins such as apolipoprotein A-I, LCAT, and PAF-AH (3, 16).

By using several PON inhibitors, we were able to confirm previous observations (3) that HDL-associated PON protects LDL from oxidation. The ability of PON inhibitors to stimulate LDL oxidation when added together with HDL is probably related to both PON inactivation and the contribution of HDL lipids to total lipid peroxide formation.

Under oxidative stress, LDL and other serum lipoproteins, including HDL, are prone to lipid peroxidation (18). Oxidation of HDL may essentially lower the effective level of this lipoprotein. Reduced levels of serum HDL is an independent risk factor for atherosclerosis (55, 56), and oxidized HDL perhaps increases this risk. This possibility is related to the fact that native HDL functions in the process of reverse cholesterol transport (20, 21, 57, 58), and also as an inhibitor of LDL oxidation (3). Previous studies demonstrated that HDL oxidation substantially reduces the ability of this lipoprotein to function as a potent acceptor for cholesterol efflux (20, 21). The present study indeed shows that under oxidative stress, the inhibitory effect of PON on HDL oxidation was associated with preservation of the HDL ability to induce cellular cholesterol efflux from macrophages. Although the relevance of this phenomenon to the physiological situation is not clear, it suggests a plausible mechanism by which PON protects HDL for cholesterol efflux function. In our experiments, we used whole HDL rather than an enriched apoA-I HDL fraction. Since PON is associated with phospholipids and apoA-I in HDL, it is possible that HDL-mediated cholesterol efflux from macrophages did not reach its maximal capacity in our study.

As in vivo serum PON is predominantly associated with HDL, its major role might be to protect the HDL, rather than LDL, from the harmful effects of oxidative stress. The present study indeed demonstrated for the first time that HDL-associated PON inhibited not only LDL oxidation (39), but also HDL oxidation when induced by different modes of oxidation (transition metal ions, free radicals). This effect could be related to the ability of PON to hydrolyze lipoprotein-associated peroxides. PON is an esterase capable of hydrolyzing the O-P ester bond in paraoxon (2). A similar type of bond may exist in lipoprotein-associated phospholipid peroxides, and in cholesteryl ester peroxides. Such a chemical structure in oxidized lipoproteins may serve as the physiopathological substrate for HDL-associated PON. The ability of PON to hydrolyze peroxides in oxidized HDL suggests that PON can act on an already-produced oxidized lipoprotein, and thus reverse potential atherogenic effects. PON peroxidase activity is suggested since PON reduced cholesteryl linoleate hydroperoxides levels in oxidized HDL, and in parallel produced cholesteryl linoleate hydroxides, but not fatty acid hydroperoxides. The absence of fatty acid hydroperoxide accumulation after PON action on oxidized HDL, however, does not necessarily indicate an absence of PON esterase activity, as these products can be rapidly metabolized.

In addition to the earlier evidence that paraoxonase and arylesterase activities are purified together during enzyme purification (29), the demonstration that both activities are found with the recombinant expressed human enzyme (59) leaves no doubt that a single gene product has both activities. It is not absolutely established whether the active sites for these respective activities are identical, but the fact that they are competitive (29), suggests that some features of the same active center must be shared. However, it has been observed that some inhibitors do not have equal effects on both of these activities. For example, we have found chloropromazine to be more potent against arylesterase activity than paraoxonase activity (B. La Du, unpublished observation). Thus, the present findings on the differences between these two enzymatic activities in response to the inhibitors does not negate the evidence that both activities as well as protection against LDL oxidation (60) are catalyzed by the same enzyme.

PON was found to use efficiently not only lipoprotein-associated peroxides (including cholesteryl linoleate hydroperoxides), but also hydrogen peroxide (H_2O_2). PON may act to hydrolyze specific lipid peroxide(s) or serve as a target for peroxides (60). This later effect can result in PON autoinactivation (M. Aviram, unpublished observation). H_2O_2 is a major ROS produced by arterial wall cells during atherogenesis, and it is converted under oxidative stress into a more potent ROS leading to LDL oxidation (61, 62). The ability of HDL-associated PON to hydrolyze H_2O_2 (in addition to peroxides) may thus play an important role in eliminating potent oxidants that are involved in atherosclerosis.

Of special importance are the findings of the inverse relationships between serum PON activity and the serum lipid peroxidation state in the apoE-deficient mouse. As the mice age, their atherosclerotic lesions became progressively worse, and their basal state of serum lipids peroxidation rose. In these mice, accelerated atherosclerosis is associated with an enhanced state of basal serum lipid peroxidation (34). Likewise, we show that the induced lipid peroxidation (lipoprotein oxidizability induced by copper ions or by AAPH) results in a similar inverse association with PON activity. This result suggests that induced lipoprotein oxidation mimics the in vivo lipid peroxidation conditions in this system.

We do not know whether the lack of an expressed activity polymorphism in the apolipoprotein E–deficient mice may possibly affect oxidative stress in these mice. It is difficult to anticipate how variations in PON structure in these mice may affect the oxidative state. Mice strains so far examined were shown to contain arginine at position 191, like the human PON R allozyme.

In conclusion, the present study demonstrated that HDL-associated PON can exert a protective effect on HDL functions. This effect may be due to PON's peroxidase-like activity and may contribute to HDL's antiatherogenic properties.

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