Paraoxonase Inhibits High-density Lipoprotein Oxidation and Preserves its Functions

A Possible Peroxidative Role for Paraoxonase

Michael Aviram,* Mira Rosenblat,* Charles L. Bisgaier,† Roger S. Newton,* Sergio L. Primo-Parmo,* and Bert N. La Du‡

*The Lipid Research Laboratory, Technion Faculty of Medicine, the Rappaport Family Institute for Research in the Medical Sciences and Rambam Medical Center, Haifa, Israel 31096; †Vascular and Cardiac Diseases, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105; and the ‡Department of Pharmacology and Department of Anesthesiology, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0572

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 = 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 (H₂O₂), 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 anti-atherogenicity 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) 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-

1. Abbreviations used in this paper: AAPH, 2,2 azobis-2-aminopropane 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 demonstrates 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.

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 oxidizing 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 mM CuSO₄ or with 5 mM AAPH for 6 h at 37°C. The kinetic of lipid peroxidation 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) Na₂ EDTA, and refrigerating at 4°C. The extent of lipid peroxidation 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).

Macrophase cholesterol efflux. The J-774 A.1 murine macrophase-like cell line was purchased from American Type Culture Collection (Rockville, MD). Cells were plated at 1 × 10⁶ 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 2μCi/ml, 55 Ci/nmol, added in ethanol; Amersham Corp., Arlington Heights, IL). With this procedure, [³H]cholesterol is selectively incorporated 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 CaCl₂ (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 CaCl₂ and 5 μM EDTA as previously described (28–30). PON was eluted with 0.1% deoxycholate as previously described (29–30). 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 tertigol (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 Centricron 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 \( \mu \)g of PON protein/ml. Arylesterase 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 nmol 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. 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 \( \mu \)g 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.

**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 \( \mu \)M 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).

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 \( \mu \)M 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 \( \mu \)M CuSO\( _4 \))-induced oxidation was then initiated. Without inhibitor, copper-induced formation of conjugated dienes (the
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 CuSO₄ 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 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 [³H]cholesterol for 1 h at 37°C. Next, the cells were challenged with 100 μg of

<table>
<thead>
<tr>
<th>Table I. The Effect of HDL Enrichment with Paraoxonase on its Subsequent Oxidation by Copper Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL paraoxonase HDL oxidation</td>
</tr>
<tr>
<td>Specific activity Lag time MDA Peroxides</td>
</tr>
<tr>
<td>nmol/HDl protein/min</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>+ Paraoxonase</td>
</tr>
</tbody>
</table>

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 CuSO₄, 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).

<table>
<thead>
<tr>
<th>Table II. The Effect of PON Inhibitors on Serum Paraoxonase and Arylesterase Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor added</td>
</tr>
<tr>
<td>nmol/ml/min</td>
</tr>
<tr>
<td>Control (none)</td>
</tr>
<tr>
<td>+ PD-113487</td>
</tr>
<tr>
<td>+ PD-92770</td>
</tr>
<tr>
<td>+ PD-11612</td>
</tr>
<tr>
<td>+ PD-65950</td>
</tr>
</tbody>
</table>

Human serum was incubated for 30 min with 100 μM of each of the PON inhibitors. Paraoxonase and arylerase activities were measured as described in Methods. Results are given as mean±SD (n = 3). * P < 0.01 (vs. control).
protein/ml of native HDL or oxidized HDL (prepared by incubation with 5 μM CuSO₄ 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 CuSO₄ 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 alone 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**

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

HDL (100 μg of protein/ml) was incubated with 5 μM CuSO₄ 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).

**Table IV. Dose Effect of the Paraoxonase Inhibitor PD-65950 on CuSO₄-induced HDL Oxidation**

<table>
<thead>
<tr>
<th>Inhibitor concentration (μM)</th>
<th>HDL paraoxonase Specific activity (nmol/mg HDL protein/min)</th>
<th>HDL oxidation MDA Peroxides (nmol/mg HDL protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27±4</td>
<td>11.7±0.2</td>
</tr>
<tr>
<td>10</td>
<td>24±2</td>
<td>12.5±0.3</td>
</tr>
<tr>
<td>25</td>
<td>21±3*</td>
<td>12.9±0.1*</td>
</tr>
<tr>
<td>50</td>
<td>19±2*</td>
<td>13.3±0.2*</td>
</tr>
<tr>
<td>100</td>
<td>17±2*</td>
<td>21.2±0.2*</td>
</tr>
</tbody>
</table>

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 CuSO₄ 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).
Peroxidase-like activity of paraoxonase. PON’s inhibitory effect on HDL oxidation may be related to a possible hydrolytic action of PON on lipoprotein 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 linoleate hydroperoxides (CL-OOH) by HPLC revealed that reduction in the levels of HDL-associated CL-OOH was paralleled by the formation of cholesteryl linoleate 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 (H₂O₂) 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 µM/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 H₂O₂ (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).
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 alloszyme 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 chlorpromazine 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 mouse 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.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Mr. Arnold Eisenburg of Parke-Davis Pharmaceuticals and Mr. Scott Billieck of the University of Michigan. The authors also thank Dr. Jacob Vaya and Mrs. Paula Belinski for their HPLC analyses of lipoprotein peroxides, and Dr. Tony Hayek for his help with the E- mice analyses.

This research was supported by a grant from the Rappaport Institute for Research in the Medical Sciences, Technion, Haifa, Israel.

References

4. Nevin, D.N., A. Zambon, C.E. Furlong, R.J. Richter, R. Humbert, J.E.
molecular on the molecular size and kinetic properties of bovine plasma aroy-
erosclerotic apolipoprotein E-deficient transgenic mice and in humans is oxi-
2752.
36. Aviram, M. 1993. Modified forms of low density lipoprotein and athero-
genetics of paraoxonase and effectiveness of high-density lipoproteins in pro-
tecting low-density lipoprotein against lipid peroxidation [letter]. Lancet. 354:851–
852.
44. Navab, M., S. Hama, A.M. Fogelman, J.A. Berliner, J.I. Van Lenten, G.C. Fonarow, C.J. Car-
dent oxidative modification of human low density lipoprotein. J. Lipid Res. 34:1745–
1753.
47. Steinberg, D., S. Parthasarathy, T.E. Carew, J.C. Khoo, and J.L. Witz-
tum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that in-
51. Levy, Y., M. Kaplan, A. Ben Amotz, and M. Aviram. 1996. Effect of di-
citry supplementation of beta-carotene on human monocyte-macrophage-
tect low-density lipoprotein against lipid peroxidation: in vitro and ex vivo stud-


