High density lipoprotein (HDL)-associated paraoxonase-1 (PON1) anti-atherogenic properties in macrophages, i.e. inhibition of cell-mediated oxidation of low density lipoprotein (LDL) and stimulation of cholesterol efflux, were studied using recombinant variants of PON1 and apoA-I expressed in *Escherichia coli* and reconstituted HDL (rHDL) particles composed of phosphatidylcholine/free cholesterol (PC/FC) and apoA-I. PON1 lactonase activity is stimulated by apoA-I by 7-fold relative to PC/FC particles. Wild-type (WT) PON1 bound to rHDL inhibited macropage-mediated LDL oxidation and stimulated cholesterol efflux from the cells to 2.3- and 3.2-fold greater extents, respectively, compared with WT PON1 bound to PC/FC particles without apo-A-I. We also tested PON1 catalytic histidine dyad mutants (H115Q and H134Q) with WT PON1 bound to PC/FC particles without apo-A-I. The His dyad mutants did not, suggesting that the above anti-atherogenic properties of HDL-associated PON1 involve LPC release. Indeed, enrichment of macrophages with increasing concentrations of LPC resulted in inhibition of the cells' capability to oxidize LDL and in stimulation of HDL-mediated cholesterol efflux from the macrophages in an LPC dose-dependent manner. Thus, we provide the first direct indication that the anti-atherogenic properties of PON1 are related to its lipolactonase activity and propose a model in which PON1 acts as a lipolactonase to break down oxidized lipids and to generate LPC.
of the structure and catalytic mechanism of PON1 (6,30) lead to a better understanding of the in vivo biochemical functions of PON1 and how these functions may mediate its anti-atherogenic activities. The main features of the PON1 active site are a catalytic calcium ion and a histidine dyad composed of His115 and His134 (30) that mediates the lactonase activity of PON1 (as well as other mammalian PONs, e.g. PON3) (31,55). The promiscuous arylesterase activity is mediated by the same dyad, but the paraoxonase activity is not (55).

In this study, we examined, for the first time, the biological functions of PON1 in a reconstituted in vitro system based entirely on purified lipid components and recombinant PON1 (32) and apoA-I expressed in Escherichia coli (5), thus ruling out the participation of any contaminating serum components. We have previously shown that recombinant PON1, which exhibits catalytic properties that are essentially identical to those of serum-purified human PON1 (32), also binds to HDL and is stimulated by apoA-I (5). This stimulation increases PON1 stability by well over 300-fold and its lipolactonase activity by ∼20-fold relative to the delipidated form or by ∼10-fold relative to the detergent-associated form. We now show that, under these conditions, recombinant PON1 also inhibits copper ion-induced LDL oxidation and stimulates macrophage cholesterol efflux, two major anti-atherosclerotic activities. We also show that the H115Q and H134Q mutants of PON1, which exhibit diminished lactonase activity, also exhibit greatly reduced or even no anti-atherogenic activities. These results therefore provide the first direct evidence for the hypothesis that the anti-atherogenic functions of PON1 are mediated by its lipolactonase activity.

**EXPERIMENTAL PROCEDURES**

**Recombinant PON1 Variants**

A recombinant PON1 variant named G3C9 (WT PON1) generated by directed evolution for expression in E. coli was used in this study. PON1 G3C9 is almost identical in sequence to rabbit PON1 (32) and exhibits kinetic parameters and HDL binding properties that are essentially identical to those of serum-purified rabbit and human PON1 (5,32). The preparation and characterization of recombinant PON1 G3C9 and its active-site histidine mutants have been described (5,31,55). The promiscuous arylesterase activity is mediated by the same histidine dyad, but the paraoxonase activity is not (55).

**Preparation of Reconstituted HDL (rHDL) and Phosphatidylcholine/Free Cholesterol (PC/FC) Particles**

Rabbit apoA-I containing an N-terminal His6 tag was expressed in E. coli and purified to homogeneity as described (5). Discoidal HDL containing apoA-I and PC/FC particles with no apolipoprotein component were prepared by the cholate dialysis method as described previously (5) using an egg PC/FC/recombinant apoA-I starting molar ratio of 100:5:1. The concentration of rHDL was determined on the basis of apoA-I concentration, assuming an apoA-I/rHDL ratio of 2:1 (33,34). The homogeneity of the preparations was assessed by nondenaturing gradient gel electrophoresis, which indicated the formation of ∼10-nm particles. rHDL particles were used without further purification.

**Binding of PON1 Mutants to rHDL**

Dialyzed PON1 samples were delipidated using Bio-Beads SM-2 (Bio-Rad) as described (5) to minimize the amount of detergent present in the protein samples. Delipidated proteins were diluted in activity buffer (to 0.2 μM for enzyme assays and to 8 μM for LDL oxidation and cholesterol efflux studies) and incubated with rHDL (10 μM) for 3 h at 37 °C.

**PON1 Enzyme Assays**

The catalytic activities of recombinant PON1 and its histidine mutants were determined in detergent-containing buffer or in the presence of rHDL particles as described (5,6). Briefly, wild-type (WT) PON1 and its mutants were diluted in activity buffer to 0.01−1 μM or incubated with a large excess (50-fold) of rHDL, and enzyme activities were detected spectrophotometrically with various substrates at 0.4−1 mM: paraoxon (1 mM), phenyl acetate (1 mM), 5-(thiobutyyl)-butyrolactone (0.4 mM), and γ-nonanoic lactone (0.4 mM). Hydrolysis of paraoxon and phenyl acetate was detected in activity buffer at 405 and 270 nm, respectively. Hydrolysis of 5-(thiobutyyl)butyrolactone was assayed in activity buffer using 5,5′-dithiobis(2-nitrobenzoic acid) for product detection at 412 nm (35). Hydrolysis of γ-nonanoic lactone was assayed in 2.5 mM Bicine (pH 8.3), 0.2 M NaCl, and 1 mM CaCl2 in the presence of 0.2 mM of cresol purple indicator. Proton release by the carboxylic acid product was detected at 577 nm (6).

**Cells**

J774A.1 murine macrophage cells were purchased from American Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum.

**Human LDL and HDL Preparation**

LDL and HDL were prepared from the serum of fasted normolipemic human volunteers by discontinuous density gradient ultracentrifugation (36). The HDLs were then dialyzed against 150 mM NaCl and 1 mM CaCl2 (pH 7.4), and their protein content was determined with the Folin phenol reagent (37).

**LDL Oxidation Studies**

**Cell-free System**—LDL (100 μg/ml protein) in phosphate-buffered saline was preincubated with no addition (control LDL) or with the above PON1 preparations for 15 min at room temperature. Then, 5 μM CuSO4 was added to all test tubes for further incubation for 1.5 h at 37 °C. The extent of LDL oxidation was measured by the thiobarbituric acid-reactive substance (TBARS) assay (38) and by the lipid peroxide assay (39).

**Macrophage-mediated Oxidation of LDL**—Before oxidation, LDL (1 mg/ml protein) was dialyzed against phosphate-buffered saline. LDL (100 μg/ml protein) in RPMI 1640 medium (without phenol red) was incubated without cells or with J774A.1 cells (2 × 10⁵) in the presence of 2.5 μM CuSO₄ for 5 h at 37 °C or 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 h. WT PON1 and its histidine mutants (either in free form or bound to rHDL or PC/FC) were added to the incubation medium of LDL with the cells. The extent of cell-mediated oxidation was then determined by the TBARS assay (38).
cells were washed three times with phosphate-buffered saline, and 0.1 N NaOH was added. Cellular and medium 3H labels were quantitated, and the percentage of cholesterol efflux was calculated as the ratio of (3H label in the medium × 100) to (3H label in the medium + 3H label in the cells) (25). HDL- or PC/FC-mediated cholesterol efflux is the value obtained in the presence of HDL or PC/FC minus the value obtained in cells incubated without HDL or PC/FC. The extent of cholesterol efflux with no addition of HDL or PC/FC was ~5%.

**Macrophage Lysophosphatidylcholine (LPC) Content**

J774A.1 macrophages (6 × 10⁵) were incubated for 3 h at 37 °C with control rHDL (1.3 μg/ml cholesterol) or with rHDL preincubated with WT PON1 or its mutants. The cells were then washed and lipid-extracted with hexane/isopropanol alcohol (3:2, v/v). The hexane phase was collected and dried under nitrogen. The identification and quantitative determination of LPC were performed by liquid chromatography-mass spectrometry (Micromass Quattro Ultima mass spectrometer) using a direct injection method at positive mode electrospray ionization. The identification of LPC was based on the characteristic mass spectra of standard LPC constructed from a 2:1 mixture of palmitate/stearate esters (25).

**Statistics**

Statistical analysis was performed using Student’s paired t test when comparing the mean of two groups. Analysis of variance was used when more than two groups were compared, and the results are given as the means ± S.D.

**RESULTS**

**Catalytic Activities of PON1 and Its His¹¹⁵ and His¹³⁴ Mutants**

PON1 exhibits a wide range of hydrolytic activities that can be broadly defined under three categories: lactonase (which is the primary native function of mammalian PONs, including PON1), arylesterase, and phosphotriesterase. We have found that the first two activities are mediated by a His dyad composed of His¹¹⁵ and His¹³⁴, in which His¹¹⁵ acts as a general base to deprotonate a water molecule and to prompt the attack of the lactone or aryl ester carbonyl, and His¹³⁴ acts as a proton shuttle to increase the basicity of His¹¹⁵ (55). Consequently, even the most conserved mutation of His¹¹⁵ (H115Q) reduced PON1 lactonase and arylesterase activities by 45–500-fold in terms of k_cat/K_m and by 30–110-fold at substrate concentrations >S<K_m (Table 1). The H134Q mutation had a milder effect (6–20-fold decrease), consistent with its auxiliary role. The double mutant H115Q/H134Q exhibited almost no lactonase or arylesterase activity (100-fold lower compared with WT PON1). In contrast, the phosphotriesterase activity of all three mutants was barely affected (2-fold decrease for H115Q and ~2-fold increase for H134Q and the double mutant) (Table 1).

We have previously demonstrated that binding of PON1 to HDL in the presence of apoA-I dramatically stabilizes PON1 and stimulates its lactonase activity by <20-fold relative to the delipidated form in buffer (5). In the presence of saturating concentrations of HDL and apoA-I, the His mutants described above showed essentially identical stability compared with WT PON1, indicating that their ability to bind HDL has not been compromised (data not shown). However, as expected, removal of the His dyad residues that mediate the catalysis of lactone hydrolysis reduced the stimulation of the lactonase activity (2–3-fold stimulation for the mutants relative to 8.5-fold for WT PON1 under the same conditions) (Table 1). Thus, when bound to HDL in the presence of apoA-I, the His mutants showed a further decrease in their lactonase activity relative to WT PON1 (~100-fold for H115Q and 1000-fold for the

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**TABLE 1**

| PON1 Hydrolytic Activity and Anti-atherogenic Functions |
|-------------------------------------------------------|
| **Catalytic activities of PON1 and its His dyad mutants in the lipid-poor form, in micellar detergent solution, or in apoA-I-containing HDL particles** |
| **Delipidated PON1 and its His115 and His134 mutants (H115Q, H134Q, and the double mutant H115Q/H134Q) were incubated in activity buffer (lipid-poor form) with 0.1% Tergitol or apoA-I-containing rHDL, and the hydrolytic activity was tested with paraoxon (1 mM), phenyl acetate (1 mM), and two lactones (5-(thiobutyl)butyrolactone (TBBL), 0.4 mM; and 7-nonenolic lactone, 0.1 mM).** |
| **Table 1** |
| **HDL** |
| **Lipid-poor** |
| **Tergitol** |
| **ApoA-I-containing HDL** |
| **Km** |
| **Values represent mean ± S.D. of two to three independent measurements.** |
| **WT PON1** |
| **H115Q** |
| **H134Q** |
| **H115Q/H134Q** |
| **S.E.** |
| **Values indicate the percent change relative to the activity of WT PON1 under the same conditions.** |
| **Phenyl acetate** |
| **TBBL** |
| **6.7** |
| **0.7** |
| **0.3** |
| **0.02** |
| **0.02** |
| **0.01** |
| **52.6** |
| **0.2** |
| **12.0** |
| **0.2** |
| **9.1** |
| **0.3** |
| **HDL** |
| **Lipid-poor** |
| **Tergitol** |
| **ApoA-I-containing HDL** |
| **Activity** |
| **Values indicate the percent change relative to the activity of WT PON1 under the same conditions.** |
| **Phenyl acetate** |
| **TBBL** |
| **6.7** |
| **0.7** |
| **0.3** |
| **0.02** |
| **0.02** |
| **0.01** |
| **52.6** |
| **0.2** |
| **12.0** |
| **0.2** |
| **9.1** |
| **0.3** |
| **HDL** |
| **Lipid-poor** |
| **Tergitol** |
| **ApoA-I-containing HDL** |
| **Activity** |
| **Values indicate the percent change relative to the activity of WT PON1 under the same conditions.** |
| **Phenyl acetate** |
| **TBBL** |
| **6.7** |
| **0.7** |
| **0.3** |
| **0.02** |
| **0.02** |
| **0.01** |
| **52.6** |
| **0.2** |
| **12.0** |
| **0.2** |
| **9.1** |
| **0.3** |
| **HDL** |
| **Lipid-poor** |
| **Tergitol** |
| **ApoA-I-containing HDL** |
| **Activity** |
| **Values indicate the percent change relative to the activity of WT PON1 under the same conditions.** |
| **Phenyl acetate** |
| **TBBL** |
| **6.7** |
| **0.7** |
| **0.3** |
| **0.02** |
| **0.02** |
| **0.01** |
| **52.6** |
| **0.2** |
| **12.0** |
| **0.2** |
| **9.1** |
| **0.3** |
| **HDL** |
| **Lipid-poor** |
| **Tergitol** |
| **ApoA-I-containing HDL** |
| **Activity** |
| **Values indicate the percent change relative to the activity of WT PON1 under the same conditions.** |
| **Phenyl acetate** |
| **TBBL** |
| **6.7** |
| **0.7** |
| **0.3** |
| **0.02** |
| **0.02** |
| **0.01** |
| **52.6** |
| **0.2** |
| **12.0** |
| **0.2** |
| **9.1** |
| **0.3** |
| **HDL** |
| **Lipid-poor** |
| **Tergitol** |
| **ApoA-I-containing HDL** |
| **Activity** |
| **Values indicate the percent change relative to the activity of WT PON1 under the same conditions.** |
| **Phenyl acetate** |
| **TBBL** |
| **6.7** |
| **0.7** |
| **0.3** |
PON1 Hydrolytic Activity and Anti-atherogenic Functions

**FIGURE 1. Effect of WT PON1 and its His<sup>115</sup> and His<sup>134</sup> mutants on LDL oxidation.** A and B: LDL (100 μg/ml protein) was incubated for 15 min with Tris buffer (without glycerol) alone (control LDL) or with increasing concentrations (5, 10, and 20 μg/ml) of WT PON1 or its histidine mutants. A similar volume of PON buffer was present in all test tubes. Then, 5 μM CuSO<sub>4</sub> was added to initiate oxidation, followed by a 5-h incubation at 37 °C. The extent of LDL oxidation was determined by the TBARS assay (A) and by the lipid peroxide assay (B). C and D: LDL (100 μg/ml protein) was incubated with J774A.1 macrophages with no addition (control LDL) or in the presence of 20 μg/ml WT PON1, H115Q (H115Gln), or H134Q (H134Gln). The above samples were incubated with the cells for 5 h at 37 °C in the presence of 5 μM CuSO<sub>4</sub> (C) or for 24 h at 37 °C in the presence of 100 ng/ml PMA. The extent of LDL oxidation was measured by the TBARS assay. The results are expressed as the means ± S.D. of three different experiments. *p < 0.01 versus 0 concentration; #, p < 0.01 versus control LDL.

Cultured J774A.1 macrophages were incubated with LDL (100 μg/ml protein) in the presence of 2.5 μM CuSO<sub>4</sub> for 5 h at 37 °C with no addition (control LDL) or with WT PON1 or its His<sup>115</sup> and His<sup>134</sup> mutants (20 μg/ml). This incubation time was chosen based on preliminary experiments performed to obtain significant LDL oxidation by the cells above the levels obtained following LDL incubation under similar conditions in a cell-free system (data not shown). Although WT PON1 significantly inhibited cell-mediated LDL oxidation (as measured by the TBARS levels) by 62%, H115Q and H134Q inhibited LDL oxidation by only 19 and 35%, respectively (Fig. 1C). Similar results were observed after macrophage incubation with LDL in the presence of the PON1 samples and 100 ng/ml PMA for 24 h (Fig. 1D). WT PON1 significantly reduced macrophage-mediated LDL oxidation by 65%, whereas H115Q and H134Q reduced cell-mediated LDL oxidation by only 22 and 37%, respectively (Fig. 1D). These results further indicate the importance of both PON1 His<sup>115</sup> and His<sup>134</sup> in PON1 protection against macrophage-mediated LDL oxidation, with His<sup>115</sup> being more important in this respect.

**Effect of WT PON1 and Its His<sup>115</sup> and His<sup>134</sup> Mutants on LDL Oxidation—**We first analyzed the possible role of PON1 His<sup>115</sup> and His<sup>134</sup>, which mediate the lactonase activity of PON1, in the protection of LDL against copper ion-induced oxidation using HDL-free PON1 in a cell-free system. The addition of increasing concentrations (0–20 μg/ml) of WT PON1 to LDL significantly inhibited LDL oxidation induced by 5 μM CuSO<sub>4</sub> in a PON1 dose-dependent manner (Fig. 1A, A and B). LDL oxidation measured by the TBARS assay was reduced by up to 46% (Fig. 1A), and lipid peroxide analysis revealed up to a 60% decrease in LDL oxidation (Fig. 1B). The PON1 mutants H134Q and H115Q also inhibited LDL oxidation in a dose-dependent manner, although to a much lesser extent than observed with WT PON1. The TBARS level in LDL was reduced by the H134Q mutant by up to 18% (Fig. 1A), and the lipid peroxide content was decreased by up to 21% (Fig. 1B). H115Q at a concentration of up to 10 μg/ml had no inhibitory effect at all on LDL oxidation (Fig. 1, A and B), and at 20 μg/ml, it reduced the TBARS level by only 10% (Fig. 1A) and the lipid peroxide content by only 14% (Fig. 1B). These results suggest that both PON1 His<sup>115</sup> and His<sup>134</sup> are required for the PON1-induced decrease in LDL oxidation, with His<sup>115</sup> being more important for this PON1 biological activity.

As in vivo LDL is oxidized by arterial wall cells, including macrophages (21), we next determined the effect of the PON1 His<sup>115</sup> and His<sup>134</sup> in a more physiological system i.e. LDL oxidation by macrophages. Because metal ions are present in the arterial wall (40) and because arterial “activated macrophages” oxidize LDL at enhanced rates (41), we performed the macrophage-mediated LDL oxidation studies in the presence of copper ions (Fig. 1C) or the macrophage activator PMA (Fig. 1D).
that both PON1 His^{115} and His^{134} are required to maintain this inhibitory effect.

**Contribution of ApoA-I to Ability of rHDL-associated PON1 to Protect LDL from Oxidation**—To determine the contribution of apoA-I in rHDL to the inhibitory effect of PON1 on macrophage-mediated LDL oxidation, we compared the effect of PC/FC particles (no apoA-I present) with that of rHDL containing PC/FC and also recombinant apoA-I (Fig. 2). In contrast to rHDL (Fig. 2A), PC/FC particles did not inhibit LDL oxidation by the cells (Fig. 2B). The addition of WT PON1 to rHDL reduced cell-mediated LDL oxidation by 60% versus rHDL with no PON1, whereas the addition of WT PON1 to PC/FC particles resulted in a 26% decrease in LDL-associated TBARS levels compared with the effect of PC/FC particles with no PON1 (Fig. 2A versus B). The addition of the PON1 double histidine mutant to rHDL (Fig. 2A) or to PC/FC particles (Fig. 2B) had no inhibitory effect on macrophage-mediated LDL oxidation compared with the effect of WT PON1 bound to rHDL or PC/FC particles. Similar results were observed in a cell-free LDL oxidation system (data not shown). These results suggest that apoA-I in HDL significantly contributes to PON1 protection against macrophage-mediated oxidation of LDL.

**Macrophage Cholesterol Efflux Studies**

**Effect of PON1 and Its His^{115} and His^{134} Mutants on Human HDL-mediated Macrophage Cholesterol Efflux**—In addition to its protection against oxidized LDL accumulation, another important anti-atherogenic property of PON1 is its ability to stimulate cholesterol efflux from macrophages (25). Enrichment of human HDL (100 μg/ml protein) with increasing concentrations (0–20 μg/ml) of WT PON1 resulted in a significant stimulation of HDL-mediated cholesterol efflux from J774A.1 macrophages of up to 41% in a PON1 dose-dependent manner (Fig. 3A). We next compared the extent of macrophage cholesterol efflux stimulation by human HDL enriched with H115Q and H134Q versus WT PON1 (Fig. 3B). Although enrichment of human HDL with WT PON1 at 20 μg/ml significantly increased human HDL (100 μg/ml protein)-mediated cholesterol efflux from J774A.1 macrophages by 41%, H134Q increased it by only 23%, and H115Q showed only a 6% stimulatory effect (Fig. 3B). These results suggest that both these histidine residues are required also for PON1 stimulation of macrophage cholesterol efflux, with His^{115} being again more important in this biological effect.

**Effect of rHDL-bound WT PON1 and Its His^{115} and His^{134} Mutants on Ability of PON1 to Stimulate Macrophage Cholesterol Efflux**—WT PON1 associated with rHDL substantially stimulated cholesterol efflux from J774A.1 macrophages by 45% compared with cholesterol efflux induced by rHDL alone, with no PON1 (Fig. 4A). In contrast, H115Q and H115Q/H134Q had no stimulatory effect at all compared with rHDL alone, with no PON1 (Fig. 4A). The addition of the PON1 inhibitor 2-hydroxyquinoline (200 μM) (6, 25) to rHDL containing WT PON1 completely abolished the rHDL-bound PON1 stimulatory effect on macrophage cholesterol efflux (Fig. 4A). These results clearly indi-
PON1 Hydrolytic Activity and Anti-atherogenic Functions

**FIGURE 4. Contribution of PON1 His\(^{115}\) and His\(^{134}\) and of apoA-I when present in rHDL to ability of PON1 to stimulate macrophage cholesterol efflux**. rHDL at 16 \(\mu\)g/ml cholesterol (A) or PC/FC at a similar cholesterol concentration (B) was preincubated for 3 h at 37 °C with 100 \(\mu\)g/ml WT PON1, H115Q (H115Gln), or H115Q/H134Q (H115Gln-H134Gln), with a similar volume of Tris buffer (rHDL or PC/FC); or with WT PON1 and 200 \(\mu\)M 2-hydroxyquinoline (PON1 inhibitor). Then, rHDL at 1.3 \(\mu\)g/ml cholesterol (A) or PC/FC (B) was added to J774A.1 macrophages that were prelabeled with \(\[^{3}H\]\)cholesterol. The extent of rHDL-mediated (A) or PC/FC-mediated (B) macrophage cholesterol efflux was measured as described under “Experimental Procedures.” The results are expressed as the means ± S.D. of three different experiments. *, \(p < 0.01\) versus rHDL; #, \(p < 0.01\) versus PC/FC.

cate that His\(^{115}\) is required for the rHDL-bound PON1 stimulatory effect on macrophage cholesterol efflux. **Contribution of ApoA-I to Ability of rHDL-associated PON1 to Stimulate Macrophage Cholesterol Efflux**—We next compared the effect of WT PON1 bound to PC/FC particles (with apoA-I) with that of WT PON1 bound to rHDL (containing recombinant apoA-I) on macrophage cholesterol efflux. The extent of macrophage cholesterol efflux stimulation by PC/FC particles was significantly (\(p < 0.01\)) lower by 37% compared with the effect of rHDL (Fig. 4, B versus A). Although WT PON1 in rHDL significantly stimulated macrophage cholesterol efflux by 45% versus rHDL with no PON1 (Fig. 4A), WT PON1 in PC/FC particles increased the extent of cholesterol efflux from the cells by only 10% compared with PC/FC particles with no PON1 (Fig. 4B). In contrast, rHDL- or PC/FC-associated H115Q and H115Q/H134Q had no stimulatory effect on macrophage cholesterol efflux (\(p < 0.01\)) compared with rHDL- or PC/FC-associated WT PON1 (Fig. 4). The addition of the PON1 inhibitor to rHDL or PC/FC particles containing WT PON1 completely abolished (\(p < 0.01\)) the stimulatory effect of rHDL- or PC/FC-associated WT PON1 on macrophage cholesterol efflux (Fig. 4). These data further demonstrate that PON1 His\(^{115}\) is essential for PON1-induced stimulation of cholesterol efflux from macrophages and that apoA-I contributes to this stimulatory effect of rHDL-associated PON1.

**Possible Mechanism for PON1 Anti-atherogenicity in Macrophages**

**Effect of rHDL-bound WT PON1 and its His\(^{115}\) and His\(^{134}\) Mutants on Ability of PON1 to Induce LPC Formation in Macrophages**—We have previously shown that free PON1 and HDL-associated PON1 induce the formation of LPC in macrophages (21, 25). Thus, we next questioned the possible role of PON1 His\(^{115}\) and His\(^{134}\) (in its active site) in PON1-induced macrophage LPC formation. Incubation of J774A.1 macrophages with rHDL-associated WT PON1 increased cellular LPC content by 184% compared with the levels observed in control cells that were incubated with medium alone (Fig. 5). In contrast, H115Q/H134Q in rHDL did not significantly affect cellular LPC levels compared with rHDL alone (Fig. 5). Similarly, the addition of the PON1 inhibitor to rHDL-associated WT PON1 completely abolished (\(p < 0.01\)) the stimulatory effect of rHDL-associated PON1 (Fig. 5). These results suggest that the PON1 active-site residues His\(^{115}\) and His\(^{134}\) are required for PON1-induced macrophage LPC formation.

The addition of WT PON1 in association with PC/FC particles (no apoA-I) to the cells did not induce LPC formation compared with the effect of WT PON1 in association with rHDL (Fig. 5). These results indicate that apoA-I stimulates not only PON1 lactonase activity and anti-atherogenic functions, but also the ability of the enzyme to induce the formation of LPC in macrophages.

**Direct Effect of Macrophage LPC on Cell-mediated LDL Oxidation and rHDL-mediated Cholesterol Efflux**—We finally analyzed the direct effect of macrophage LPC accumulation on cell-mediated oxidation of LDL. Incubation of J774A.1 macrophages for 2 h at 37 °C with increasing concentrations (0–20 \(\mu\)M) of LPC, followed by a cell wash and an additional incubation with LDL (100 \(\mu\)g/ml protein) in the presence of 2.5 \(\mu\)M CuSO\(_4\) for 5 h, resulted in a decreased capability of these cells to oxidize LDL of up to 27% in a cellular LPC dose-dependent manner (Fig. 6A).
We have previously shown that macrophage LPC accumulation stimulates human HDL-mediated cholesterol efflux from the cells (25). To find out whether cellular LPC stimulates also rHDL-mediated macrophage cholesterol efflux, we incubated J774A.1 macrophages with increasing concentrations (0–20 μM) of rHDL for 2 h at 37 °C and then determined the extent of rHDL-mediated macrophage cholesterol efflux (Fig. 6B). Incubation of the cells with LPC resulted in an LPC dose-dependent stimulatory effect on rHDL-mediated macrophage cholesterol efflux of up to 35% (Fig. 6B). These results thus suggest that cellular LPC formed by PON1 could have contributed to the PON1 anti-atherogenic effects.

**DISCUSSION**

This study has demonstrated, for the first time, that the hydrolytic active site of PON1 mediates at least two of its major anti-atherogenic functions, i.e. protection of LDL from oxidation and stimulation of HDL-mediated macrophage cholesterol efflux. We also show that apoA-I in HDL stimulates PON1 lactonase activity and the above anti-atherogenic functions. It therefore appears that the anti-atherogenic effects of PON1 on macrophages could have resulted from its lactonase action on macrophage phospholipids, producing LPC in these cells. Previous studies of PON1 were performed with enzymes and/or HDL particles purified from human sera and yielded conflicting results (26, 27). In contrast, in this study, we used recombinant PON1 and apoA-I that were expressed in and purified from E. coli, and the HDL particles were reconstituted from pure lipids (5). Recombinant WT PON1 appears to preserve both the enzymatic properties of native PON1 (6, 32) as well as its LDL binding properties (5), thus making it a useful tool for mechanistic and structural studies of PON1 (5, 6, 31, 32). By ruling out contamination of serum components that are co-purified with PON1 or the HDL particles, this study assigns anti-atherogenic functions to PON1, including the protection of LDL from oxidation (in a cell-free system as well as by macrophages) and the stimulation of macrophage cholesterol efflux, in an unambiguous manner.

Our results also indicate that these two key anti-atherogenic properties of PON1, when present either in its free form and, more so, when present in the more physiological system, i.e. bound to apoA-I-containing HDL, are mediated by the PON1 hydrolytic active site and appear to stem from its lipolactonase activity. The key evidence for this is that impairment of the lactonase activity through mutations of His115 and His134 (Table 1), which compose the catalytic dyad that mediates the lactonase activity in PON1 and other mammalian PONs (30, 31, 55), has a parallel effect on PON1 protection of LDL from oxidation (Figs. 1 and 2) and on PON1 stimulation of macrophage cholesterol efflux (Figs. 3 and 4).

There are several indications that the PON1 His115 and His134 mutations are indeed local and restricted to the catalytic dyad. (i) The effect of a range of mutations of these residues has been studied, and mutations to Gln were found to be the least impairing (55). (ii) The undiminished paraoxonase activity of these mutants (Table 1), which takes place at the same active site (6), indicates that, apart from the His dyad, the hydrolytic active site is essentially intact. (iii) These mutants have the ability to bind to and to be stabilized by apoA-I in HDL. It is also notable that the effect of these mutations on the lactonase activity indicates that His134 serves to assist His115 (Table 1) (55). The PON1 effect on the biological functions (Figs. 1–4) is in accordance; namely, the H134Q mutant is partially active, whereas the H115Q mutant shows almost no activity, and once His115 is knocked out, mutation of His134 has little additional effect (i.e. the H115Q/H134Q double mutant is as inactive as the H115Q mutant). We ascribe the loss of anti-atherosclerotic functions in the His mutants (and in the presence of the active-site inhibitor 2-hydroxyquinoline) (Fig. 4) to the loss of lactonase activity. Although the H115Q and H134Q mutants are also defective in their arylesterase activity (Table 1), it is highly unlikely that the arylesterase activity mediates the anti-atherosclerotic functions. PON1 is incapable of efficiently hydrolyzing aliphatic esters and, in particular, aliphatic esters derived from secondary alcohols that resemble phospholipase A2 substrates (6) or phospholipase A2 substrates themselves (27). Yet if PON1 has no phospholipase A2 activity, how can the increased levels of LPC and other hydrolytic products of lipids (42) consistently observed upon cell incubation with PON1 (21, 25) be explained? We suggest a model in which PON1 lactonase activity could be responsible for the hydrolysis of oxidized lipids in macrophages, leading to the formation of LPC in cells. Our results clearly indicate that, when bound to rHDL (with apoA-I), recombinant PON1 induces significant LPC formation in macrophages and that cellular LPC further stimulates HDL-mediated macrophage cholesterol efflux (Fig. 6). Furthermore, knocking out the His dyad and the use of the PON1 active-site inhibitor 2-hydroxyquinoline (6, 25) clearly demonstrate that LPC formation and stimulation of macrophage cholesterol efflux.
PON1 Hydrolytic Activity and Anti-atherogenic Functions

Inhibition of LDL oxidation by recombinant WT PON1 in the cell-free system (Fig. 1) is the result of an increase in the lag phase and also a decrease in the slope and plateau phase (17, 18). PON1 hydrolyzes "seeded" lipid peroxides in LDL and those formed during the oxidation period (17, 19, 21, 42), and while doing so, the enzyme is inactivated (44). Inhibition of macrophage-mediated oxidation of LDL by PON1 could have resulted not only from the hydrolytic activity of PON1 on LDL oxidized lipids, but also from PON1 action on the cells. We have previously demonstrated that PON1 can hydrolyze oxidized lipids in macrophages (13, 16, 20, 21), resulting in a decrease in the cells’ ability to oxidize LDL (13, 16, 21). Furthermore, PON1-induced LPC formation can lead to a significant reduction in cell-mediated oxidation of LDL. We therefore propose a mechanism for PON1-induced LPC formation based on the proven lactonizing (lactone formation) (43) and lactonase (lactone hydrolysis) activities of PON1 (6, 7, 45), by which PON1 can hydrolyze certain oxidized lipids in macrophages to yield LPC (Fig. 7).

The mechanism by which PON1 lactonase activity inhibits copper ion-induced LDL oxidation in both the cell-free system and the macrophage system involves the hydrolysis of oxidized fatty acid lactones (7) as well as the hydrolysis of oxidized phospholipids in LDL and/or in cells, as shown previously (42) and as suggested in this study (Fig. 7).

Inhibition of copper ion-induced LDL oxidation by WT PON1 is not the result of PON1 copper ion chelating properties because a similar inhibitory effect of WT PON1 was observed upon LDL oxidation by PMA-activated macrophages (with no copper ions present) (Fig. 1D).

Our studies comparing the anti-atherogenic effects of WT PON1 bound to rHDL (composed of PC/FC and apoA-I) and bound to PC/FC particles (without apoA-I) clearly indicate that the interaction of PON1 with apoA-I contributes to the anti-atherogenic effects. ApoA-I is the major structural protein in HDL, and it has a role in determining the structure and composition of HDL (46, 47). The structure of PON1 implies that HDL anchoring can modify its active site, and its enzymology clearly indicates that it is an interfacial activated enzyme (5). ApoA-I stabilizes PON1 (1, 2, 5), stimulates its hydrolytic activities and its lactonase activity in particular (5), and provides an optimal environment for the interaction of PON1 with its natural substrates. Several lines of evidence demonstrate the preferential association of PON1 with apoA-I-containing HDL particles in vivo and in vitro (5, 48). PON1 co-purifies with apoA-I (49), and immunosassays of sera have shown that PON1 is found mainly in association with apoA-I-containing HDL particles (50). Finally, both native and reconstituted HDL particles carrying apoA-I promote the release of PON1 from cells, stabilize it, and increase its lactonase activity (1, 5, 51). In this study, we have demonstrated that, in the presence of apoA-I in HDL, PON1 protects LDL from oxidation by macrophages and stimulates macrophage cholesterol efflux to a significantly higher degree than in the presence of PC/FC particles (with no apoA-I), and these results are in accordance with LPC formation (Fig. 5).

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