The 192R/Q polymorphs of serum paraoxonase PON1 differ in HDL binding, lipolactonase stimulation, and cholesterol efflux[S]

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Abstract Serum paraoxonase (PON1) is a HDL-associated enzyme exhibiting potentially antiatherogenic properties. Here, we examined the common PON1-192R/Q human polymorphism. Despite numerous studies, the effect of this polymorphism on the antiatherogenic potential of PON1 is yet unresolved. Our structural model suggests that amino acid 192 constitutes part of the HDL-anchoring surface and active site of PON1. Based on our findings that PON1 is an interfacially activated lipolactonase that selectively binds HDL carrying apolipoprotein A-I (apoA-I) and is thereby greatly stabilized and catalytically activated, we examined the interaction of the PON1-192 isozymes with reconstituted HDL-apoA-I particles. We found that PON1 position 192 is indeed involved in HDL binding. The PON1-192Q binds HDL with a 3-fold lower affinity than the R isozyme and consequently exhibits significantly reduced stability, lipolactonase activity, and macrophage cholesterol efflux. We also observed the lower affinity and stability of the 192Q versus the 192R isozyme in sera of individuals belonging to the corresponding genotypes. The observed differences in the properties of PON1-192R/Q isozymes provide a basis for further analysis of the contribution of the 192R/Q polymorphism to the susceptibility to atherosclerosis, although other factors, such as the overall levels of PON1, may play a more significant role.—Gaidukov, L., M. Rosenblat, M. Aviram, and D. S. Tawfik. **The 192R/Q polymorphs of serum paraoxonase PON1 differ in HDL binding, lipolactonase stimulation, and cholesterol efflux.** ***J. Lipid Res.*** **2006.** 47: **2492–2502.**

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Serum paraoxonase (PON1) is a HDL-associated enzyme belonging to a family of calcium-dependent hydrolases (1). Although its physiological functions and endogenous substrates are unknown, there is ample evidence linking PON1 with the prevention of atherosclerosis (2). Serum PON1 levels appear to be inversely related to the risk of coronary heart disease (3, 4), and PON1 knockout mice are susceptible to atherosclerosis (5, 6). It has been suggested that HDL-bound PON1 can protect against oxidative stress by hydrolyzing oxidized lipids in lipoproteins (7–9) and in atherosclerotic lesions (10), including macrophages (6, 11). PON1 was shown to inhibit macrophage cholesterol biosynthesis (12) and to stimulate cholesterol efflux from macrophages (12, 13). The structure and enzymology of PON1 were resolved only recently. PON1 hydrolyzes a broad range of substrates and has been traditionally described as paraoxonase/arylesterase (14). However, it recently became apparent that PON1 is in fact a lactonase with lipophylic lactones constituting its primary substrates (15–17) and that the arylesterase and paraoxonase activities are merely promiscuous (18).

The crystal structure of PON1 has enabled better understanding of its mechanism (19, 20) and provided a model for its anchoring onto HDL (21). This model indicated that PON1 might be an interfacially activated enzyme (21). Indeed, it has recently been shown that HDL particles carrying apolipoprotein A-I (apoA-I) bind PON1 with high affinity (nM) and thereby dramatically stabilize the enzyme (>100-fold) and stimulate its lipolactonase activity (≤20-fold relative to the delipidated form). In contrast, the promiscuous paraoxonase and arylesterase activities are barely affected by HDL binding (22). It has also been shown that impairing the lactonase activity of PON1, through mutations of its catalytic dyad (19), diminishes PON1's ability to prevent LDL oxidation and stimulate macrophage cholesterol efflux, in-

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Abbreviations: apoA-I, apolipoprotein A-I; HSL, homoserine lactone; NTA, nitrilotriacetic acid; PON1, serum paraoxonase; rePON1, recombinant serum paraoxonase; rHDL, reconstituted high density lipoprotein.

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

dicating that these potentially antiatherogenic functions of PON1 are likely mediated by its lipolactonase activity (20).

These new findings, and the fact that position 192 constitutes part of PON1's active site (21), called for a reexamination of PON1's intensively studied polymorphism at this position. Indeed, the 192R/Q polymorphism is known to alter PON1's substrate specificity, in particular toward organophosphates (23–25). The R allele exhibits several-fold higher activity toward paraoxon, whereas the arylesterase activity levels are similar in both isozymes (25). The paraoxonase activity of the R isozyme is also stimulated at high salt concentrations (26). These differences provide the basis for the determination of the R/Q polymorphs in individuals (27). However, to date, no clear indications exist regarding the differences in the properties of the 192R/Q isozymes in relation to atherosclerosis, neither in vitro nor in vivo. Numerous case-control studies have been conducted in the attempt to relate the PON1 R/Q polymorphism with the incidence of cardiovascular disease (reviewed in 28–30). These reports are conflicting, however, with some studies showing that the RR genotype is more closely associated with cardiovascular disease (31–34) and others indicating association with neither allele (35–37). None of the previous studies, however, examined PON1 in light of it being an interfacially activated lipolactonase that undergoes a substantial stimulation of enzymatic activity and a dramatic increase in stability conferred by binding to HDL (22). Our structural model suggested that HDL anchoring is mediated by the N-terminal helix of PON1 (dubbed H1) and another amphipathic helix that constitutes part of the active site (dubbed H2). That parts other than H1 are involved in HDL binding was confirmed by the fact that engineered PON1 variants lacking H1 can still bind HDL, although with lower affinity, and are stabilized and stimulated (22). Because amino acid 192 is part of H2, we sought to examine whether the HDL binding properties of the 192R/Q isozymes differ.

Described below are our results showing that the R isozyme binds HDL with higher affinity and consequently exhibits much higher stability and lipolactonase activity as well as higher stimulation of HDL-mediated macrophage cholesterol efflux. These conclusions were drawn from the in vitro system of reconstituted high density lipoprotein (rHDL) and recombinant serum paraoxonase (rePON1) (38) and verified with the human PON1 isozymes. Differences in HDL binding and stability were also observed in sera samples obtained from individuals belonging to the QQ, QR, and RR genotypes.

MATERIALS AND METHODS

Production of rePON1

A recombinant, wild-type PON1 variant dubbed G3C9 (gi:40850544), fused to a His_8 tag at the C terminus [dubbed rePON1-192K], was used for the in vitro system (22). Its 192R and 192Q isozymes were generated by PCR (19) and cloned into a modified pET32b vector (pET32-trx) as described (38). The truncated (Δ20-rePON1) variants of rePON1 and its 192R and 192Q isozymes were prepared as described (22). All rePON1 variants were expressed in *Escherichia coli* and purified as described previously (22).

Preparation of rHDL-apoA-I

Human apoA-I gene in pET20b vector (Novagen) (39) was kindly provided by Michael Oda (Oakland Research Institute). Rabbit apoA-I was cloned into pET20b vector as described (22). Both apoA-Is were expressed in *E. coli* and purified as described (22). Discoidal rHDL containing egg L-α-phosphatidylcholine (Avanti Polar Lipids), free cholesterol (Sigma), and apoA-I at a starting molar ratio of 100:5:1 was prepared by the cholate dialysis method as described previously (22) with the following variations. Purified apoA-Is were resuspended in 3 M guanidine hydrochloride solution, briefly dialyzed against TBS (10 mM Tris, pH 8.0, and 150 mM NaCl), and immediately added to the phosphatidylcholine/free cholesterol mixture. Anion-exchange resin (diethyl aminoethyl; Whatman) was added to the three last buffer exchanges to remove residual sodium deoxycholate.

Stability of rePON1 isozymes

Inactivation rates of rePON1s were measured as described (22). Briefly, rePON1 samples were delipidated using Bio-Beads SM-2 (Bio-Rad), diluted to 0.2 μM, and incubated in activity buffer (50 mM Tris, pH 8.0, and 1 mM $CaCl_2$) or with a 50-fold molar excess of rHDL-apoA-I in a final volume of 100 μl. An equal volume of inactivation buffer (10 mM EDTA and 20 mM β-mercaptoethanol in 50 mM Tris, pH 8.0) was added, and samples were incubated at 37°C. Residual activity at different time points was determined with 2 mM phenyl acetate, and inactivation rates were fitted to either mono or double exponentials (22). The inactivation assay was repeated at least twice with independent preparations of rHDL and rePON1 isozymes.

Surface plasmon resonance

rHDL-apoA-I particles containing 0.7% *N*-biotinyl-dipalmitoylphosphatidylethanolamine (Avanti Polar Lipids) were prepared and purified as described (22). Surface plasmon resonance was performed on BIAcore 3000 (Biacore AB, Uppsala, Sweden) as described (22). Briefly, the biotinylated rHDL particles were adsorbed on a streptavidin (SA5) chip, and Δ20-rePON1 isozymes were injected (at a flow rate of 20 μl/min at 25°C) over the immobilized and blank surfaces to obtain the net binding response. Binding rate constants were obtained by fitting the association and dissociation phases to single exponentials as described (22).

Stimulation of PON1 activity by rHDL

Delipidated rePONs at 0.2 μM were incubated with a range of rHDL concentrations (0.1–10 μM) for 3 h at 37°C. Enzymatic activity toward δ-nonanoic lactone, γ-dodecanoic lactone, phenyl acetate, and paraoxon was determined in activity buffer with substrates at 1 mM concentrations as described (22). Activity toward *N*-acyl-homoserine lactones (HSLs), including *N*-butyryl-DL-HSL, *N*-(3-oxooctanoyl)-L-HSL, and *N*-(β-ketocaproyl)-DL-HSL, was determined in activity buffer with substrates at 0.25 mM concentrations using the pH-sensitive colorimetric assay as described previously (17).

Stability and activity stimulation of human isozymes on rHDL

Human PON1-192R and -192Q isozymes purified from pooled blood samples (40) were kindly provided by Dr. Dragomir Draganov (University of Michigan, Ann Arbor, MI) and stored in the

presence of 0.1% tergitol and 20% glycerol. Before assay, these samples were briefly delipidated (22) and dialyzed against activity buffer to remove the tergitol and glycerol that interfere with HDL binding. Dialyzed samples (0.2 μM) were incubated with rHDL (10 μM) in activity buffer in a final volume of 100 μl. Inactivation was initiated by adding an equal volume of 50 mM Tris (pH 8.0) buffer supplemented with nitrilotriacetic acid (NTA) and β-mercaptoethanol (both at 10 mM) and incubating the samples at 25°C. Data analysis was performed as described above for rePON1s. Stimulation of enzymatic activities was measured with various substrates at 1 mM concentration. Each experiment was repeated at least twice with independent preparations of rHDL and PON1 isozymes.

PON1 phenotyping in human sera

Human sera were collected from 54 healthy individuals at Rambam Medical Center with the approval of the institute's Helsinki committee. Sera were divided into aliquots and stored frozen at −20°C. After thawing, sera were immediately supplemented with β-mercaptoethanol (5 mM) to prevent oxidation and stored for the duration of the assays at 4°C (maximum of 1 weak). Phenotyping of sera for PON1 was performed by a two-substrate method as described (27). Briefly, sera were diluted 20-fold, and arylesterase activity was measured in activity buffer containing 1 mM phenyl acetate by monitoring the absorbance at 270 nm in a final volume of 200 μl (ε = 700 OD/M). Paraoxonase activity was measured in buffer containing 50 mM glycine (pH 10.5), 1 mM $CaCl_2$, and 1 mM paraoxon, either supplemented or not with 1 M NaCl, by monitoring the absorbance at 405 nm in a final volume of 200 μl (ε = 11,725 OD/M). The initial rates of product release derived from the two measurements were expressed as U/ml (1 unit = 1 μmol of phenyl acetate or 1 nmol of paraoxon hydrolyzed per minute per milliliter of serum). The paraoxonase-arylesterase activity ratio was calculated by dividing the paraoxonase activity of a sample in the presence of 1 M NaCl by its arylesterase activity. Stimulation by salt corresponds to the rate of paraoxonase activity in the presence of 1 M NaCl and its absence.

PON1 inactivation assays in human sera

Sera samples (5 μl) were diluted 10-fold in TBS. Inactivation was initiated by adding an equal volume of inactivation buffer (TBS supplemented with 0.5 mM NTA and 2 mM β-mercaptoethanol) at 25°C. Residual activity was determined with 2 mM phenyl acetate. Inactivation rates were fitted well to a monoexponential fit for all RR sera, and a double exponential fit was necessary only for RQ and QQ sera. It should be noted that the reproducibility of these inactivation assays was low. Although the differences between R and Q sera were observed in all assays, the inactivation rates varied from one assay to another. It appears that the sera inactivation kinetics are very sensitive to oxidation. Indeed, supplementing sera with β-mercaptoethanol (5 mM) immediately after defrosting and storing the β-mercaptoethanol-supplemented sera at 4°C for 12 h before the experiment yielded more reproducible results.

Biological activities of the R/Q isozymes

Delipidated rePON1 isozymes were incubated with a 2.5-fold molar excess of rHDL-apoA-I. Cholesterol efflux from macrophages and macrophage-mediated oxidation of LDL in the presence of copper ions and HDL-bound rePON1s were performed as described (20). For the cholesterol efflux studies, rHDL (60 μg of apoA-I) was used, with the final PON1 concentration (16 μg/ml) in a final volume of 0.5 ml. For the macrophage-mediated oxidation of LDL studies, rHDL (30 μg of apoA-I) was used, with the final PON1 concentration (4 μg/ml) in a final volume of 0.5 ml. Each assay was repeated at least twice with independent preparations of rHDL and rePON1 isozymes.

RESULTS

We addressed the properties of the 192R/Q isozymes in an in vitro system, using rHDL-apoA-I particles and either rePON1 or human PON1. The rePON1 variant was generated by directed evolution for expression in *E. coli* (38). It comprises a close homolog of rabbit PON1 (95% amino acid identity) as well as human PON1 (86% identity) and exhibits essentially identical enzymatic and HDL binding properties (18, 22, 38). In wild-type rabbit PON1 and rePON1, position 192 is lysine (K), which is similar in size and charge to the arginine found in the human PON1-192R isozyme. Therefore, we generated and examined both the rePON1-192Q and -192R isozymes of the wild-type-like rePON1-192K. The results obtained with rePON1 isozymes were then confirmed in assays of the 192Q and 192R isozymes of human PON1 and by analyses of human sera.

PON1 stability and HDL binding

It has been shown previously that determining the rate of PON1 inactivation provides a measure of the level of HDL-bound versus HDL-free enzyme and thereby reflects PON1's affinity to HDL (22). The rate of inactivation of the rePON1-192K, -192R, and -192Q isozymes, either in buffer or in the presence of a saturating concentration of HDL-apoA-I, was followed by measuring the residual enzymatic activity in the presence of EDTA, which chelates PON1's essential calcium atoms. The inactivation profiles were fitted to either one or two exponentials to derive the inactivation rate constants (**Fig. 1**, **Table 1**).

As reported previously (22), in the presence of HDL-apoA-I, inactivation of wild-type-like rePON1-192K followed monoexponential kinetics with a rate constant ($k^{inactiv}$) of 0.01 h^{-1}, indicating a complete (100%) association with HDL. In contrast, inactivation of rePON1-192Q followed a double exponential regime, with the first (fast) phase ($k_1^{inactiv}$ = 1.1 h^{-1}) corresponding to 30% "free" PON1 and the second (slow) phase corresponding to 70% HDL-bound PON1 (Table 1). The latter exhibits 100 times greater stability and an inactivation rate identical to that of the wild type (rePON1-192K; $k_2^{inactiv}$ = 0.01 h^{-1}). The difference between the two isozymes, therefore, is in the degree of HDL binding, whereas the rate of inactivation of the HDL-bound form is similar for both isozymes. The two isozymes also exhibit identical inactivation rates in buffer. As expected, the effect of replacing K192 by R was much milder, with 90% of rePON1-192R bound to HDL.

Affinity measurements

These results were supported by affinity measurements using surface plasmon resonance. We have shown previously that the HDL-apoA-I affinity of the intact rePON1 is

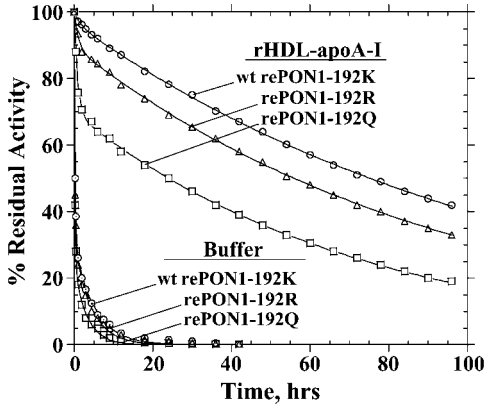

Fig. 1. Inactivation kinetics of the wild-type (wt) recombinant serum paraoxonase (rePON1)-192K, -192R, and -192Q isozymes bound to reconstituted high density lipoprotein-apolipoprotein A-I (rHDL-apoA-I) or in buffer. Delipidated enzymes were incubated with a 50-fold excess of rHDL-apoA-I or in activity buffer and subjected to inactivation in the presence of EDTA (5 mM) and β-mercaptoethanol (10 mM) at 37°C. Residual activity at various time points was determined by initial rates of phenyl acetate hydrolysis (2 mM) and plotted as a percentage of the activity at time zero. Data were fitted to a single exponential for wild-type rePON1 on rHDL-apoA-I and to double exponentials for the remaining samples. Inactivation rate constants and amplitudes were derived from this fit (see Table 1).

very high (subnanomolar) and could not be determined accurately (22). Truncation of the hydrophobic N-terminal helix significantly reduced PON1's affinity for HDL, although the truncated enzyme (Δ20-rePON1) binds HDL with relatively high affinity ($K_d \sim 10^{-7}$ M) (22). The latter observation supported our model for HDL binding that indicated a role for parts of PON1 other than the N-terminal helix, most notably the amphipathic helix H2, which residue 192 is part of. Therefore, we produced the truncated versions of the different isozymes and measured their binding affinities. Biotinylated rHDL-apoA-I particles were immobilized onto the surface of the streptavidin sensor chip, and PON1 samples were injected in a series of concentrations over the immobilized particles, yielding the binding sensorgrams (**Fig. 2**). Binding rate constants were derived by fitting the association and dissociation phases to single exponentials (**Table 2**).

The wild-type Δ20-rePON1 (i.e., the truncated 192K isozyme) shows the highest affinity (10^{-7} M). The affinity of its R equivalent (Δ20-rePON1-192R) is reduced only slightly (1.1×10^{-7} M), but that of the Q isozyme is 3-fold lower (3×10^{-7} M) as a result of a decrease of the association rate and an increase of the dissociation rate (Table 2). As demonstrated previously (22), the 3-fold difference in affinity correlates well with the 3-fold lower fraction of the HDL-bound Q isozyme revealed by the inactivation assay (Fig. 1, Table 1).

Stimulation of PON1's enzymatic activities

The rePON1 isozymes were incubated with increasing concentrations of HDL-apoA-I, and the rate of enzymatic catalytic activity was measured with various substrates, including the hydrophobic lactones δ-nonanoic lactone and γ-dodecanoic lactone, as well as phenyl acetate and paraoxon (**Fig. 3**). Data were fitted to the Langmuir saturation curve to give the stimulation factor relative to the delipidated PON1 (V_{max}; **Table 3**). The wild-type rePON1-192K exhibited the highest stimulation levels (16–18-fold with lactones), the R isozyme exhibited slightly reduced stimulation levels (12–14-fold stimulation of V_{max}), whereas stimulation of the Q isozyme was reduced significantly (4–8-fold stimulation). The promiscuous arylesterase and paraoxonase activities exhibited correspondingly lower stimulations (2–4-fold with the wild type and 1.3–1.7-fold with rePON1-192Q). Together with the stability measurements, these results indicate that the 192Q mutation of rePON1 significantly disrupts the ability of PON1 to associate with HDL-apoA-I, whereas the 192R substitution, which involves minor changes in size and charge, exerts a much smaller effect.

We also tested the ability of HDL-bound PON1 isozymes to degrade *N*-acyl-HSLs, bacterial quorum-sensing signal molecules that were shown to be substrates for PON1 (16, 41). However, stimulation levels were quite low (2–4-fold), and we could not detect significant differences between PON1 isozymes (data not shown). Low HDL stimulation, together with the fact that PON1 activity toward *N*-acyl-HSLs is

TABLE 1. Kinetic and equilibrium constants for the inactivation of the rePON1-192 isozymes in buffer and on rHDL-apoA-I

	rHDL-apoA-I				Activity Buffer			
Isozyme	A_1	$k_1^{inactiv}$	A_2	$k_2^{inactiv}$	A_1	$k_1^{inactiv}$	A_2	$k_2^{inactiv}$
	%	h^{-1}	%	h^{-1}	%	h^{-1}	%	h^{-1}
Wild-type rePON1-192K	0	–	100	0.01	69	4.5	31	0.2
rePON1-192R	10	0.9	90	0.01	69	5.5	31	0.2
rePON1-192Q	30	1.1	70	0.01	76	5.2	24	0.3

apoA-I, apolipoprotein A-I; rePON1, recombinant serum paraoxonase; rHDL, reconstituted high density lipoprotein. Amplitudes (A) and kinetic rates of inactivation ($k^{inactiv}$) were derived by fitting the data to a monoexponential curve in the case of wild-type rePON1-192K interacting with rHDL-apoA-I and to double exponential curves for all the rest. Suffixes 1 and 2 designate the first (fast) and second (slow) phases of the inactivation, respectively. Each value represents the mean of two independent experiments. Standard deviations were <10% of parameter values.

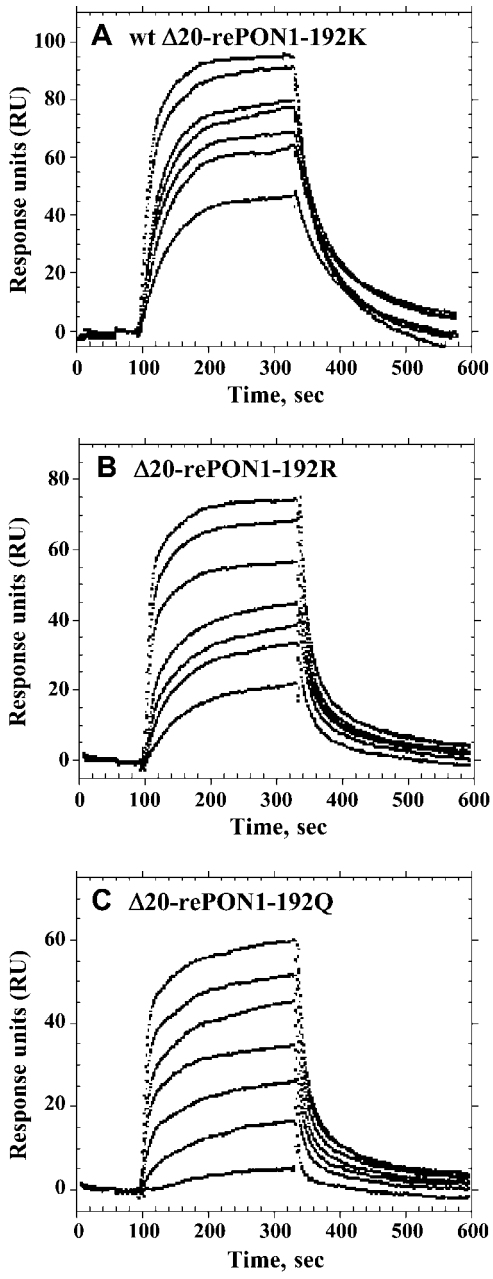

Fig. 2. Sensorgrams for the binding of wild-type (wt) Δ20-rePON1-192K (A), Δ20-rePON1-192R (B), and Δ20-rePON1-192Q (C) isozymes to rHDL-apoA-I. Biotinylated rHDL-apoA-I particles were immobilized onto the streptavidin surface (SA chip), and a series of serum paraoxonase (PON1) concentrations were injected over the immobilized and blank surfaces to obtain the net binding response. Binding was performed at 25°C. Association and dissociation phases were fitted to a single exponential, from which kinetic rate constants were derived (see Table 2). PON1 concentrations in all sensorgrams are (from bottom to top) 25, 40, 60, 80, 100, 150, and 200 nM.

500–20,000-fold weaker than with the lipolactones, such as δ-nonanoic lactone and 5(*S*)-hydroxy-6*E*,*Z*,11*Z*,14*Z*-eicosatetraenoic acid 1,5-lactone (5-HETEL) (16, 22), suggest that *N*-acyl-HSLs do not resemble PON1's native substrates.

Stability and stimulation of human PON1 isozymes

These results were validated with the human PON1-192R/Q isozymes purified from pooled blood samples (40). Compared with rePON1, which is analogous to rabbit PON1, human PON1 exhibits >10-fold lower affinity for the "structural calcium," the chelation of which induces irreversible inactivation (42). Therefore, we applied a low-affinity calcium chelator, NTA, rather than EDTA. Inactivation of both isozymes followed two-phase kinetics, with similar inactivation rates but different partitioning between the stable and unstable phases, which corresponds to the partitioning between the HDL-bound and unbound forms (**Fig. 4A**, **Table 4**). The bound phase constituted 87% and 72% for the R and Q isozymes, respectively, indicating the more efficient HDL binding of human PON1-192R. HDL-mediated stimulation of the enzymatic activity of human PON1 isozymes was determined by incubating PON1 at saturating HDL-apoA-I concentrations (Fig. 4B). For all of the lactones tested, human PON1-192R exhibited ~2-fold higher stimulation levels than 192Q, whereas the weak stimulation of the promiscuous paraoxonase and arylesterase activities was equally low with both isozymes.

Overall, the differences in stability and stimulation of human PON1 resembled those observed with rePON1, indicating the lower affinity of the 192Q isozymes in both rabbit and human PON1. However, the stimulation levels of human PON1 with most substrates were lower than those observed with rePON1. This could result from the lower affinity of human PON1 to HDL or, more likely, from residual glycerol and tergitol in the samples and other impurities, including proteins that copurify with PON1 (43).

Inactivation of the 192R/Q isozymes in human sera

After the observations made with 192R/Q isozymes in the reconstituted systems described above, we tested PON1 stability in sera of 54 healthy individuals bearing the QQ, RQ, or RR genotype. Sera were phenotyped by determining the ratio of paraoxonase to arylesterase activity, and the degree of paraoxonase activity stimulation by 1 M NaCl, as described (27). Of 54 samples, 34 were phenotyped as QQ, 14 as RQ, and 6 as RR (see supplementary Fig. I).

The inactivation assay was applied to serum samples under conditions similar to those used with the purified human isozymes. PON1's stability differed markedly among the three types of sera (**Fig. 5A**). As observed with the reconstituted system, inactivation of QQ and RQ sera followed a two-phase regime with the fast and slow phases of inactivation, whereas the RR sera followed a monoexponential decay (**Table 5**). Interestingly, in RQ sera, the inactivation rate of the fast phase was similar to that of the QQ sera, whereas the rate of the slow phase was similar to the single-phase inactivation rate of RR sera. Thus, in the heterozygote RQ sera, the fast initial phase of the Q isozyme is followed by the slow inactivation of the R isozyme. Marked differences in the stability of the three genotypes were also observed by measuring PON1's residual activity

TABLE 2. Kinetic and affinity constants for the binding of Δ20-rePON1-192 isozymes to rHDL-apoA-I

Isozyme	k_{on}	k_{off}	K_d	A_2[a]
	$s^{-1}\ M^{-1}$	s^{-1}	M	%
Wild-type Δ20-rePON1-192K	$(2.0 \pm 0.3) \times 10^5$	$(2.0 \pm 0.2) \times 10^{-2}$	$(1.0 \pm 0.2) \times 10^{-7}$	100
Δ20-rePON1- 192R	$(2.6 \pm 0.3) \times 10^5$	$(2.9 \pm 0.2) \times 10^{-2}$	$(1.1 \pm 0.2) \times 10^{-7}$	90
Δ20-rePON1- 192Q	$(1.4 \pm 0.5) \times 10^5$	$(4.2 \pm 0.2) \times 10^{-2}$	$(3.0 \pm 0.6) \times 10^{-7}$	70

Binding experiments were performed at 25°C. Association and dissociation phases were fitted to a single exponential to give k^{obs}. k_{on} was derived from the linear fit of $k_{on}{}^{obs}$ versus concentration ($k_{on}{}^{obs} = [\text{rePON1}]k_{on} + k_{off}$). k_{off} was derived directly from $k_{off}{}^{obs}$ independent of serum paraoxonase (PON1) concentration. Each value represents the mean ± SD of two independent experiments.

[a] The amplitude of the slow (HDL-bound) phase of inactivation (A_2) was derived from the stability measurements (see Table 1).

at a single time point (9 h) (Fig. 5B, Table 5). Notably, the largest heterogeneity in these values was observed in Q-type sera, probably because of the large differences in PON1 concentration.

Biological activities of the R/Q isozymes

Two potential antiatherogenic activities of HDL-bound rePON1 isozymes were examined with respect to the stimulation of HDL-mediated cholesterol efflux from macrophages and the protection against cell-mediated oxidation of LDL in the presence of copper ions (20). The rePON1 isozymes were incubated with HDL-apoA-I (molar ratio, 1:2.5; final PON1 concentration, 16 μg/ml) and then added to J774A.1 macrophages that were preincubated with the labeled cholesterol. The degree of HDL-mediated cholesterol efflux was subsequently determined after 3 h of incubation at 37°C (**Fig. 6**). The wild-type rePON1-192K increased cholesterol efflux from macrophages (relative to HDL-apoA-I alone) by 93%, rePON1-192R yielded a slightly milder increase (67%), and

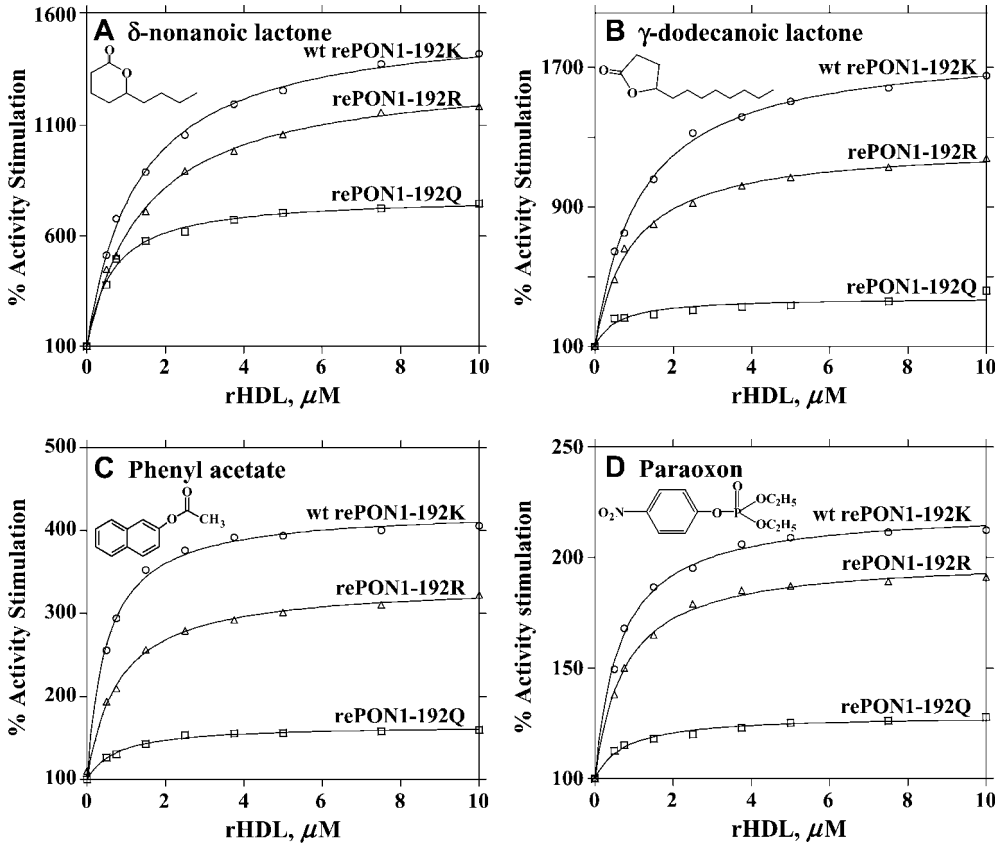

Fig. 3. Stimulation of the lipolactonase activity (A, B) and promiscuous enzymatic activities (C, D) of the rePON1-192 isozymes by rHDL-apoA-I. Delipidated enzymes (0.2 μM) were incubated with increasing concentrations of rHDL-apoA-I, and enzymatic activity was determined with 1 mM δ-nonanoic lactone (A), γ-dodecanoic lactone (B), phenyl acetate (C), or paraoxon (D). The activity is presented in relation to the initial activity of the delipidated enzymes (percentage stimulation). Data were fitted to the Langmuir saturation curve, from which the activation factor (V_{max}) and the apparent affinity (K_{app}) were derived (see Table 3). wt, wild-type.

TABLE 3. Enzymatic activation of rePON1-192 isozymes by rHDL-apoA-I

	Wild-Type rePON-192K		rePON1-192R		rePON-192Q	
Substrate[a]	V_{max}[b]	K_{app}[c]	V_{max}	K_{app}	V_{max}	K_{app}
	%	μM	%	μM	%	μM
δ-Nonanoic lactone	1,580 ± 19	1.3 ± 0.1	1,351 ± 20	1.5 ± 0.1	778 ± 12	0.6 ± 0.1
γ-Dodecanoic lactone	1,830 ± 22	1.2 ± 0.1	1,258 ± 21	0.9 ± 0.1	382 ± 20	0.6 ± 0.2
Phenyl acetate	425 ± 4	0.5 ± 0.1	336 ± 4	0.8 ± 0.1	165 ± 1	0.8 ± 0.1
Paraoxon	222 ± 2	0.6 ± 0.1	200 ± 1	0.8 ± 0.1	128 ± 1	0.8 ± 0.1

Data were fitted to the Langmuir saturation curve, from which the V_{max} and K_{app} values were directly derived. All values represent the derived parameters with the standard error of the fit.

[a] All of the substrates were at 1 mM ($\geq K_M$ for all substrates).

[b] V_{max} values are presented as percentages relative to the delipidated enzyme (designated as 100%).

[c] K_{app} is the apparent affinity for HDL stimulation.

rePON1-192Q exhibited the lowest effect (30%). These results correlate well with the degree of HDL binding observed with the three isozymes.

We did not, however, detect significant differences in the antioxidative activity of the HDL-apoA-I-bound R and Q isozymes on macrophage-mediated LDL oxidation in the presence of copper ions (2.5 μM). The thiobarbituric acid-reactive substance levels after 3 h of incubation at 37°C were 60.2 ± 2.1 for control LDL, 34.4 ± 0.5 for LDL + rHDL (no PON1), 16.0 ± 0.5 for LDL + HDL and wild-type rePON1-192K, 22.0 ± 0.3 for LDL + HDL and rePON1-192R, and 21.0 ± 3.4 for LDL + HDL and rePON1-192Q. Similarly, no significant differences in the antioxidative activity of R and Q izozymes in buffer against macrophage-mediated LDL oxidation were noted (data not shown). These results could be attributable to the fact that the R/Q isozymes do not differ in their antioxidative capabilities. A more likely explanation, however, is that assay volume restrictions do not allow the assay to be performed at HDL/PON1 ratios > 5:1, whereas complete binding of PON1 to HDL requires a ⩾50-fold molar excess of HDL (Fig. 3) (22). Thus, it is possible that the assay was performed with a mixture containing substantial amounts of free PON1, thus resulting in the R/Q isozymes exhibiting similar properties. It should also be noted that several recent studies demonstrated that human PON1 showed no inhibition of copper-induced LDL oxidation (16, 44). However, the consistent results obtained with this assay using purified, directly evolved recombinant PON1 variants (20), and in particular the lack of inhibitory activity by PON1 mutants that lost their lactonase activity (19), suggest that this assay reflects the capability of our recombinant PON1 variants to inhibit LDL oxidation.

DISCUSSION

The 192R/Q and other polymorphisms of human PON1 have attracted much interest as a result of PON1's potential impact on atherosclerotic disease and resistance to organophosphate toxicity (24). However, until now, no clear indications were available regarding the biochemical differences between the two isozymes that relate to their antiatherogenic potential. The recent realization that PON1 is an interfacially activated lipolactonase that selectively binds apoA-I-containing HDL, and is thereby stabilized (>100-fold) and catalytically activated (>10-fold) toward lipophylic lactones (16, 17, 22), and the proposed structural model of PON1's anchoring to HDL (21) led us to hypothesize that the R/Q isozymes might differ in their HDL binding properties and, as a result, in their stability and lipolactonase activity. The results of this study clearly indicate that this is the case. The marked effect of the substitutions at position 192 on PON1's HDL binding strongly supports the structural model according to which the H2 helix of the active site is involved in HDL anchoring. By most studied parameters, 192R/Q isozymes appear identical in buffer or in the presence of detergent. However, the affinity of the 192Q isozyme toward HDL is ~3-fold lower than that of 192R (or 192K in the case of recombinant and rabbit PON1s). As a result, in the presence of HDL-apoA-I, the stability and stimulation of lactonase activity of the HDL-anchored 192R (or 192K) are significantly higher. The higher lactonase activity is also manifested by increased antiatherogenic potency: the observed rate of HDL-mediated cholesterol efflux from macrophages is 2.2-fold higher for the 192R than for the 192Q isozyme (Fig. 6).

The conclusions drawn from the in vitro, reconstituted system of PON1 and HDL proved strikingly valid in human sera. The three genotypes of human sera (QQ, RQ, and RR) clearly exhibit different stabilities of the expected order. Inactivation of RR sera proceeds with a single slow-rate kinetics, whereas two phases (fast and slow) are observed in QQ and RQ sera. As observed in vitro, the rates of both the fast and slow inactivation phases were similar in all sera types, and the main difference regards the distribution between the two phases. Indeed, the two inactivation phases appear to correspond to two PON1 populations and thereby provide an insightful analytical tool (22): the slow phase corresponds to PON1 tightly bound to HDL-apoA-I, and the fast phase corresponds to "free" PON1 [or PON1 that is loosely bound to possibly other HDL types, or other lipoprotein particles (45, 46)]. The differences in phases of the inactivation assay are entirely consistent with the direct binding measurements by surface plasmon resonance for different types of HDL particles as well as for different PON1 variants (22), including the 192R/Q isozymes (Tables 1, 2). In RR sera, PON1

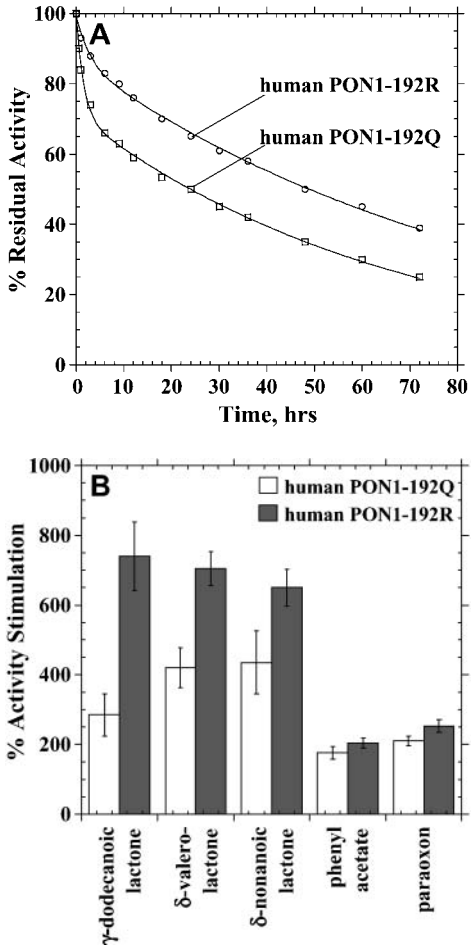

Fig. 4. Inactivation and catalytic stimulation of the human PON1-192R/Q isozymes. A: Inactivation kinetics of human PON1-192R and -192Q in the presence of rHDL-apoA-I. The delipidated isozymes (0.2 μM) were incubated with a 50-fold excess of rHDL-apoA-I in activity buffer and subjected to inactivation by nitrilotriacetic acid (NTA) and β-mercaptoethanol (each at 5 mM) at 25°C. Residual activity was determined as described for Fig. 1, and data were fitted to a double exponential function (see Table 4). B: Stimulation of the enzymatic activity of human PON1-192R and -192Q by rHDL-apoA-I. The delipidated isozymes were incubated with a 50-fold molar excess of rHDL-apoA-I, and enzymatic activity was determined with various substrates (at 1 mM for all substrates). The activity is presented in relation to the initial activity of the delipidated enzymes (percentage stimulation). Each bar represents the mean ± SD of at least two independent measurements.

TABLE 4. Kinetic and equilibrium constants for the inactivation of human PON1-192 isozymes on rHDL-apoA-I

Isozyme	A_1	$k_1^{inactiv}$	A_2	$k_2^{inactiv}$
	%	h^{-1}	%	h^{-1}
Human PON1-192R	13	0.4	87	0.01
Human PON1-192Q	28	0.6	72	0.01

Amplitudes (A) and kinetic rates of inactivation ($k^{inactiv}$) were derived by fitting the data to a double exponential curve. Suffixes 1 and 2 designate the first (fast) and second (slow) phases of the inactivation, respectively. Each value represents the mean of two independent experiments. Standard deviations were <10% of parameter values. Human PON1 isozymes were purified from pooled blood samples (40) and stored in activity buffer with glycerol (20%) and tergitol (0.1%). Before the incubation with HDL, PON1 samples were briefly dialyzed and delipidated to remove the glycerol and tergitol that might interfere with HDL binding.

seems to be tightly associated with HDL-apoA-I, whereas QQ and RQ sera exhibit significant fractions of "free" PON1 (18–46%). Even in the very small sample tested here (54 individuals), large heterogeneity was observed in the stability of QQ sera (Fig. 5, Table 5). This heterogeneity is also related to variations in PON1 concentrations. Indeed, variations on the order of 10–40-fold in PON1 paraoxonase activity were routinely observed (25). We observe similar variations with all activities tested. Moreover, we observe that individuals with higher serum PON1 concentrations, and in particular Q polymorphs, also exhibit higher stability (L. Gaidukov and D. S. Tawfik, unpublished observations). Increased PON1 levels seem to shift the binding equilibrium and increase the levels of tightly-bound PON1, thus increasing PON1's stability. Increased PON1 stability might also result from the possible dimerization of PON1 (47), which in turn might promote a more efficient HDL association.

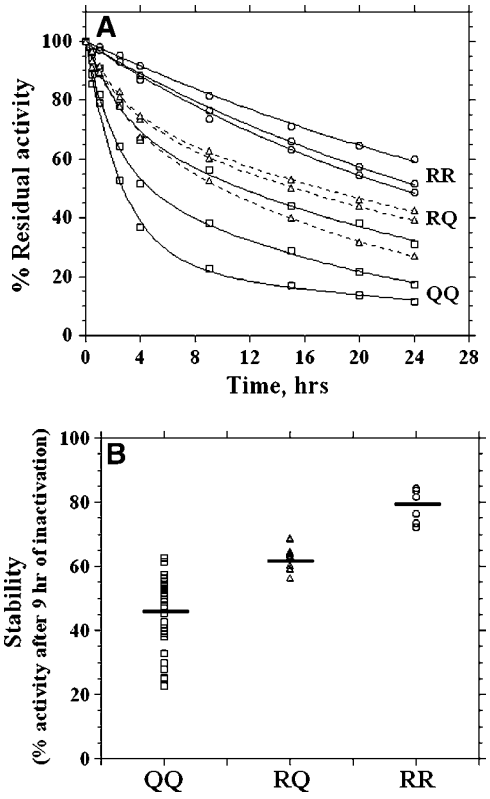

Fig. 5. PON1 inactivation assays with human sera. A: Kinetics of human PON1 inactivation in three representative sera of each genotype. Human sera from healthy individuals were diluted 10-fold in TBS and subjected to inactivation by 0.25 mM NTA and 1 mM β-mercaptoethanol at 25°C. Residual activity was determined by initial rates of phenyl acetate hydrolysis (2 mM) and plotted as a percentage of the rate at time zero. Data were fitted to monoexponentials for RR sera (circles with solid lines) and double-exponentials for RQ sera (triangles with dashed lines) and QQ sera (squares with solid lines), from which the inactivation rate constants and amplitudes were derived (see Table 5). B: PON1 stability, expressed as the percentage of residual activity after 9 h of inactivation, for 54 samples of human sera belonging to the QQ, RQ, and RR genotypes. Horizontal bars represent the mean stability of each group.

TABLE 5. Kinetic and equilibrium constants for PON1 inactivation in human sera of 54 healthy individuals

Sera	A_1	$k_1^{inactiv}$	A_2	$k_2^{inactiv}$	Stability
	% fast phase	h^{-1}	*% slow phase*	h^{-1}	*% activity after 9 h*
RR sera (n = 6)	0	—	100	0.027 ± 0.003	79 ± 5
RQ sera (n = 14)	22 ± 4	0.45 ± 0.14	78 ± 4	0.029 ± 0.005	62 ± 4
QQ sera (n = 34)	33 ± 13	0.44 ± 0.15	67 ± 13	0.043 ± 0.014	46 ± 11

Human sera from 54 healthy individuals were phenotyped and subjected to inactivation by nitrilotriacetic acid (0.25 mM) and β-mercaptoethanol (1 mM) at 25°C. Inactivation kinetics were fitted to a monoexponential function for RR sera and to a double exponential function for RQ and QQ sera. The amplitudes (A), inactivation rate constants ($k^{inactiv}$), and residual activity after 9 h of inactivation were derived from these fits. All values represent means ± SD for each serum type.

The results of in vitro and sera tests presented here clearly indicate the superior HDL binding, stability, lipolactonase, and antiatherogenic activities of the PON1-192R isozyme. Why, then, have numerous epidemiological studies (reviewed in 28–30) provided no clear correlation between PON1 polymorphism and the susceptibility to atherosclerosis? The answer to this discrepancy may lie in the fact that atherosclerosis is a complex disease that depends on multiple factors, including genetic, environmental, dietary, and pharmacological factors (48). Although the 192Q isozyme of human PON1 exhibits inferior HDL binding and antiatherogenic activity, this is not enough to make this polymorph a risk factor for atherosclerotic disease on its own. In addition, most epidemiological studies were entirely genetic, whereas more recent studies concluded that the genotype, as well as enzyme levels and activity, are important variables (49, 50). Therefore, it is reasonable that susceptibility to atherosclerosis is affected not only by PON1's genotype at position 192 but also by other factors related to the PON1 "phenotype" as well as HDL status. The phenotype incorporates numerous factors that affect PON1 levels and activity, including polymorphisms in the regulatory region (51, 52), environmental and dietary factors (53–55), ethnic differences (56, 57), and HDL status (58–60). Indeed, the application of newly developed human sera tests indicated that the variations in total PON1 levels are so large (~20-fold) that they mask the differences in HDL binding of the R/Q isozymes. Hence, our preliminary results indicate that the mean values of PON1 HDL levels are similar for all three genotypes (QQ, RQ, and RR) (L. Gaidukov and D. S. Tawfik, unpublished observations). This may explain why previous attempts to correlate the 192R/Q genotype with a predisposition for atherosclerosis failed and opens the road for new PON1 phenotyping methodologies that may provide better correlation.

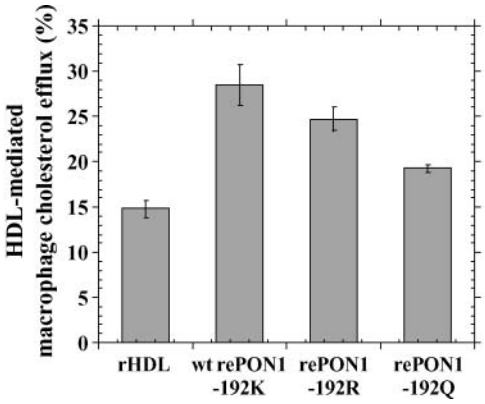

Fig. 6. Macrophage cholesterol efflux rates by HDL-bound rePON1 isozymes. rHDL containing apoA-I was incubated for 3 h at 37°C with activity buffer (rHDL) or with the delipidated PON1 samples: wild-type (wt) rePON1-192K, rePON1-192R, or rePON1-192Q (at a PON1/HDL molar ratio of 1:2.5). Then, the rHDL samples (60 μg apoA-I/ml) were added to J774A.1 macrophages that were prelabeled with [^{3}H]cholesterol (final PON1 concentration, 16 μg/ml). The extent of rHDL-mediated cholesterol efflux was determined after 3 h of incubation at 37°C. Each bar represents the mean ± SD of two independent measurements.

NOTE ADDED IN PROOF

As noted, the fast-inactivating PON1 fraction observed in sera, and dubbed "free" PON1, does not necessarily correspond to PON1 molecules that are not bound to lipoproteins. Under physiological conditions, only a small fraction of PON1 is usually associated with lipoprotein deficient serum (LPDS) fraction. The fast-inactivating fraction is therefore more likely to correspond to PON1 which is bound to different types of HDL particles, and/or other lipoprotein particles. While further investigations are required to elucidate the exact nature of the two PON1 populations, at this stage the appropriate terminology might be "loosely" and "tightly" bound PON1, rather than "free" and "bound".

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