methods

The development of human sera tests for HDL-bound serum PON1 and its lipolactonase activity[S]

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Abstract **Serum paraoxonase (PON1) is a lipolactonase that associates with HDL-apolipoprotein A-I (HDL-apoA-I) and thereby plays a role in the prevention of atherosclerosis. Current sera tests make use of promiscuous substrates and provide no indications regarding HDL-PON1 complex formation. We developed new enzymatic tests that detect total PON1 levels, irrespective of HDL status and R/Q polymorphism, as well as the degree of catalytic stimulation and increased stability that follow PON1's tight binding to HDL-apoA-I. The tests are based on measuring total PON1 levels with a fluorogenic phosphotriester, measuring the lipolactonase activity with a chromogenic lactone, and assaying the enzyme's chelator-mediated inactivation rate. The latter two are affected by tight HDL binding and thereby derive the levels of the serum PON1-HDL complex. We demonstrate these new tests with a group of healthy individuals (n = 54) and show that the levels of PON1-HDL vary by a factor of 12. Whereas the traditionally applied paraoxonase and arylesterase tests weakly reflect PON1-HDL levels (R = 0.64), the lipolactonase test provides better correlation (R = 0.80). These new tests indicate the levels and activity of PON1 in a physiologically relevant context as well as the levels and quality of the HDL particles with which the enzyme is associated.—** Gaidukov, L., and D. S. Tawfik. **The development of human sera tests for HDL-bound serum PON1 and its lipolactonase activity. *J. Lipid Res.* 2007.** 48: **1637–1646.**

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Serum paraoxonase (PON1) is a HDL-associated enzyme playing an important role in organophosphate detoxification and the prevention of atherosclerosis (1, 2). HDL-bound PON1 inhibits LDL oxidation (3–5) and stimulates cholesterol efflux from macrophages (6, 7). PON1 knockout mice are highly susceptible to atherosclerosis (8, 9). Accordingly, serum PON1 levels seem to be inversely related to the level of cardiovascular disease, although this correlation is weak (10–12). PON1 hydrolyzes a broad range of substrates and has been traditionally described as paraoxonase/arylesterase (2). However, it recently became apparent that PON1 is in fact a lactonase with lipophilic lactones constituting its primary substrates (13, 14). Impairing the lactonase activity of PON1, through mutations of its catalytic dyad (15), diminishes PON1's ability to prevent LDL oxidation and stimulate macrophage cholesterol efflux, indicating that the antiatherogenic functions of PON1 are likely to be mediated by its lipolactonase activity (16).

PON1 is synthesized in the liver and secreted into the blood, where it associates with HDL complexes carrying apolipoprotein A-I (apoA-I) (17). The structural model of PON1 indicated that the HDL surface lies in close proximity to PON1's active site, thus providing an optimal environment for the enzyme's interaction with its lipophilic substrates (18). Indeed, it has been shown that PON1 binds HDL-apoA-I particles with nanomolar affinity (19). HDL-apoA-I binding stabilizes the enzyme and selectively stimulates its lipolactonase activity (19).

The impact of PON1 on atherosclerotic disease and resistance to organophosphate toxicity led to intensive investigations of its natural polymorphisms. These include 192R/Q, which alters PON1's substrate specificity toward organophosphates (20–22), M55L (23, 24), and polymorphisms in the promoter region that affect PON1's expression levels (25, 26). We recently showed that the 192R/Q polymorphs differ in their HDL binding properties, with the R isozyme exhibiting higher affinity, stability, lipolactonase activity, and macrophage cholesterol efflux (27). However, several studies concluded that PON1's phenotype, namely the total enzyme levels and activity, are better predictors of the risk of atherosclerotic disease than its genotype (28, 29). However, to date, blood tests measure phosphotriesterase and arylesterase activities to examine PON1's levels and activity (30–34). Because these promis-

Abbreviations: apoA-I, apolipoprotein A-I; DEPCyMC, 7-*O*-diethyl phosphoryl 3-cyano 4-methyl 7-hydroxycoumarin; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; PON1, serum paraoxonase; TBBL, 5-thiobutyl butyrolactone.

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cuous activities of PON1 are hardly stimulated by HDL (19) and obviously bear no physiological relevance, these tests cannot predict the levels of the PON1-HDL complex or its antiatherogenic potential. In addition, these activities are affected by the 192R/Q polymorphism and thus can only provide a measure of PON1's total levels within the same genotype (35). Although the paraoxonase activity differs significantly between the R/Q polymorphs (20–22), the arylesterase activity of the R polymorph undergoes a 2-fold higher level of catalytic stimulation by HDL (27). Therefore, there is a need for the development of new sera tests that provide a facile measure of total PON1 levels irrespective of its genotype and HDL status (for ELISA tests of total PON1 protein, see Ref. 35) and also examine PON1 in light of it being a lipolactonase. Moreover, PON1 is primarily endowed with efficient lipolactonase and antiatherogenic activity when associated with HDL-apoA-I (16, 19). In diabetic patients, PON1 is dissociated from HDL to the lipoprotein-free serum fraction, where it is less biologically active (36), and in vitro studies showed a good correlation between the degree of HDL binding and the stimulation of cholesterol efflux by PON1 (27). Therefore, we aimed at developing sera assays that would also address the levels of the HDL-PON1 complex.

The new sera tests presented here are based on a chromogenic lactone substrate that resembles PON1's favorable lipolactone substrates (37) and was applied in high-throughput assays. A new chromogenic phosphotriester substrate was applied to measure total PON1 levels. In addition, we applied a previously described assay that differentiates between two PON1 forms that are either "tightly" or "loosely" bound to HDL (19, 27). We describe the combined application of these assays for testing the degree of HDL binding, stability, and lipolactonase activity of PON1 in human sera. Comparison with current assays that measure the levels of paraoxonase and arylesterase activities in sera indicated that the former are poor predictors of the levels of PON1-HDL in sera. In contrast, we show that the lipolactonase activity correlates significantly better with the levels of the PON1-HDL complex and may thus provide a better indication of the levels and quality of the HDL particles onto which the enzyme is anchored.

MATERIALS AND METHODS

Lactonase activity in sera

Human sera were kindly provided by Michael Aviram. The samples were collected from 54 healthy individuals at Rambam Medical Center (Haifa, Israel) with the approval of the institute's Helsinki Committee. Phenotyping sera for the PON1 192R/Q polymorphism was performed by a two-substrate method (33) as described previously (27). Sera were divided into aliquots, supplemented with β-mercaptoethanol (5 mM) to prevent oxidation, and stored frozen at −20°C. All assays were performed on 96-well plates (Nunc) using an automated microplate reader (Bio-Tek; optical length, ~0.5 cm). Lactonase activity was measured in activity buffer (50 mM Tris, pH 8.0, and 1 mM $CaCl_2$) containing 0.25 mM 5-thiobutyl butyrolactone (TBBL) (37) and 0.5 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) by monitoring the absorbance at 412 nm in a final volume of 200 μl (ϵ = 7,000 OD/M). The serum was diluted 400-fold in 100 μl of activity buffer complemented with 1 mM DTNB. DTNB was used from 100 mM stock in DMSO. TBBL was used from 250 mM stock in acetonitrile. TBBL was diluted 500-fold in activity buffer containing 2% acetonitrile. The reaction was initiated by adding 100 μl of TBBL (0.5 mM) to 100 μl of sera dilution. The final sera dilution was 800-fold. All of the reaction mixtures contained a final 1% acetonitrile. Rates of spontaneous hydrolysis of TBBL in buffer were subtracted from all measurements. Activities are expressed as U/ml (1 unit = 1 μmol of TBBL hydrolyzed per minute per 1 ml of undiluted serum).

Measurements of PON1 levels in sera

Total PON1 levels in human sera were assessed by measuring the activity with 7-*O*-diethyl phosphoryl 3-cyano 4-methyl 7-hydroxycoumarin (DEPCyMC), synthesized as follows: 3-cyano 4-methyl 7-hydroxycoumarin (604 mg, 3 mmol) was dispersed in dichloromethane (50 ml). Diethyl phosphorochloridate (0.61 ml, 4.2 mmol) was added, followed by triethylamine (0.6 ml, 4.3 mmol), and the mixture was stirred overnight at room temperature. The reaction mixture was washed with HCl at pH ~ 1 (2 × 50 ml), brine (saturated NaCl solution) (1 × 50 ml), and dried over Na_2SO_4. The organic solvent was evaporated, and the product was purified by chromatography on silica (2% methanol in dichloromethane). Recrystallization from dichloromethane ether gave a yellowish solid (410 mg, 40.5% yield). ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm): 7.71–7.74 (d, 1H), 7.31–7.35 (d, 1H), 7.27 (s, 1H), 4.21–4.30 (m, 4H), 2.77 (s, 3H), 1.36–1.42 (m, 6H). ^{31}P NMR (250 MHz, $CDCl_3$) δ (ppm): 6.00 (s). Electrospray ionization mass spectrometry: *m/z* 336 $[M-1]^-$.

For the enzymatic measurements, DEPCyMC was used from 100 mM stock in DMSO, and all of the reaction mixtures contained a final 1% DMSO. The activity was measured with 10 μl of serum and 1 mM substrate in 50 mM bis-trispropane, pH 9.0, with 1 mM $CaCl_2$, by monitoring the absorbance at 400 nm in a final volume of 200 μl (ϵ = 22,240 OD/M). Activities are expressed as mU/ml (1 mU = 1 nmol of DEPCyMC hydrolyzed per minute per 1 ml of undiluted serum). The normalized lactonase activity was calculated by dividing the TBBLase activity of each sample by its DEPCyMC activity.

The DEPCyMC activity of human PON1 192R and Q polymorphs

Purified human PON1 R and Q polymorphs were kindly provided by Michael Aviram. DEPCyMC hydrolysis at 1 mM was measured in the pH range of 7–10 in 50 mM buffers (Tris, bis-trispropane, and CAPS) containing 1 mM $CaCl_2$. Protein concentrations were verified by activity measurements with phenyl acetate using the reported specific activities of the R and Q polymorphs (38). The extinction coefficients of the 3-cyano 4-methyl 7-hydroxycoumarin product at different pH levels were determined spectrophotometrically. Specific activities are expressed in units (1 unit = 1 μmol of DEPCyMC hydrolyzed per minute per 1 mg of protein).

Inhibition of TBBL and DEPCyMC activity in sera

Inhibition of TBBL and DEPCyMC activity in sera samples was measured with EDTA (5 mM) and 2-hydroxyquinoline (0.1 mM), as described (37).

Paraoxonase and arylesterase activity in sera

Paraoxonase activity in sera samples was measured in activity buffer with 1 mM paraoxon by monitoring the absorbance

at 405 nm in a final volume of 200 μl (ϵ = 10,515 OD/M). Arylesterase activity was measured in activity buffer with 1 mM phenyl acetate by monitoring the absorbance at 270 nm in a final volume of 200 μl (ϵ = 700 OD/M). Activities are expressed as mU/ml for paraoxon and U/ml for phenyl acetate (1 nmol of paraoxon or 1 μmol of phenyl acetate hydrolyzed per minute per 1 ml of undiluted serum).

Sera inactivation assays

Sera inactivation assays were performed as described (27). Briefly, sera were diluted 10-fold in TBS (10 mM Tris, pH 8.0, and 150 mM NaCl), and inactivation was initiated by adding an equal volume of inactivation buffer (TBS supplemented with 0.5 mM nitrilotriacetic acid and 2 mM β-mercaptoethanol) at 25°C. Residual activity at various time points was determined with 2 mM phenyl acetate in activity buffer. Inactivation rates were fitted well to a monoexponential or a double exponential fit. It should be noted that the reproducibility of these inactivation assays was low and that 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 12–24 h before the experiment, yielded more reproducible results.

Estimation of PON1-HDL levels

PON1-HDL levels in sera were derived from the inactivation assay and the measurements of total PON1 concentrations with DEPCyMC. For each serum sample, the amplitude of the slow phase of inactivation, A_2 [which corresponds to the fraction of PON1 tightly bound to HDL (19, 27)], was multiplied by the units of DEPCyMC activity (which corresponds to the total concentration of PON1).

Stimulation of TBBL activity by reconstituted HDL

Recombinant PON1 polymorphs were incubated with a range of reconstituted HDL-apoA-I concentrations, as described previously (27). TBBLase activity was determined in activity buffer with 0.25 mM TBBL and 0.5 mM DTNB.

RESULTS

Lipolactonase activity in human sera

Based on the observations that the lactonase is the native activity of serum paraoxonases (13, 14, 39) and mediates the antiatherogenic functions of PON1 (16), we tested the lactonase activity in human sera of 54 healthy individuals of the QQ, RQ, and RR PON1 192 genotypes (see supplementary Fig. I). Although the effect of the 192R/Q polymorphism on the levels of the PON1-HDL complex in sera appears to be largely masked by other factors (see below), we used the observations made with 192R/Q polymorphs in the reconstituted system (27) to validate the results of the sera tests described below. Lactonase activity was assayed using TBBL (see supplementary Fig. II), which was designed for chromogenic lactonase assays of PON1 (37). This lipophilic lactone resembles the most favorable PON1 lactone substrates in terms of activity (k_{cat}/K_M values) and HDL stimulation (37) and is applicable for testing PON1's lactonase activity in sera. The distribution of the TBBLase activity in 54 human sera samples is shown in **Fig. 1A** and **Table 1**. Overall, the TBBLase activity varied 7-fold in this sample. The mean lactonase activity in RR sera was found to be 1.5-fold higher than in QQ sera (5.4 U/ml, on average, vs. 3.5 U/ml; Fig. 1A, Table 1), in agreement with the observation that PON1 R lactonase activity is better stimulated by HDL than PON1 Q (27).

Total PON1 levels in human sera

The differences in TBBLase activity are the combined outcome of two factors: differences in the absolute concentrations of PON1, and different levels of stimulation in each serum. To separate these factors, we searched for a substrate that would have exactly the same specific activity with PON1 R and Q polymorphs and would not undergo any stimulation by HDL, thus reflecting the total levels of the PON1 protein in sera, as in the previously described PON1 ELISA (35). Phenyl acetate, which is usually used as a surrogate marker for PON1 concentration, is not a suitable substrate because it undergoes ~2-fold stimulation upon HDL binding (27). Paraoxonase activity, although unaffected by HDL binding, differs between the R and Q polymorphs. We screened a large number of potential substrates and identified a new chromogenic/fluorogenic phosphotriester, DEPCyMC (see supplementary Fig. II), that is ideal for measuring total PON1 concentrations. We found that, like other phosphotriesters, DEPCyMC is not stimulated by HDL binding. The DEPCyMC activity of the two polymorphs is pH-dependent and thus can be tuned (**Fig. 2A**). At pH 7.0, PON1 Q hydrolyzes DEPCyMC with a 2-fold higher activity, but the activity of both polymorphs becomes identical at pH 9.0. Both TBBL and DEPCyMC activities in sera appear to be highly specific to PON1, because both are efficiently inhibited (≥92%) by the calcium chelator EDTA (5 mM) and the selective competitive inhibitor of PON1, 2-hydroxyquinoline (0.1 mM).

Therefore, we surmised that DEPCyMC activity can provide a reliable measure of total PON1 concentration in sera regardless of its polymorphism and HDL status. This was further supported by comparing the measurements of PON1 activities in sera with DEPCyMC (at pH 7 and 9), phenyl acetate, and paraoxon (Fig. 2B; see supplementary Table I). DEPCyMC at pH 9.0 showed the least variable distribution (as revealed by the lowest standard deviation around the mean activity): the 54 sera samples exhibited variations in PON1 activities in the range of 5-fold (Fig. 2B; see supplementary Table I). The mean DEPCyMC activity at pH 9 was also found to be essentially identical for the R and Q genotypes (Fig. 1B, Table 1). As expected, DEPCyMC hydrolysis at pH 7.0 exhibited higher mean activity and much greater variations between the sera samples (in the range of 10-fold) as a result of differences in the specific activities of PON1 Q compared with PON1 R (Fig. 2A). The arylesterase and paraoxonase activities also revealed large variations (6- to 23-fold) between the samples, with large standard deviations around the mean activity and differences between the R and Q genotypes (Fig. 2B; see supplementary Table I). These large variations between individuals result not only from differences in total PON1 levels but also from differences in the R and

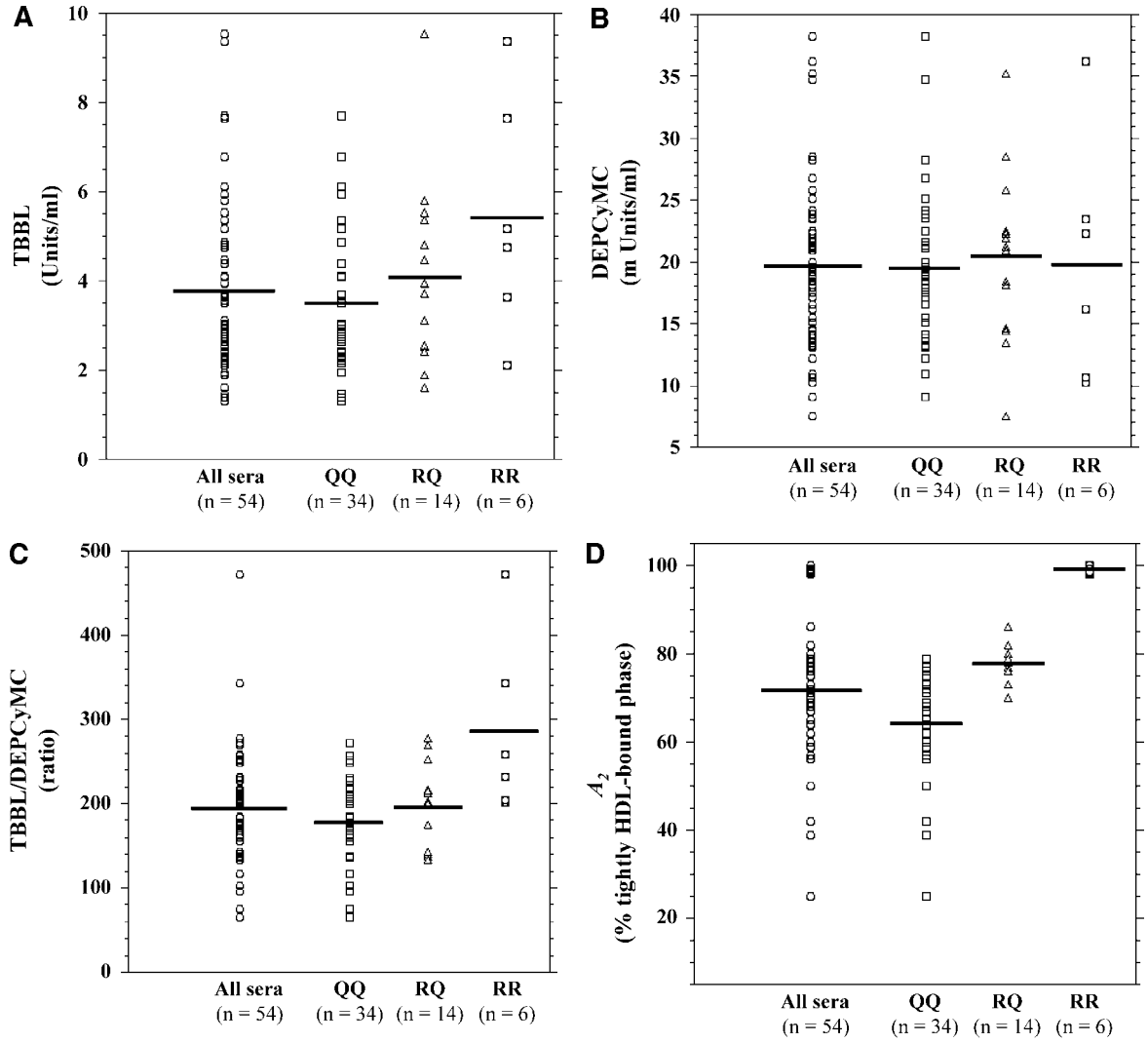

Fig. 1. Lactonase activity, PON1 levels, lactonase stimulation, and fraction of tightly HDL-bound serum paraoxonase (PON1) in human sera from 54 healthy individuals. Horizontal bars represent mean values for each group. A: Levels of lactonase activity, measured with 5-thiobutyl butyrolactone (TBBL; 0.25 mM) and expressed in U/ml (1 μmol of TBBL hydrolyzed per minute per 1 ml of undiluted serum). B: Total PON1 levels, measured with 7-*O*-diethyl phosphoryl 3-cyano 4-methyl 7-hydroxycoumarin (DEPCyMC; 1 mM) and expressed in mU/ml (1 nmol of DEPCyMC hydrolyzed per minute per 1 ml of undiluted serum). C: Stimulation of lactonase activity, expressed as the ratio of TBBL to DEPCyMC activity for each individual serum. D: Amplitude of the slow phase of inactivation (A_2; %) was derived from inactivation assay (see Fig. 3A below) and corresponds to the fraction of tightly HDL-bound PON1.

Q genotypes (DEPCyMC at pH 7 and paraoxon) and from differences in the degree of HDL stimulation (phenyl acetate). In contrast, the 5-fold variations in DEPCyMC at pH 9 reflect differences only in total enzyme concentration, as observed previously in measures of PON1 protein by ELISA (35).

The ratio of TBBL to DEPCyMC activity provides the normalized lactonase activity and therefore corresponds to the degree of HDL stimulation. In the absence of HDL, purified human PON1 polymorphs exhibit the same specific activity for TBBL (1.0 ± 0.1 μmol/min/mg protein at 0.25 mM TBBL); thus, the differences in lactonase activity between individual sera result solely from differences in lactonase stimulation by HDL. In the in vitro system of recombinant PON1 or human PON1 R/Q polymorphs and reconstituted HDL, the lipolactonase activities differed by a factor of ~2-fold in the degree of stimulation by HDL-apoA-I (see supplementary Fig. III) (27). In agreement with these observations, the RR sera exhibited 1.6-fold higher mean normalized lactonase activity than the QQ sera (Fig. 1C, Table 1).

Inactivation assays of PON1 in human sera

We showed previously that determining the rate of PON1's chelator-mediated inactivation provides a measure of the level of tightly versus loosely HDL-bound enzyme both in the reconstituted in vitro system (19) and in sera samples (27). The human sera examined here were previously subjected to inactivation by a low-affinity calcium chelator, nitrilotriacetic acid, which chelates PON1's essential calcium ions, and the reducing agent β-mercaptoethanol (27). The rate of inactivation was monitored by

TABLE 1. Lactonase activity, PON1 levels, lactonase stimulation, and PON1-HDL levels in human sera of 54 healthy individuals

Sera	TBBL[a]	DEPCyMC[b]	TBBL/DEPCyMC[c]	PON1-HDL[d]
	U/ml	*mU/ml*	*ratio*	*arbitrary units*
All sera (n = 54)	3.8 ± 1.9	19.7 ± 6.7	194.3 ± 65.6	14.3 ± 6.2
QQ sera (n = 34)	3.5 ± 1.6	19.5 ± 6.3	177.4 ± 49.9	12.8 ± 5.2
RQ sera (n = 14)	4.1 ± 2.1	20.4 ± 6.9	196.4 ± 48.5	15.8 ± 5.4
RR sera (n = 6)	5.4 ± 2.7	19.8 ± 9.8	285.4 ± 105.5	19.6 ± 9.6

DEPCyMC, 7-*O*-diethyl phosphoryl 3-cyano 4-methyl 7-hydroxycoumarin; PON1, serum paraoxonase; TBBL, 5-thiobutyl butyrolactone. All values represent means ± SD for each 192R/Q genotype.

[a] Lactonase activity was measured with TBBL (0.25 mM) and expressed as μmol of TBBL hydrolyzed per minute per 1 ml of undiluted serum.

[b] PON1 levels were measured with DEPCyMC (1 mM) at pH 9.0 and expressed as nmol of DEPCyMC hydrolyzed per minute per 1 ml of undiluted serum.

[c] Lactonase stimulation was calculated as the ratio of TBBL to DEPCyMC activity for each individual serum.

[d] PON1-HDL levels were obtained by multiplying, for each serum sample, the amplitude of the slow phase of inactivation, which corresponds to the fraction of tightly HDL-bound PON1 (A_2 values; Fig. 1D), by the DEPCyMC activity (Fig. 1B), which corresponds to the total concentration of serum PON1.

measuring the residual arylesterase activity at different time points and comparing it to the initial activity. To illustrate the results of these assays, inactivation profiles of representative sera are depicted in **Fig. 3A**.

PON1's inactivation rates differed markedly between different sera samples. Inactivation kinetics followed either a monoexponential slow rate decay (Fig. 3A, RR sample) or a double exponential regime in which a first (fast) inactivation phase was followed by a second (slow) phase (RQ and QQ samples). As shown previously (19, 27), the stable phase corresponds to PON1 that is tightly, or effectively, bound to HDL, whereas the unstable phase corresponds to the loosely bound PON1 population. Thus, by following PON1's inactivation in sera, the fractions of the tightly and loosely HDL-bound PON1 population can be derived (A_2 and A_1, respectively). As reported elsewhere (27), the monoexponential decay observed primarily with the homozygous RR individuals reflects a favorable partitioning of PON1 in the tightly bound phase (i.e., the percentage of the slow inactivation phase, A_2, is 100%). In other sera, in which inactivation obeys a double exponential regime, A_2 ranges from 25% to 86%. The distribution of the tightly HDL-bound fractions derived from the inactivation assay for the 54 sera samples is shown in Fig. 1D.

Notably, the lactonase stimulation measurements (Fig. 1C) and the inactivation rates (Fig. 1D) appear to cor-

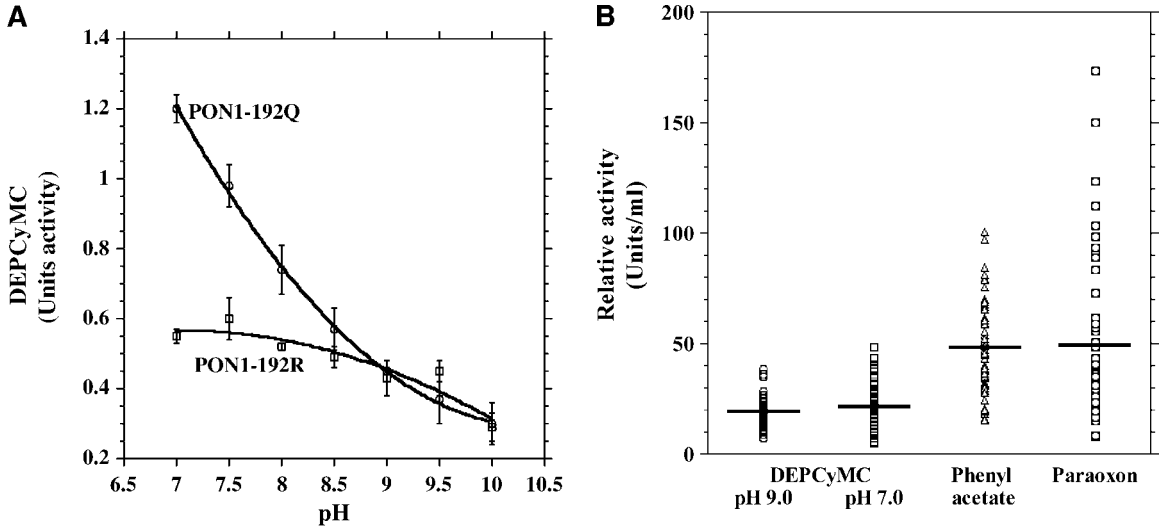

Fig. 2. A: pH rate profile of human PON1 R and Q polymorphs with DEPCyMC. Specific activity of DEPCyMC hydrolysis at 1 mM was measured with the purified human PON1 R and Q polymorphs in the pH range 7–10. Protein concentrations were verified by enzymatic measurements with phenyl acetate using the reported specific activities of the R and Q polymorphs (38). Specific activities were expressed in units (1 unit = 1 μmol of DEPCyMC hydrolyzed per minute per 1 mg of protein). Each value represents the mean of three measurements, and error bars indicate SD values of these measurements. B: Levels of activity with DEPCyMC, phenyl acetate, and paraoxon in human sera from 54 healthy individuals. DEPCyMC activity was measured in 50 mM bis-trispropane at pH 9.0 and 7.0 with 1 mM $CaCl_2$. Phenyl acetate and paraoxonase activities were measured in 50 mM Tris, pH 8.0, with 1 mM $CaCl_2$. All activities were measured with 1 mM substrate and expressed in U/ml for phenyl acetate (1 μmol of phenyl acetate hydrolyzed per minute per 1 ml of undiluted serum) or mU/ml for other activities (1 nmol of substrate hydrolyzed per minute per 1 ml of undiluted serum). Horizontal bars represent mean values for each group.

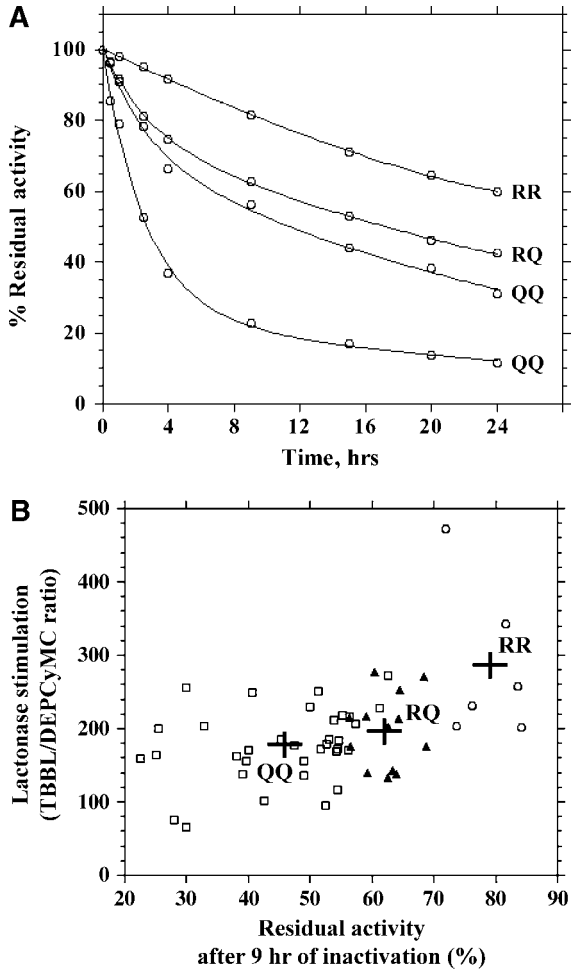

Fig. 3. PON1 inactivation assays in human sera. A: Kinetics of PON1 inactivation in selected human sera. Human sera from healthy individuals were diluted 10-fold in TBS (10 mM Tris, pH 8.0, and 150 mM NaCl) and subjected to inactivation by 0.25 mM nitrilotriacetic acid and 1 mM β-mercaptoethanol at 25°C. 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 either to monoexponential (for RR sera) or to double exponential (for RQ and QQ sera) curves, from which inactivation rate constants and amplitudes of the phases were derived. Note the large variation in the stability observed mainly with QQ sera. This figure was adapted from Ref. 27. B: Correlation between the rate of PON1 inactivation, expressed as the residual activity after 9 h of incubation with a calcium chelator, and lactonase stimulation, expressed as the ratio of TBBL to DEPCyMC activity, for 54 samples of human sera belonging to the QQ (open squares), RQ (closed triangles), and RR (open circles) genotypes. The boldface crosses correspond to mean values of residual activity and lactonase stimulation for each genotype.

relate and cluster in accordance with the 192R/Q genotypes (Fig. 3B).

Estimation of PON1-HDL levels

The levels of the PON1-HDL complex can be derived by combining the inactivation measurements (Fig. 1D) with the measurements of PON1's total concentration using DEPCyMC (Fig. 1B). The amplitude of the second inactivation phase, A_2, corresponds to the fraction of tightly HDL-bound PON1. Multiplying A_2 by the total PON1 concentration (derived from the DEPCyMC rates) yields the level of PON1 that is tightly, or efficiently, associated with HDL, namely the level of the PON1-HDL complex. The derived HDL-associated PON1 levels in 54 human sera are shown in **Fig. 4** and Table 1. These levels vary considerably between individuals (between 3 and 36 in arbitrary units). The mean HDL-PON1 levels are 1.5-fold higher for the RR sera compared with the QQ sera, mainly as a result of the higher A_2 values. However, there is a large overlap in the estimated PON1-HDL levels between PON1 polymorphs, and the differences between the individuals go well beyond the effect of the 192R/Q genotype. Thus, even in the small sample examined here, there are many QQ individuals with higher estimated PON1-HDL levels than some RR individuals.

DISCUSSION

The newly developed chromogenic lactone TBBL (37) enabled us to establish a throughput assay of PON1's lipolactonase activity in sera. The assay is specific to PON1, requires a small amount of serum (<5 μl), is highly reproducible (no changes in sera activity were detected during a year of storage of the frozen sera samples), and is applicable in a high-throughput manner. The lactonase activity varied by ~7-fold in a sample (n = 54) of healthy individuals (Fig. 1A). To reveal whether these variations result from the differences in enzyme levels, or in the levels of catalytic stimulation by HDL, we searched for a substrate that would not be affected by HDL binding or by the R/Q polymorphism and thus could be applied to determine PON1's total concentration. We identified a new phosphotriester substrate, dubbed DEPCyMC, that meets

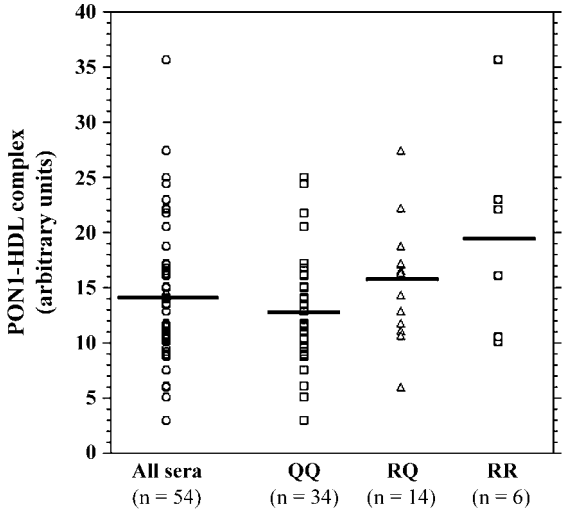

Fig. 4. Estimated levels of the PON1-HDL complex. These levels (in arbitrary units) were obtained from the amplitude of the slow phase of inactivation (A_2), multiplied by the levels of DEPCyMC activity (Table 1). Horizontal bars represent mean values for each group.

these demands. At pH 9.0, PON1-192R/Q polymorphs hydrolyze DEPCyMC at the same rate, with no effects of HDL binding. DEPCyMC activity is also specific to PON1, requires a small amount of serum (10 μl), and can be assayed in a high-throughput manner by both absorbance and fluorescence.

A large variability (10- to 40-fold) in PON1 sera activity has been observed with many substrates (21, 22). However, direct measurements of PON1 protein levels by ELISA showed variations of only 5-fold (35). This discrepancy results from the fact that most activities reflect not only the differences in total PON1 levels but also the differences in the specific activities of various genotypes and/or in the degree of catalytic stimulation by HDL. DEPCyMC activity, on the other hand, solely reflects the enzyme concentrations and can be compared across PON1 genotypes. Thus, similarly to the direct PON1 quantification by ELISA (35), measurements of DEPCyMC activity showed variations of 5-fold in PON1 concentrations in different sera (Fig. 1B).

The ratio of lipolactonase (measured with TBBL) to DEPCyMC activity yields the levels of lactonase stimulation and indicates clear differences between the three types of sera (Fig. 1C, Table 1). The RR sera exhibit, on average, 1.6-fold higher stimulation levels than the QQ sera. This difference is in agreement with the levels of stimulation observed with lipolactones in vitro (see supplementary Fig. III) (27). However, the overall differences in lipolactonase activity (Fig. 1A) and stimulation (Fig. 1C) of PON1's R/Q genotypes seem to be masked by much greater variations in total enzyme concentrations as well as by other factors such as the degree of HDL binding.

The measure of catalytic stimulation (TBBL/DEPCyMC; Fig. 1C) and the fraction of tightly bound PON1 from the inactivation assays (Fig. 1D) appear to correlate (Fig. 3B). Thus, as observed in vitro with recombinant PON1 polymorphs and reconstituted HDL (27), the tightly HDL-associated fraction of PON1 is more stable and exhibits higher lactonase activity. Therefore, this fraction may represent the "biologically active" population of PON1, whereas the loosely bound PON1 is much less stable and largely nonactive. Interestingly, large heterogeneity is observed in the fraction of tightly bound PON1 (Figs. 1D, 3) (27). This heterogeneity is also related to variations in PON1's concentration. In particular, we observed that QQ individuals with higher serum PON1 concentrations also tend to exhibit higher stability (slopes for linear regression are 0.62 for the QQ sera and 0 for the RQ and RR sera; see supplementary Fig. IVA). Higher serum PON1 concentrations also lead to higher lactonase activity (slopes for linear regression are 0.19, 0.26, and 0.21 for the QQ, RQ, and RR sera, respectively; see supplementary Fig. IVB). This observation might be related to the positive correlation between serum PON1 and HDL levels (40, 41). Therefore, increased PON1 levels seem to shift the binding equilibrium and increase the levels of tightly HDL-bound PON1, thus increasing PON1's stability and lactonase activity.

By determining the percentage of PON1 that is tightly bound to HDL and the total PON1 concentration, we were able to assess the levels of HDL-associated PON1 (Fig. 4). Our results show that these levels vary between individuals (3–36 arbitrary units, or ~12-fold; Fig. 4, Table 1) to a much larger degree than total enzyme concentrations (5-fold; Fig. 1B). Although the mean values for the estimated HDL-PON1 complex in human sera are 1.5-fold higher for the RR than the QQ genotype, the effect of the R/Q polymorphism appears to play a minor role and might result from the small sample examined here (n = 54), with only six RR sera samples. The intragenotype variability (4- to 8-fold) is significantly larger than the mean intergenotype differences (1.2- to 1.5-fold); thus, even in the small sample examined here, we could observe many individuals with the inferior QQ genotype that exhibit higher levels of HDL-associated PON1, and subsequently higher lipolactonase activity, than most RR individuals. Numerous case-control studies that tried to relate the PON1 R/Q polymorphism with the risk of cardiovascular disease yielded conflicting results, with some studies indicating the RR genotype as a risk factor (42–44) and others indicating no association between the disease and either allele (45–48). Our tests show that the PON1 R/Q polymorphism plays a relatively minor role in determining the levels of the HDL-PON1 complex and may explain why previous attempts to correlate the 192R/Q phenotype with a predisposition for atherosclerosis failed. Similar conclusions were derived from several previous studies that suggested that PON1's phenotype may be more important than its genotype (28).

Paraoxonase and arylesterase activities have traditionally been used to test PON1 levels and activity and have been suggested as markers for the prediction of cardiovascular disease (11, 29, 49, 50). Indeed, we found that the phosphotriesterase and arylesterase activities of PON1 are positively correlated with total PON1 concentrations [the Pearson correlation coefficients for linear regression (R) for paraoxon are 0.36, 0.78, and 0.94 for QQ, RQ, and RR sera, respectively, and those for phenyl acetate are 0.53, 0.80, and 0.97 for QQ, RQ, and RR sera, respectively; see supplementary Fig. VA, B]. PON1 concentrations, in turn, are positively correlated with PON1-HDL levels and stability (see supplementary Fig. IVA). However, the correlation between the paraoxonase and arylesterase activities and HDL-PON1 levels is quite poor (R = 0.64 and 0.62, respectively) (**Fig. 5A, B**). Indeed, PON1-HDL levels at around the mean paraoxonase and arylesterase activities (50 units) vary by as much as 8-fold for paraoxon (between 3 and 25 arbitrary units) and 5-fold for phenyl acetate (between 5 and 23 arbitrary units). This may explain why previous attempts to correlate PON1 phosphotriesterase and arylesterase activities with the risk of atherosclerosis did not yield significant results (28). Moreover, we now know that these activities are promiscuous, nonphysiological functions of PON1. In contrast, the lactonase activity is the primary function of PON1 (13, 14), is greatly stimulated by HDL (19), and appears to mediate at least two of PON1's antiatherogenic functions (16). Indeed, the TBBLase activity exhibits a significantly better correlation with the levels of PON1-HDL (R = 0.80), with only 2.6-fold variations around the mean activity (between 9 and 23

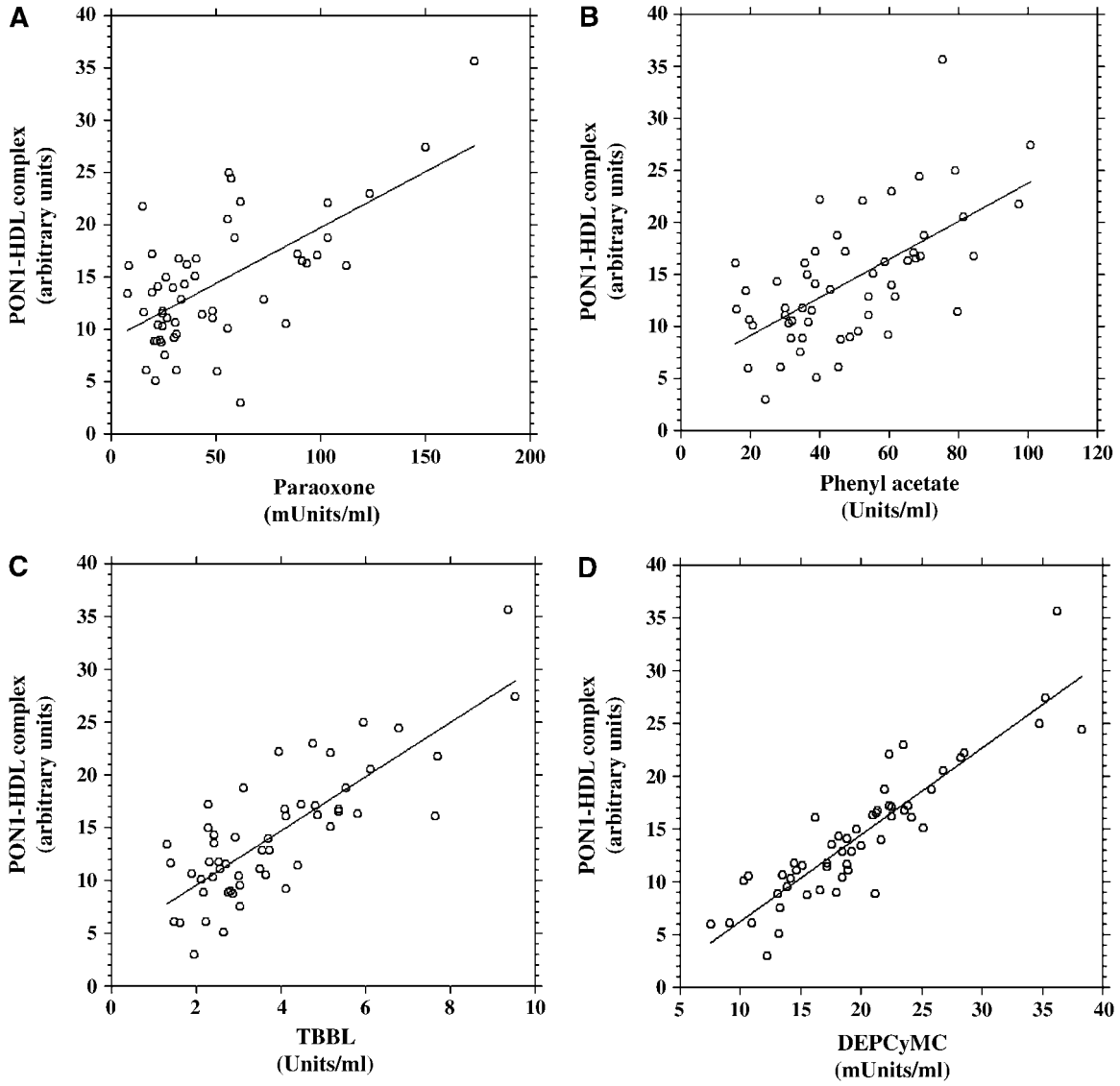

Fig. 5. Correlation between activity levels with paraoxon (A), phenyl acetate (B), TBBL (C), and DEPCyMC (D) and levels of the PON1-HDL complex (in arbitrary units) in human sera from 54 healthy individuals. Levels of the PON1-HDL complex were calculated as described for Fig. 4. The activities were measured either in activity buffer (50 mM Tris, pH 8.0, and 1 mM $CaCl_2$) with 1 mM paraoxon, 1 mM phenyl acetate, and 0.25 mM TBBL or in 50 mM bis-trispropane, pH 9.0, and 1 mM $CaCl_2$ with 1 mM DEPCyMC. The activities are expressed in U/ml for phenyl acetate and TBBL (1 μmol of substrate hydrolyzed per minute per 1 ml of undiluted serum) and mU/ml for paraoxon and DEPCyMC (1 nmol of substrate hydrolyzed per minute per 1 ml of undiluted serum). Pearson correlation coefficients for linear regression (R) are 0.64 for paraoxon (A), 0.62 for phenyl acetate (B), 0.80 for TBBL (C), and 0.90 for DEPCyMC (D).

arbitrary units; Fig. 5C). As expected, DEPCyMC activity is strongly correlated with PON1-HDL levels (R = 0.90; Fig. 5D), because the latter was deduced by multiplying DEPCyMC activity (total PON1 levels) by A_2 (the fraction of tightly bound PON1). Thus, the variation in this plot reflects variation in A_2 only (see also Fig. 1D).

In conclusion, the new sera tests involve the measurements of PON1's absolute levels, lipolactonase activity, and the degree of catalytic stimulation. The minimal test measures the total PON1 protein levels with DEPCyMC and the lipolactonase activity with TBBL. Total TBBLase activity appears to be in reasonable correlation with the levels of HDL-associated PON1 (Fig. 5C), and the normalized TBBLase activity (TBBL/DEPCyMC ratio; Fig. 1C) reflects the efficiency of catalytic stimulation by HDL. A more comprehensive measure may involve the inactivation assay to derive the fraction of tightly bound PON1 (A_2; Fig. 1D). This fraction is a marker of the degree of HDL binding, and in combination with PON1 DEPCyMC activity, it reflects the levels of HDL-associated PON1. Together, these tests provide integrative measures of the activities and levels of PON1 and the HDL particles onto which it is bound. Future studies may reveal whether these new tests of sera PON1, perhaps in conjunction with other assays that address the levels of various types of HDLs, LDLs, apolipoproteins, and other proteins and factors related to

atherosclerosis (51), constitute reliable indicators as well as predictors of atherosclerosis.

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