Determination of Paraoxonase 1 Status Without the Use of Toxic Organophosphate Substrates

Rebecca J. Richter, BS; Gail P. Jarvik, MD, PhD; Clement E. Furlong, PhD

Paraoxonase 1 (PON1) is a member of a tandem 3-gene family localized on human chromosome 7q21-22. High-density lipoprotein-associated PON1 and PON3 are ubiquitously expressed. PON1 was initially characterized and named for its ability to hydrolyze paraoxon, the toxic oxon metabolite of parathion. Although Aldridge proposed in 1953 that serum paraoxonase (POase) and arylesterase (AREase) activities were carried out by the same enzyme, controversy about 1 versus 2 enzymes persisted for many years, resulting in a reclassification of POase/AREase from EC 3.1.1.2 to EC 3.1.8.1 for PON1 as an example of an organophosphorus (OP) hydrolase. The controversy was finally settled when Sorenson et al demonstrated both activities in recombinant PON1. However, the revised nomenclature remains in place. Early studies of plasma PON1 found a large variability of POase activity among different species and in different tissues. Serum POase activity distribution studies in human populations revealed an activity polymorphism of high versus low POase activity. Studies on the polymorphic distribution of PON1 in human populations using a variety of different assays revealed bi or trimodal distributions of plasma POase activity (reviewed in Ref.9).

Our initial characterization of the human PON1 cDNA clones revealed 2 coding region polymorphisms Q192R and L55 M. Subsequently, it was shown that the Q192R polymorphism determined high versus low rates of paraoxon hydrolysis by the enzyme, with the PON1_R192 alloform specifying high activity. After the demonstration that high-density lipoprotein-associated PON1 was implicated in reducing low-density lipoprotein oxidation and high-density lipoprotein oxidation, epidemiological studies were undertaken to explore the possible role of genetic variability of PON1 in cardiovascular disease (reviewed in Ref.19). Several meta-analyses of studies that examined only the association of PON1 genotypes with risk of vascular disease have been published in recent years. The first meta-analysis in 2001 by Mackness et al examined the 19 studies carried out up to that time as part of a study of PON1 status in 417 coronary heart disease subjects and 282 controls. A second meta-analysis examined studies in addition to their own, whereas a third analyzed 43 previous studies. Unfortunately, the majority of the epidemiological studies examined only PON1 genotypes using DNA methodologies and ignored the large individual variability in plasma PON1 activity levels. Fundamental biochemical and physiological principles dictate that rates of detoxication or metabolism depend on the quantity of enzyme present. Thus, it is not surprising that many analyses examining disease or exposure risk using only single-nucleotide polymorphism (SNP) analysis and not enzyme activity levels have been inconclusive. Several of the most experienced investigators in PON1 research have pointed out the inadequacy of examining PON1 genotype alone as a risk factor for disease or exposure. We introduced the term PON1 status to include both plasma PON1 activity levels and PON1/R192 genotypes. The few studies that have examined PON1 status have found that plasma PON1 activity level is indeed a risk factor for vascular disease, whereas there was no association observed with PON1 genotypes.

The importance of plasma PON1 activity level in protecting against OP exposure has been clearly demonstrated in the mouse and genetically modified mouse model systems. Resistance to diazoxon exposure is modulated primarily by PON1 plasma activity level, whereas both PON1 activity level and PON1 genotype are important in modulating exposures to chlorpyrifos oxon, due to substrate-specific differences in catalytic efficiency between the PON1Q192 and the PON1_R192 alloforms.

The most convenient protocol for determining PON1 status—plasma activity levels as well as functional position 192 genotype—makes use of a 2-substrate assay, 2-dimensional enzyme activity plot that displays rates of diazoxonase activity versus POase activity under high salt conditions. The high salt conditions are used to separate the PON1Q192R and PON1Q192Q data points from the PON1Q192R data points. Unfortunately, this protocol involves the use of 2 highly toxic OP substrates. We report here a 2-substrate assay/analysis protocol that makes use of non-OP substrates and is convenient for general laboratory use. A third assay that measures rates of phenyl acetate (PA) hydrolysis (AREase activity) at low salt concentration reveals plasma PON1 activity levels for all 3 PON1Q192 genotypes.
Factors are provided to allow the conversion of rates of hydrolysis of one substrate to another for each PON1\textsubscript{192} phenotype.

**Methods**

**Subjects**
The plasma samples used for this study came from an institutional review board-approved project investigating the role of PON1 in vascular disease. Plasma samples were drawn into lithium-heparin tubes, and the cells were separated from the plasma by centrifugation for 15 minutes at 1800 g.

**4-(Chloromethyl)phenyl Acetate Assay**
CMPA [4-(chloromethyl)phenyl acetate] was obtained from Sigma Chemical Co (St Louis, Mo). Rates of CMPA hydrolysis were determined in a SPECTRAmax PLUS Microplate Spectrophotometer (Molecular Devices, Sunnyvale, Calif) using ultraviolet transparent 96-well microplates from Costar (Cambridge, Mass.). Rates of hydrolysis were measured at 280 nm for 4 minutes at 25°C. Only initial linear rates were used for calculations, and results were normalized using the path-length correction software supplied by the system manufacturer. Replicates that varied by >10% were repeated. AREase assays used plasma dilutions (in dilution buffer) of 1:40 for assays run at high salt concentration and 1:80 for assays run at low salt concentration. The assay used 20 μL of diluted plasma per well to which 200 μL of 3.26 mmol/L PA substrate was added in either high salt assay buffer, or no salt assay buffer. High salt PA buffer contained 2 mol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 1.0 mmol/L CaCl\textsubscript{2}, and low salt assay buffer contained 20 mmol/L Tris-HCl (pH 8.0), 1.0 mmol/L CaCl\textsubscript{2}. Activities were expressed in Units/mL, based on the molar extinction coefficient of 1.31 mmol/L \textminus 1 cm\textminus 1 for phenol.

**Paraoxonase and Diazoxonase Assays**
Plasma PON1 activities toward paraoxon (PO) and diazoxon (DZO) and were determined as described previously.\textsuperscript{24,38} Paraoxon and diazoxon were obtained from Chem Service (West Chester, Pa.). Rates of paraoxon and diazoxon hydrolysis were determined in the SPECTRAMax PLUS Microplate Spectrophotometer using either ultraviolet transparent 96-well microplates from Costar for UV diazoxonase readings (270 nm) or standard flat bottom 96-well microplates from Greiner One (Monroe, N.C.) for visible wavelength POase readings (405 nm). All assays were carried out in triplicate using a multi-channel pipette (Matrix, Hudson, N.H.). Outlier samples were reassayed. Rates of hydrolysis were measured for 4 minutes, with only initial linear rates used for calculations and results normalized using path-length correction. PO hydrolysis rates (POase) were expressed in Units/liter (U/L), based on the molar extinction coefficient of 18 mmol/L \textminus 1 cm\textminus 1 for p-nitrophenol. DZO hydrolysis (DZOase) activities were expressed in Units/liter (U/L), based on the molar extinction coefficient of 3 mmol/L \textminus 1 cm\textminus 1 for the diazoxon hydrolysis product, 2-isopropyl-4-methyl-6-hydroxypyrimidine.

**Results**

**Identification and Characterization of Nontoxic Discriminatory Substrates**
The aim of this study was to develop assays for the determination of PON1 status (plasma PON1 activity levels and functional position 192 genotype) that do not use the highly toxic organophosphate substrates paraoxon and diazoxon. More than 70 compounds with many assay conditions (vari-
ation of salt concentration and pH) were examined in attempts to find 2 substrates and assay conditions that would provide the same resolution of the PON1<sub>192</sub> phenotypes as the toxic DZO/PO substrate pair.<sup>24</sup> Of the many substrates and conditions tried, hydrolysis of PA at 2 mol/L salt and CMPA at low salt provided the best resolution of functional PON1<sub>192</sub> phenotypes. The primary requirement for useful substrates is that the substrate and assay conditions reveal different rates of hydrolysis between the PON1<sub>Q192</sub> and the PON1<sub>R192</sub> alloforms.<sup>24</sup>

Figure 1 shows the structures of the 4 substrates used to determine PON1 status. The first step in the design of a spectrophotometric assay for substrate hydrolysis is to examine the spectra of the unhydrolyzed ester and the released alcohol. Aromatic alcohols in general provide useful spectral shifts on hydrolysis. Figure 2 shows the spectra of CMPA and 4-(chloromethyl)phenol. The wavelength of 280 nm was chosen for continuous monitoring of the hydrolysis of CMPA.

In designing assays that best discriminate between the 2 PON1<sub>192</sub> alloforms, it was necessary to find conditions where the kinetic properties of the 2 alloforms differed sufficiently to separate the 3 PON1<sub>192</sub> phenotypes or “functional genotypes” (QQ, QR, and RR). Previous experience with PON1 assays has shown that either pH and/or salt conditions are most conveniently used to generate optimal assay conditions that will resolve the 3 PON1<sub>192</sub> phenotypes. Our earlier work also showed that it is necessary to measure PON1 activity at or below pH 8.5 to avoid interference from the esterase activity of albumin, which catalyzes a higher rate of OP hydrolysis than PON1 at high pH values in plasma samples with low PON1 activity levels.<sup>9</sup> A pH value of 8.0 proved to be optimal for measuring rates of hydrolysis of both PA and CMPA (data not shown).

The effects of varying NaCl concentration on rates of PA hydrolysis are shown in Figure 3A. As with diazoxon hydrolysis,<sup>24</sup> PON1<sub>R192</sub> was more sensitive to inhibition by NaCl than was PON1<sub>Q192</sub>. Because most of the currently used assays are run at 2 mol/L NaCl, and this level of salt provided a good differentiation of the activity of the 2 PON1<sub>192</sub> alloforms, 2 mol/L NaCl was selected for optimizing the spread of the data points for the “y axis substrate” PA. Because we have previously shown that rates of PA hydrolysis in absence of NaCl may be used to compare levels of plasma PON1 across PON1<sub>192</sub> genotypes, rates of PA hydrolysis were also determined in the absence of salt.

Figure 3B shows the dependence of rates of hydrolysis of CMPA by the 2 PON1<sub>192</sub> alloforms as a function of salt. Interestingly, PON1<sub>R192</sub> had higher rates of CMPA hydrolysis than PON1<sub>Q192</sub> in the absence of salt, indicating that it would be a useful “x axis substrate.” Figure 4A shows the substrate dependence of PA hydrolysis by PON1<sub>Q192</sub> (K<sub>m</sub>=443 μmol/L), and Figure 4B by PON1<sub>R192</sub> (K<sub>m</sub>=279 μmol/L) at pH 8.0 in the presence of 2 mol/L NaCl. Figure 4C shows the substrate dependence of rates of hydrolysis of CMPA for PON1<sub>Q192</sub> (K<sub>m</sub>=341 μmol/L), and Figure 4D by PON1<sub>R192</sub> (K<sub>m</sub>=454 μmol/L) at pH 8.0 in the absence of NaCl. K<sub>m</sub> values were determined from plots of substrate concentration/velocity versus substrate concentration.<sup>39</sup>

**Comparison of the 2 Protocols for Determining PON1 Status**

Figure 5 compares the population distribution of rates of hydrolysis of the substrate pair DZO/PO (Figure 5A) with those of the substrate pair PA/CMPA (Figure 5B) for 183 individuals (PON1 status). Both distributions clearly resolve the 3 functional PON1<sub>192</sub> phenotypes (QQ, QR, and RR). These plots reveal not only the PON1<sub>192</sub> alloform(s) present in an individual’s plasma but also the relative levels of each individual’s plasma PON1 activity within each PON1<sub>192</sub> phenotype, data that is for most considerations much more relevant for estimating risk than the PON1 SNP data. All PON1<sub>192</sub> genotypes were correctly inferred by both 2-substrate analyses as verified by polymerase chain reaction assays.<sup>12</sup> We have reported previously, mutations discovered by discrepancies observed between the PON1 status determinations and polymerase chain reaction analyses.<sup>27</sup>

**Generation of Assay Conversion Factors**

To facilitate comparison of data obtained with this new protocol for establishing PON1 status with data generated with the DZO/PO substrate pair, plots of rates of hydrolysis of a given substrate versus rates of a second substrate for each PON1<sub>192</sub> phenotype were prepared (data not shown) to obtain
the conversion factors shown in the Table. It was necessary to
determine the conversion factors for each PON1192 phenotype separately because the catalytic efficiency of substrate
hydrolysis differs for each phenotype. Because the rates of
PA hydrolysis at low salt are not affected by PON1192 phenotype (Figure 6), they can be used to compare
PON1 plasma activity levels across genotypes.40 Factors for con-
verting rates of PA hydrolysis at high salt to low salt values
were also determined so that it would not be necessary to run
3 different substrate assays for a study of PON1 status and
plasma PON1 level determination (Table). If a laboratory
environment has high ambient temperatures, it may be
necessary to generate temperature correction factors.

Discussion

The studies on the relationship of genetic variability of PON1 to
risk of disease or exposure now number into the hundreds.
Unfortunately, most of these studies have looked for association
of PON1 SNPs with susceptibility and have ignored the more
important factor, plasma PON1 activity levels.15–19,25,26,28–31 In
our initial characterization of human PON1 cDNA sequences,
we identified 2 coding region SNPs (L55 M and Q192R).10 It
was subsequently shown that it was the Q192R polymorphism
determined the catalytic efficiency of PON1,11,12 with
PON1R192 having approximately 9-times the catalytic efficiency
for hydrolysis of paraoxon compared with PON1Q192.34 The
effects of the Q192R polymorphism are substrate dependent,
with PON1Q192 having higher activity against some of the nerve
agents and PON1R192 having higher activity against PO and
chlorpyrifos oxon.37 Both alloforms hydrolyze DZO34 and PA40
with approximately the same efficiency. Further research from
our laboratory and 2 others examined the effects of 5
PON1 promoter region polymorphisms on plasma PON1 levels.41–44
The promoter region polymorphism that had the largest effect on
PON1 activity levels was the C-108T polymorphism that occurs

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Conversion Factors</th>
<th>r²</th>
</tr>
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<tbody>
<tr>
<td>QQ</td>
<td>AREase₉₅ (U/mL) × 185 = DZ0ase (U/L)</td>
<td>0.81</td>
</tr>
<tr>
<td>QR</td>
<td>AREase₉₅ (U/mL) × 205 = DZ0ase (U/L)</td>
<td>0.90</td>
</tr>
<tr>
<td>RR</td>
<td>AREase₉₅ (U/mL) × 236 = DZ0ase (U/L)</td>
<td>0.88</td>
</tr>
<tr>
<td>QQ</td>
<td>CMPAase (U/mL) × 18.9 = POase (U/L)</td>
<td>0.92</td>
</tr>
<tr>
<td>QR</td>
<td>CMPAase (U/mL) × 36.3 = POase (U/L)</td>
<td>0.90</td>
</tr>
<tr>
<td>RR</td>
<td>CMPAase (U/mL) × 54.3 = POase (U/L)</td>
<td>0.95</td>
</tr>
<tr>
<td>QQ</td>
<td>AREase₉₅ (U/mL) × 1.6 = AREase₉₅ (U/mL)</td>
<td>0.95</td>
</tr>
<tr>
<td>QR</td>
<td>AREase₉₅ (U/mL) × 2.0 = AREase₉₅ (U/mL)</td>
<td>0.66</td>
</tr>
<tr>
<td>RR</td>
<td>AREase₉₅ (U/mL) × 3.5 = AREase₉₅ (U/mL)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

r² indicates correlation coefficient squared; AREase₉₅, arylesterase at high
salt; AREase₉₅, arylesterase at low salt.
in an Sp1 transcription factor binding site. Homozygotes for PON1C_{108} had on average twice the plasma level of PON1 activity compared with PON1T_{108} homozygotes.

There have been a number of reports linking low PON1 activity levels to the L55 M polymorphism with the PON1M_{55} allele being associated with low activity levels. However, most of this effect seems to be related to linkage disequilibrium of PON1M_{55} with the inefficient PON1T_{108} allele. Levieil et al have reported that message levels and stability of the PON1M_{55} allele may also contribute to the lower levels of PON1 activity associated with the PON1M_{55} genotype. AREase levels in a study of 1527 postmenopausal women reported by Roest et al were lower among PON1M_{55} homozygotes when compared with heterozygotes and PON1L_{55} homozygotes across all 3 PON1C_{108}T_{108} genotypes, lending support to an independent effect of the PON1M_{55} allele.

Of the 70 substrates tested, only PA (at high salt) and CMPA (in absence of salt) provided resolution of the 3 PON1 phenotypes comparable with that provided by the paraoxon/diazoxyon substrate pair. Another advantage of this substrate pair is that rates of hydrolysis can be determined at saturating substrate concentration. This was not feasible for diazoxyon, where a nonsaturating concentration of 1 mmol/L was chosen for convenience and substrate solubility.

Conclusions

The protocols described here will allow most laboratories to determine individuals’ PON1 status without the use of toxic substrates. The conversion factors presented here will also determine individuals’ PON1 activity status analysis protocol (functional PON1_{192} genotype and plasma activity levels).

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References

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