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Paraoxonase 1 (PON1) Status and Substrate Hydrolysis[a]

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Abstract

Paraoxonase 1 (PON1) hydrolyzes a number of organophosphorus (OP) compounds including insecticides and nerve agents. The *in vivo* efficacy of PON1 to protect against a specific OP exposure depends on the catalytic efficiency of hydrolysis. The Q192R polymorphism affects the catalytic efficiency of hydrolysis of some substrates and not others. While $PON1_{R192}$ hydrolyzes paraoxon approximately 9-times as efficiently as $PON1_{Q192}$, the efficiency is insufficient to provide *in vivo* protection against paraoxon/parathion exposure. The two $PON1_{192}$ alloforms have nearly equivalent but higher catalytic efficiencies for hydrolyzing diazoxon (DZO) and provide equivalent *in vivo* protection against DZO exposures. On the other hand, $PON1_{R192}$ is significantly more efficient in hydrolyzing chlorpyrifos oxon (CPO) than $PON1_{Q192}$ and provides better protection against CPO exposure. Thus, for some exposures it is only the level of plasma PON1 that is important, whereas for others it is both plasma level and the $PON1_{192}$ alloform(s) present in plasma that are important. In no case is the plasma level of PON1 unimportant, provided that the catalytic efficiency is sufficient to protect against the exposure. Two-substrate enzyme assay/analysis protocols that reveal both PON1 plasma levels and $PON1_{192}$ phenotype (QQ; QR; RR) are designed to optimize the separation of $PON1_{192}$ phenotypes; however, they have not been optimized for evaluating *in vivo* rates of OP detoxication. This study describes the adaptation of a non-OP, two-substrate determination of PON1 status to the conversion of the PON1 status data to physiologically relevant rates of DZO and CPO detoxication. Conversion factors were generated for rates of hydrolysis of different substrates.

Keywords

paraoxonase 1; PON1 polymorphism; chlorpyrifos; chlorpyrifos oxon; diazinon; diazoxon; pharmacogenetics

Introduction

Genetic variability in human plasma paraoxonase 1 (PON1) activity (POase) has been of interest since the first reports of a polymorphic distribution of POase activity in human

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plasma samples (reviewed in Diepgen and Geldmacher v. Mallinckrodt, 1986). Over the years, many different assays have been developed to explore the PON1 activity polymorphism in human populations (reviewed in Diepgen and Geldmacher-von Mallinckrodt, 1986; Ortigoza-Ferado *et al*., 1984). The observed polymorphism of high vs. low POase activity suggested that individuals with high plasma POase activity would be more resistant to paraoxon (PO)/parathion exposures than individuals with low activity. This concept persisted for over four decades. It was not until human PON1 was purified (Gan *et al*., 1991; Furlong *et al*., 1991), the human PON1 cDNA cloned and characterized (Hassett *et al*., 1991), the molecular basis of the high/low activity polymorphism identified (Humbert *et al*, 1993; Adkins *et al*., 1993) and mouse model systems developed, that the toxicological significance of the genetic variability of plasma POase was understood (Li *et al*., 1993, 1995, 2000; Shih *et al*., 1998).

Characterization of the human cDNA sequences revealed two coding region polymorphisms, M55L and Q192R (Hassett *et al*., 1991). Further characterization of the polymorphisms demonstrated that it was the Q192R polymorphism that was responsible for high vs. low POase activity (Humbert *et al*., 1993; Adkins *et al*., 1993). Eckerson *et al*. (1983) developed a two-substrate assay/analysis protocol that plotted rates of PO hydrolysis against rates of phenyl acetate hydrolysis. This analysis showed that it was possible to resolve phenotypes of high vs. low POase activity and at the same time demonstrated that within a given phenotype, there was a wide variability of activity in a given population. Davies *et al*. (1996) made use of this approach in characterizing additional substrates, where they plotted the rates of hydrolysis of a number of different OP compounds and phenyl acetate against rates of PO hydrolysis. The experiments of Davies *et al*. revealed that plots of diazoxonase activity (DZOase) vs. POase for plasma samples from a population provided a complete resolution of the three $PON1_{192}$ phenotypes in the population (QQ, QR and RR). In further refining the two-substrate assay/analysis protocols, we found that the $PON1_{R192}$ alloform was much more sensitive to salt inhibition than the $PON1_{Q192}$ alloform, explaining the clear separation of the PON1 phenotypes (Richter and Furlong, 1999). Unfortunately, a number of investigators have interpreted the lower activity of $PON1_{R192}$ at high salt concentration to indicate that $PON1_{R192}$ homozygotes would be more sensitive to diazinon (DZS)/diazoxon (DZO) exposures than would be $PON1_{Q192}$ homozygotes or $PON1_{192}$ heterozygotes. $PON1_{R192}$ protects as well as $PON1_{Q192}$ against DZO exposure, if not slightly better. Even though $PON1_{R192}$ has a lower V_{max}, it has a higher affinity (lower K_m) for DZO such that the catalytic efficiencies of the two $PON1_{192}$ alloforms are nearly the same (Li *et al*., 2000).

Experiments with *PON1* null (*PON1*$^{-/-}$) mice allowed for a determination of the ability of the two purified human $PON1_{192}$ alloforms to protect against OP exposures when injected into the *PON1*$^{-/-}$ mice (Li *et al*., 2000). The results showed that injected $PON1_{R192}$ protected better than $PON1_{Q192}$ against CPO exposure, and both $PON1_{192}$ alloforms protected nearly equivalently against DZO exposure, whereas, surprisingly, neither protected against PO exposure. The determination of the *in vitro* catalytic efficiencies of each $PON1_{192}$ alloform provided a clear explanation for these observations. The catalytic efficiency of $PON1_{R192}$ for hydrolysis of CPO was better than that of $PON1_{Q192}$, explaining the increased protection afforded by injection of purified human $PON1_{192}$ into the *PON1*$^{-/-}$ mice. The equivalent catalytic efficiencies of the two $PON1_{192}$ alloforms for hydrolysis of DZO were consistent with the equivalent protection provided by injecting each of these alloforms into *PON1*$^{-/-}$ mice prior to DZO exposure. The lack of protection against PO exposure by injection of purified $PON1_{R192}$ into the *PON1*$^{-/-}$ mice was also explained by the catalytic efficiency data. Despite the fact that the catalytic efficiency of $PON1_{R192}$ was approximately 9-times that of $PON1_{Q192}$, it was still too low to protect against *in vivo* exposures, in agreement with earlier observations in rats (Chambers *et al.*, 1994; Pond *et al.*, 1995). Experiments on the OP sensitivity of *PON1* transgenic (*PON1*-tg) mice provided

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additional confirmatory evidence for the importance of both PON1 level and catalytic efficiency in protecting against OP exposure. Cole *et al*. (2005) showed that *PON1*-tg mice expressing human $PON1_{R192}$ in place of mouse PON1 were significantly more resistant to CPO exposure than were PON1-tg mice that expressed $PON1_{Q192}$ in place of mouse PON1.

These experiments by Li *et al*. (2000) and Cole *et al*. (2005) clearly pointed out the importance of catalytic efficiency of hydrolysis in determining whether PON1 could protect against a given OP exposure. In addition to considering the catalytic efficiency of each $PON1_{192}$ alloform, it is very important to also consider the level of plasma PON1. For chlorpyrifos (CPS)/CPO exposures, both the $PON1_{192}$ phenotype as well as PON1 level are important whereas for DZS/DZO exposures, it is primarily the level of PON1 that is important. In no case is the level of plasma PON1 unimportant. For PO exposures (Li *et al*., 2000), and nerve agent exposures (Rochu *et al*., 2007; Yamada *et al*., 2001) the existing data indicate that PON1 is not effective in protecting against the exposure.

Levels of plasma PON1 are influenced by polymorphisms in the promoter region. Data from three different laboratories provided information on the promoter region polymorphisms that contribute to the large variability in PON1 levels observed among individuals with the same $PON1_{192}$ phenotypes (Brophy *et al*., 2001a, 2001b; Leviev and James, 2000; Suehiro *et al*., 2000). The C-108T polymorphism, that occurs in an Sp1 transcription factor binding site (Deakin *et al*., 2003), has a significant effect on PON1 expression with the T-108 allele expressing less PON1 than the C-108 allele.

Some concern has been expressed about the significance of measuring rates of DZO hydrolysis under non-physiological pH and salt concentration (O'Leary *et al*., 2005). The data reported here address this question and at the same time provide factors for converting data generated from PON1 status analyses either with the substrate pair DZO/PO (Davies *et al*., 1996) or a recently reported non-toxic-OP substrate pair 4-(chloromethyl)phenyl acetate/phenyl acetate (Richter *et al*., submitted to *Circulation*: *Cardiovascular Genetics*) to physiologically relevant rates of OP hydrolysis. The later assay was developed to provide laboratories not equipped to work with highly toxic OP substrates with a means of establishing an individual's PON1 status. Determining physiologically relevant rates of OP detoxication for DZO and CPO for individuals is important, since these rates can vary by 65- to 164-fold, respectively, in populations if both infants and adults are considered (Furlong *et al*., 2006).

Interest in PON1 status has expanded well beyond the toxicology community since low PON1 status has been associated with cardiovascular (reviewed in James, 2006) and other diseases (reviewed in Mackness *et al*., 2002; Marsallich *et al*., 2008). PON1 is also involved in the activation (Tougou *et al*., 1998) and inactivation (Biggadike *et al*., 2000) of specific drugs and the inactivation of bacterial quorum-sensing factors (Ozer *et al*., 2005; Stoltz *et al*., 2008).

Methods

Study Population

The plasma samples used for this study came from a University of Washington IRB-approved project investigating the role of PON1 in vascular disease. Informed consent was obtained from all study participants.

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Sampling

Blood samples were obtained by venipuncture and were drawn into lithium-heparin tubes. Cells were separated from plasma by centrifugation for 15 min at 1800 × g and the plasma samples were stored at −80 °C until assayed.

Chemicals

CMPA [4-(Chloromethyl)phenyl acetate], phenyl acetate and other reagent chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Paraoxon, chlorpyrifos oxon and diazoxon were obtained from Chem Service (West Chester, PA). The structures of the compounds used in this study are shown in Figure 1.

Precautions

Paraoxon, chlorpyrifos oxon and diazoxon are highly toxic organophosphate compounds. They are potent inhibitors of acetylcholinesterases and other important esterases and lipases, and may cause life-threatening neurologic symptoms if ingested or if they come in contact with skin. Double gloves (nitrile), a laboratory coat, and a chemical hood are used as personal protection when handling these chemicals. Organophosphate solutions are treated with alkali solution (i.e., sodium hydroxide) and broken down to non-toxic products before disposal.

Enzyme Assays

All enzyme activity assays were carried out in a SPECTRAmax® PLUS Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA) as described previously (Richter and Furlong, 1999). The assay values were corrected for path-length using software provided with the microplate reader. For continuous monitoring of hydrolysis products that absorb in the UV range, ultraviolet transparent 96-well microplates from Costar (Cambridge, MA) were used. For monitoring of hydrolysis products that absorb in the visible range, standard flat bottom 96-well microplates from Greiner One (Monroe, NC) were used. For efficiency of loading the microplate wells, a multi-channel pipette (Matrix, Hudson, NH) was used. All assays were run in triplicate. Only initial linear rates of hydrolysis were used for calculations, with results normalized using the path-length correction software provided by the manufacturer. Hydrolysis of paraoxon was monitored at 405 nm and the activity expressed in units/L (U/L) using the molar extinction coefficient 18 $mM^{-1}cm^{-1}$ for p-nitrophenol. Rates of DZO hydrolysis were monitored at 270 nm in UV transparent microplates and the activity was expressed as U/L based on the molar extinction coefficient of 3 $mM^{-1}cm^{-1}$ for the DZO hydrolysis product, 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP). Arylesterase activity (hydrolysis of phenyl acetate) was monitored at 270 nm. Activities were expressed in Units/ml, based on the molar extinction coefficient of 1.31 $mM^{-1}cm^{-1}$ for phenol. Samples with replicate values that differed by more than 10% were re-assayed. The assay conditions with respect to salt concentration and pH values are noted in the figure legends.

Results

The aim of this study was to provide protocols for converting PON1 status data generated with non-OP substrates to physiologically relevant rates of DZO and CPO hydrolysis for individuals with any of the three $PON1_{192}$ phenotypes (QQ; QR; RR). A second aim was to provide conversion factors that would allow the comparison of data generated with the new PON1 status protocol with data generated in earlier studies with other substrates.

Two protocols for determining PON1 status are shown in Figure 2. Our earlier studies (Richter and Furlong, 1999) demonstrated that a two-substrate assay/activity plot with rates

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of DZO hydrolysis plotted against rates of paraoxon hydrolysis revealed an individual's functional PON1 status—plasma PON1 activity level and $PON1_{192}$ alloform(s) present in their plasma. A comparison of the functional PON1 status protocol with SNP analyses revealed PON1 mutations that were not detectable by SNP analyses alone (Jarvik *et al*., 2003a).

The PON1 two-substrate/activity plot population distribution using the substrate pair DZO/PO is shown in Figure 2A. A recently developed protocol using the non-OP substrate pair phenyl acetate (assayed at high salt) and CMPA is shown in Figure 2B (Richter *et al*., submitted to *Circulation: Cardiovascular Genetics*). Both protocols provided a clear resolution of the three $PON1_{192}$ phenotypes (functional genotypes).

The $PON1_{192Q/R}$ alloforms can hydrolyze substrates with widely differing catalytic efficiencies (Fig. 1; Davies *et al*., 1996;Li *et al*., 2000). To develop protocols for converting rates of hydrolysis of one substrate to another, it was necessary to characterize substrate activity relationships separately for each $PON1_{192}$ phenotype. Since concern has been expressed regarding the usefulness of measuring rates of DZO hydrolysis under non-physiological conditions of salt and pH value (O'Leary *et al*., 2005), we first determined the K_m values for DZO hydrolysis by each of the $PON1_{192}$ alloforms at physiological pH, salt concentration (150 mM NaCl) and temperature (37 °C) (Fig. 3A, B). As we had observed previously when we determined K_m the values for DZO hydrolysis at high salt and pH 8.5, the $PON1_{R192}$ alloform had a higher affinity for DZO (K_m = 0.27 mM; Fig. 3A) than the $PON1_{Q192}$ alloform (K_m = 0.52 mM; Fig 3B). These values were determined using plasma samples phenotyped as homozygous for either $PON1_{R192}$ or $PON1_{Q192}$, to determine the properties of the $PON1_{192}$ alloforms while associated with high density lipoprotein (HDL).

Since PON1 is also important in modulating exposures to CPS/CPO, we also determined the K_m values for CPO hydrolysis (CPOase) by plasma from $PON1_{Q192}$ and $PON1_{R192}$ homozygotes under physiological conditions. As we had observed earlier when we determined K_m values for CPO hydrolysis with the $PON1_{192}$ alloforms purified from human plasma, $PON1_{Q192}$ had a lower affinity for CPO (K_m = 168 μM; Fig. 3C) than did $PON1_{R192}$ (K_m = 109 μM; Fig. 3D).

Many laboratories determine $PON1_{192}$ variability only by DNA analysis (PCR/restriction enzyme assays) and some additionally determine PON1 levels by measuring arylesterase activity [rates of phenyl acetate hydrolysis (AREase)] at ambient temperature. To generate factors for converting rates of hydrolysis of different substrates, rates of phenyl acetate hydrolysis at low salt (traditional AREase assay) and high salt (new PON1 status AREase assay) concentrations were plotted against rates of OP hydrolysis carried out under physiological conditions and saturating substrate for each $PON1_{192}$ phenotype. Figure 4 shows these rate plots for CPO and Figure 5 for DZO. Interestingly, the plot in Figure 4E, which shows the combined rates of phenyl acetate hydrolysis at high salt concentration plotted against rates of CPO hydrolysis, resolved all three $PON1_{192}$ phenotypes into separate groups, due to the differential effects of NaCl on the two $PON1_{192}$ alloforms.

Figure 5 shows the rates of phenyl acetate hydrolysis at low and high salt concentrations plotted against the rates of DZO hydrolysis for all three $PON1_{192}$ phenotypes. A plot of the combined rates for all three $PON1_{192}$ phenotypes with AREase assayed at high salt did not show the resolution of phenotypes observed with CPO (data not shown).

The plots shown in Figures 4 and 5 allowed us to generate factors for converting AREase rates measured at either high or low salt concentrations to physiologically relevant rates of OP hydrolysis. Conversion factors for AREase at both high and low salt concentrations were determined so that data from the non-OP PON1 status assay or data from many earlier

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studies could be converted into physiologically relevant rates of DZO or CPO hydrolysis. These conversion factors are presented in Table 1.

To examine the physiological relevance of our earlier PON1 status analysis, where rates of DZO hydrolysis determined at pH 8.5 and 2 M NaCl were plotted against rates of paraoxon hydrolysis under the same conditions (Richter and Furlong, 1999), we plotted rates of DZO hydrolysis measured at high pH and salt concentration vs. rates of DZO hydrolysis determined under physiological conditions for each of the $PON1_{192}$ phenotypes (Fig. 6). Interestingly, for the $PON1_{Q/Q192}$ and $PON1_{Q/R192}$ individuals, the rates determined under the two conditions were nearly identical and for the $PON1_{R/R192}$ individuals the conversion factor from physiological rates to rates determined at high salt, high pH rates was only 0.84 (Table 1). Since the rates of hydrolysis of DZO under physiological conditions were nearly the same as those determined under conditions of high pH and high salt, we compared both sets of data points on the same PON1 status plot (Fig. 7). These data show that whether the assay is carried out at 2 M NaCl and the pH optimum (8.5) for PON1 hydrolysis of DZO, or under physiological conditions at saturating substrate, the results are nearly identical.

Conversion factors for rates of AREase activity measured at low and high salt concentrations were determined, since there are many earlier studies on rates of phenyl acetate hydrolysis at ambient temperature and low salt concentration (Fig. 8). These conversion factors are summarized in Table 1. Since many earlier studies also have reported rates of PO hydrolysis, rates of AREase activity at high salt, were plotted against rates of POase activity for each $PON1_{192}$ phenotype to obtain conversion factors for these data. Interestingly, the high salt conditions for the AREase assay resulted in an excellent resolution of the three $PON1_{192}$ phenotypes (Fig. 8H), compared with the initial two-dimensional plot reported by Eckerson *et al*. with the low salt AREase/PO assay (1983). If assays are carried out at ambient temperatures that vary significantly from 23 °C, it may be necessary to generate temperature correction factors.

Discussion

Over the years, many different assays have been used to measure plasma PON1 activity, (reviewed in Ortigoza-Ferado *et al.*, 1984; Diepgen and Geldmacher v. Mallinckrodt, 1986). The initial interest in plasma PON1 activity was to provide an estimate of sensitivity to PO exposure prior to the demonstration that plasma PON1 level and phenotype are not significant in modulating PO exposures (Li *et al*., 2000). In more recent years, as more catalytic activities of PON1 have been revealed, genetic variability in PON1 as a risk factor for cardiovascular disease (Jarvik *et al*., 2000; reviewed in James, 2006) and other diseases (reviewed in Mackness *et al*., 2002; Marsillach *et al*., 2008) has generated increased interest. Many studies have examined only *PON1* single nucleotide polymorphisms (SNPs) (Mackness *et al*., 2001; Wheeler *et al*., 2004; Lawlor *et al*., 2006). However, since PON1 levels vary at least 15-fold in the adult population (Davies *et al*., 2006; Roest *et al*., 2007; Furlong *et al*., 2007) and the catalytic efficiencies can vary significantly between the Q192R alloforms, depending on the substrate (Davies *et al*., 1996; Li *et al*., 2000), studies that have examined only PON1 SNPs have not been informative (Jarvik *et al*., 2003b). Haplotype analysis is also not informative, due to the lack of significant linkage disequilibrium across the PON1 locus (Jarvik *et al*., 2003b). 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 (Mackness et al., 2001; Deakin and James, 2004; La Du, 2003)

The PON1 status determinations, on the other hand, provide information on both PON1 levels and the functional $PON1_{192}$ alloform(s) present in plasma. We have identified

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mutations in the PON1 gene that resulted in the inactivation of one or the other $PON1_{192}$ allele, based on discrepancies between PCR analysis and the functional PON1 status analysis (Jarvik et al., 2003a). There are many things that can happen between DNA and the final protein product. The PON1 status analysis examines the end product of all of these contributors by measuring the functional PON1 alloform(s) present in plasma (Richter and Furlong, 1999; Jarvik et al. 2000, 2003a).

It is worth mentioning problems associated with the use of POase measurements as determinants of risk of exposure or disease. The primary problem is the nearly 9-fold difference in catalytic efficiencies of the two $PON1_{192}$ alloforms in hydrolyzing PO (Li *et al*., 2000). Many studies have tried to find correlations between POase activity and risk of disease or exposure lumping subjects of all three $PON1_{192}$ phenotypes together. Useful information could be obtained comparing controls and subjects of the same $PON1_{192}$ phenotype, but the large difference in catalytic efficiency of PO hydrolysis between the two $PON1_{192}$ phenotypes coupled with different gene frequencies in different ethnic groups, make the data impossible to interpret. To date, the only substrate that is useful for comparing hydrolytic activities across $PON1_{192}$ phenotypes is phenyl acetate (AREase) when assayed at low salt concentration (Furlong, 2006). AREase activity will provide a useful surrogate measure of PON1 protein levels across $PON1_{192}$ phenotypes.

In the earlier study by O'Leary *et al*. (2005) where they looked for a correlation between DZOase activities measured at low DZO level (50 μM) vs. DZOase measured at high DZO level (500 μM), they did not find a good correlation between the rates measured under the two conditions. Two factors contributed to the lack of correlation. The most important issue is trying to correlate activities of the two $PON1_{192}$ alloforms with different kinetic properties on the same plot. A second issue is most likely related to the HPLC assay used from which it would be difficult to obtain accurate rate data at low substrate concentrations. The rate data presented here for each $PON1_{192}$ phenotype show excellent correlations for all of the different substrates examined and conditions used for the assays. These experiments also show that it is important to compare rates within each $PON1_{192}$ phenotype since each $PON1_{192}$ alloform has different catalytic properties for many of the substrates hydrolyzed by this protein.

One of the main aims of this study was to provide a means of standardizing PON1 activity measurements and to provide conversion factors that will allow comparison of rates determined by one research group with those obtained by other laboratories. A recent comparison of inter-laboratory variability between our laboratory and Dr. Nina Holland's laboratory at the University of California at Berkeley showed that inter-laboratory variability in PON1 activity measurements was less than 10% (Huen *et al*., submitted to *Clin. Chim. Acta*).

A further aim of this study was to provide physiologically relevant data with non-OP substrates that can be used in the development of PBPK/PD models (Timchalk and Poet, 2008). Appropriate conversion factors were obtained by measuring PON1 hydrolytic activities at saturating substrate with the non-OP substrates and the corresponding rates of relevant OP substrates at saturating substrate concentrations, physiological pH and temperature (Table 1). The data in Figures 4 and 5 show an excellent correlation between rates of hydrolysis for each substrate, when subjects with identical $PON1_{192}$ functional genotypes (phenotypes) are compared. Knowing the rate of substrate hydrolysis at saturating substrate concentration for a given subject allows for the determination of rate of OP hydrolysis at any given substrate concentration, based on the Michaelis-Menton relationship and using the conversion factors summarized in Table 1.

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$$v = \frac{V_{max}[S]}{K_m + [S]}$$

There are several additional points to consider in evaluating PON1 status and OP exposure. It is clear from earlier work that an individual's PON1 status plays a more important role in modulating direct exposure to the oxon component of the exposure (Shih *et al*., 1998; Li *et al*., 2000). Huff *et al*. (1995) reported that CPO inhibits acetylcholinesterase approximately 1,000 times faster than does the parent compound CPS. At this level of difference, it may well be that minor oxon contaminants in the parent compound preparations may account for the observed inhibition reported for the parent compound. It is also important to note that most if not all exposures contain an oxon component (Yuknavage *et al*., 1997; California EPA, 1998). Another important consideration is the developmental dependence of plasma PON1 levels. Newborns have only one-third to one-fourth the plasma levels of PON1 that they will have as adults (Augustinsson and Barr, 1963; Ecobichon and Stephens, 1973; Mueller *et al*., 1983; Furlong *et al*., 2006) and the time of reaching mature levels varies from individual to individual (Cole *et al*., 2003). Taking all of these factors into consideration, it is estimated that the combination of genetic and developmental variability of PON1 status in the population can result in as much as 130- to 160-fold variability in sensitivity to CPO exposure and 65-fold variability in sensitivity to DZO exposure (Furlong *et al*., 2006).

In summary, it is not possible at this time to predict plasma levels of PON1 simply from DNA analysis, even if all nearly 200 PON1 SNPs (Furlong *et al*., 2008) are analyzed. Since it is the functional level of enzyme and catalytic efficiency that determines rates of detoxication of both endogenous metabolites and xenobiotics, it is important to know both the plasma level of PON1 as well as the active PON1 alloform(s) present in plasma when estimating an individual's sensitivity to a specific OP exposure. For some exposures, e.g., DZS/DZO, it is primarily the level of plasma PON1 that is important for modulating the exposure. For other exposures such as CPS/CPO, it is both the plasma level of PON1 as well as $PON1_{192}$ alloform(s) present in plasma that are important in modulating the exposure (Li *et al*., 2000). In no case is PON1 protein level unimportant. The high throughput PON1 status determination reveals the relative plasma PON1 level within each $PON1_{192}$ phenotype and at the same time reveals the alloform(s) present in plasma. For heterozygotes, there may be some bias as to the relative levels of the two $PON1_{192}$ alloforms since expression from each of the alleles is subject to *cis*-regulation (Brophy *et al*., 2001b; Leviev and James, 2000; Suehiro *et al*., 2000). This can be seen from the larger scatter of data points in the two-dimensional analysis where rates of one substrate are plotted against rates of a second substrate (Fig. 1). La Du *et al*. (2001) pointed out the importance of considering the concentration of each PON1 alloform in heterozygotes. The high-throughput two-substrate PON1 status assay/analysis provides the information necessary for establishing an individual's PON1 status, the relative plasma level of PON1 and the $PON1_{192}$ alloform(s) present in their plasma. A determination of actual plasma PON1 level is obtained either by measuring AREase activity at low salt (Furlong, 2006) or using the conversion factors from Table 1 and the data from the non-OP PON1 status determination.

The same approach that we describe here can be used to establish conversion factors for rates of hydrolysis of drugs/drug precursors or physiological metabolites. Such factors will be particularly useful for determining rates of hydrolysis/metabolism of substrates for which there are no convenient spectral assays. Once the calibration factors are established for each $PON1_{192}$ phenotype, the high-throughput, convenient spectral assays can be used to generate the important pharmacokinetic parameters necessary for estimating metabolism of a specific drug or metabolite.

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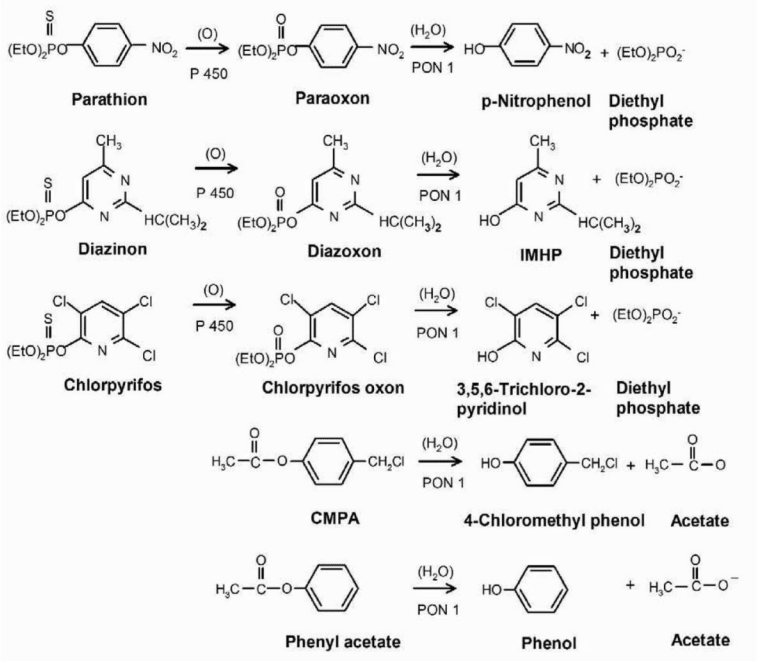

Figure 1.
Structures of the compounds used in this study.

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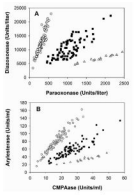

Figure 2.

Comparison of the two protocols for determining PON1 status. (**A**) Assays using the highly toxic OP substrates DZO and PO; and (**B**) assays using the non-OP substrates phenyl acetate and CMPA. The 183 plasma samples included 86 $PON1_{Q192}$ homozygotes, 79 heterozygotes and 18 $PON1_{R192}$ homozygotes with genotypes verified by PCR. From Richter *et al*. Submitted to Circulation: Cardiovascular Genetics.

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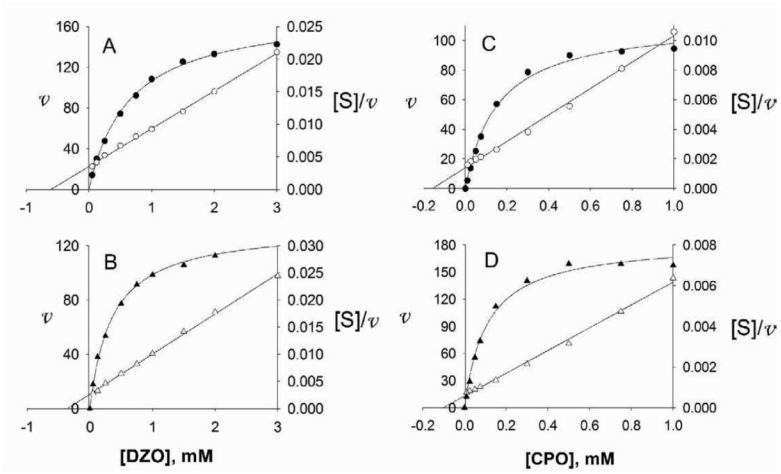

Figure 3.

Substrate dependence of DZO hydrolysis by **A**, a $PON1_{Q192}$ plasma sample (K_m = 0.52 mM); **B**, a $PON1_{R192}$ plasma sample (K_m = 0.27 mM) and for CPO hydrolysis by **C**, a $PON1_{Q192}$ plasma sample (K_m = 168 μM) and **D**, a $PON1_{R192}$ plasma sample (K_m = 109 μM). The plasma samples were matched for rates of AREase activity. The substrate concentrations are indicated in the graphs and only the initial linear rates of hydrolysis were used for kinetic analyses. The closed symbols indicate rates of hydrolysis (υ) at the indicated substrate concentration and the open symbols are the [S]/υ reciprocal values for each of the rate data points (υ). K_m values were determined from the x-axis intercepts of the [S]/υ vs. [S] plots (Dowd and Riggs, 1965).

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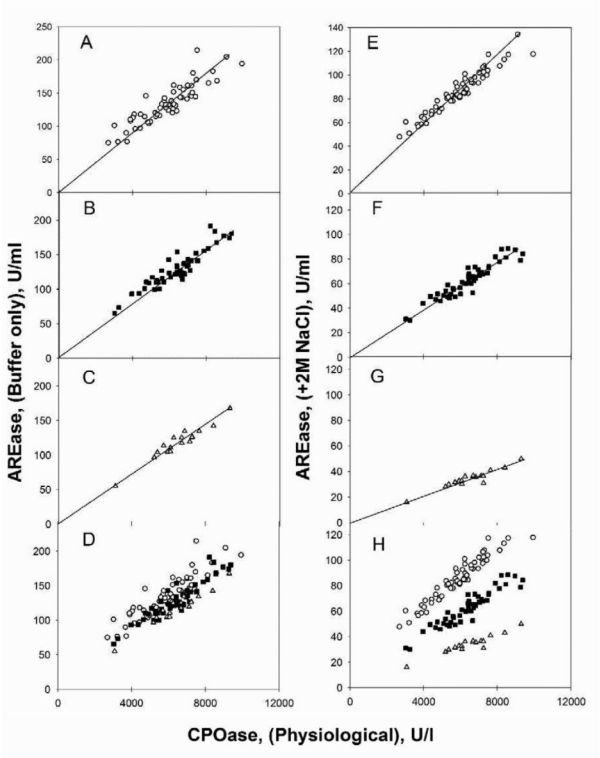

Figure 4.

Comparison of the rates of AREase hydrolysis measured at conditions of low salt vs. CPOase, at physiological conditions (ph 7.4, 150 mM NaCl, 37 °C) for samples of each $PON1_{192}$ phenotype (**A-C**), and AREase hydrolysis measured at conditions of high salt (2M NaCL)/high pH (pH 8.0) vs. CPOase, at physiological conditions (pH 7.4, 150 mM NaCl, 37 °C) for samples of each $PON1_{192}$ phenotype (**E-G**). (○-○) = $PON1_{Q/Q192}$ plasma samples, n = 61; (■-■) = $PON1_{Q/R192}$ plasma samples, n = 49; (Δ – Δ) = $PON1_{R/R192}$. plasma samples, n = 18. Panel D = combined data from **A-C**; Panel H = Combined data from panels **E-G**.

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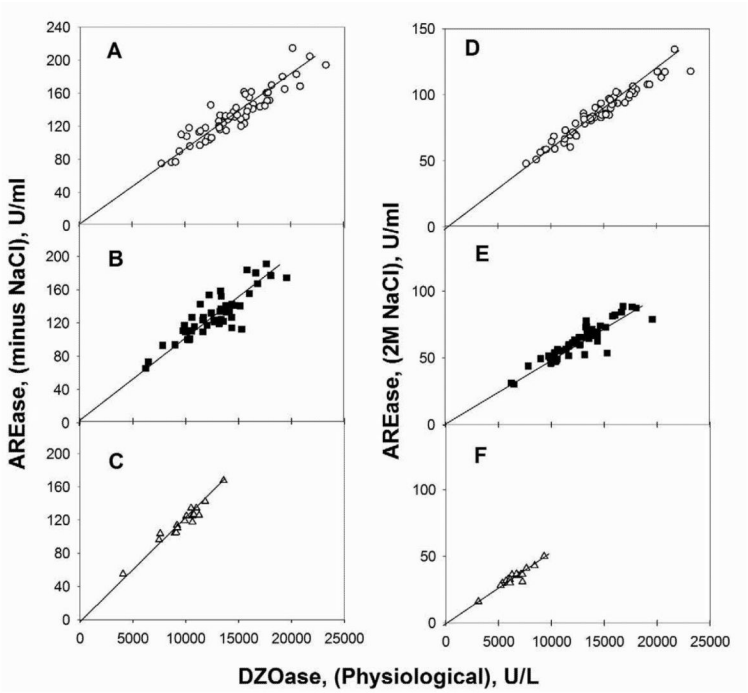

Figure 5.

Comparison of the rates of AREase hydrolysis measured at conditions of low salt vs. DZOase, at physiological conditions (ph 7.4, 150 mM NaCl, 37 °C) for samples of each $PON1_{192}$ phenotype (**A-C**), and AREase hydrolysis measured at conditions of high salt (2 M NaCL)/high pH (pH 8.0) vs. DZOase, at physiological conditions (pH 7.4, 150 mM NaCl, 37 °C) for samples of each $PON1_{192}$ phenotype (**E-G**). (○-○) = $PON1_{Q/Q192}$ plasma samples. n = 61; (■-■) = $PON1_{Q/R192}$ plasma samples, n = 49, (Δ – Δ) = $PON1_{R/R192}$. plasma samples, n = 18

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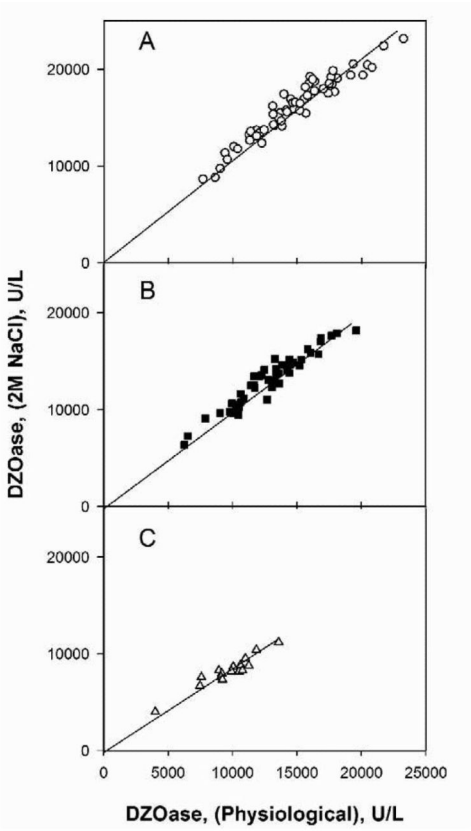

Figure 6.

Comparison of rates of DZO hydrolysis measured at high salt (2M NaCl)/high pH (8.5) vs. rates of DZO hydrolysis measured under physiological conditions (ph 7.4, 150 mM NaCl, 37 °C). (○–○) = $PON1_{Q/Q192}$ plasma samples, n = 61; (■–■) = $PON1_{Q/R192}$ plasma samples, n = 49; (Δ–Δ) = $PON1_{R/R192}$ plasma samples, n = 18.

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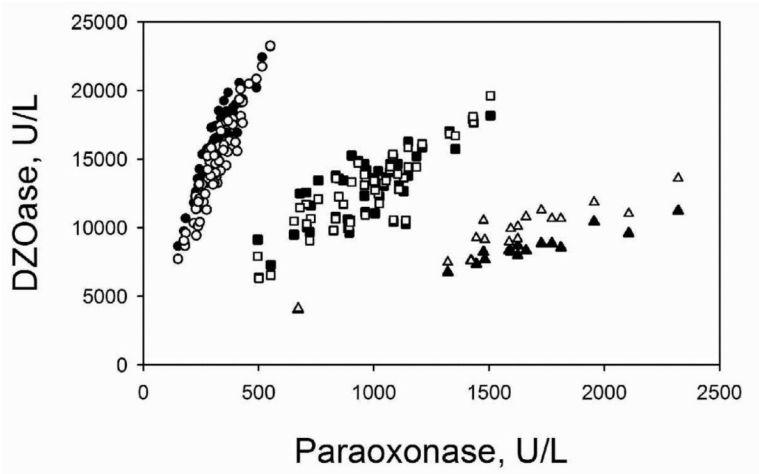

Figure 7.

PON1 status analysis determined with the high pH (8.5), high salt (2 M NaCl) DZOase assay (n = 128) and the "physiological" DZOase assay. Closed symbols = high salt/high pH DZOase data points and open symbols = the "physiological" DZOase data points. POase activity was determined at 2 M NaCl and pH 8.5, since paraoxon is not a physiologically relevant substrate (Li *et al*., 2000).

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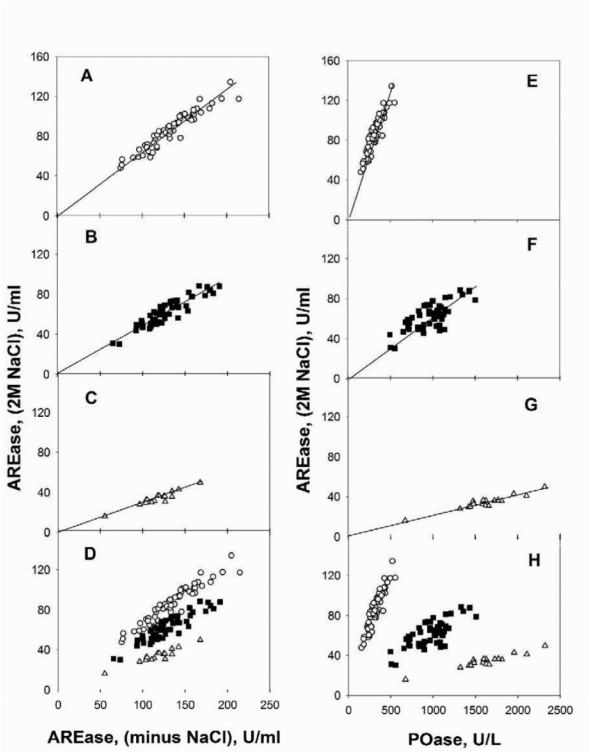

Figure 8.

A-C, Plots of AREase activity at high vs. AREase activity at low salt for the three $PON1_{192}$ phenotypes. **D**, combined data from **A-C**. **E-G**, plots of AREase measured at high salt concentration vs. POase measured at high salt concentration. **H**, combined data from **E-G**. (○–○) = $PON1_{Q/Q192}$ plasma samples, n = 61; (■–■) = $PON1_{Q/R192}$ plasma samples, n = 49; (Δ–Δ) = $PON1_{R/R192}$ plasma samples, n = 18.

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Table 1

Conversion factors for rates of substrate hydrolysis

Phenotype	Conversion Factors	$^{a}r2$
QQ	[b]$AREase_{HS}$ (U/ml) × 172 = $DZOase_{phys}$[c](U/L)	0.93
QR	$AREase_{HS}$ (U/ml) × 204 = $DZOase_{phys}$ (U/L)	0.82
RR	$AREase_{HS}$ (U/ml) × 286 = $DZOase_{phys}$ (U/L)	0.87
QQ	$AREase_{HS}$ (U/ml) × 69 = $CPOase_{phys}$[d](U/L)	0.87
QR	$AREase_{HS}$ (U/ml) × 103 = $CPOase_{phys}$ (U/L)	0.88
RR	$AREase_{HS}$ (U/ml) × 189 = $CPOase_{phys}$ (U/L)	0.89
QQ	[e]$AREase_{LS}$ (U/ml) × 110 = $DZOase_{phys}$ (U/L)	0.84
QR	$AREase_{LS}$ (U/ml) × 100 = $DZOase_{phys}$ (U/L)	0.72
RR	$AREase_{LS}$ (U/ml) × 83 = $DZOase_{phys}$ (U/L)	0.93
QQ	$AREase_{LS}$ (U/ml) × 45 = $CPOase_{phys}$ (U/L)	0.73
QR	$AREase_{LS}$ (U/ml) × 50 = $CPOase_{phys}$ (U/L)	0.84
RR	$AREase_{LS}$ (U/ml) × 55 = $CPOase_{phys}$ (U/L)	0.92
QQ	$AREase_{HS}$ (U/ml) × 3.8 = POase (U/L)	0.75
QR	$AREase_{HS}$ (U/ml) × 15.9 = POase (U/L)	0.50
RR	$AREase_{HS}$ (U/ml) × 47.6 = POase (U/L)	0.90
QQ	[f]$AREase_{HS}$ (U/ml) × 1.6 = $AREase_{LS}$ (U/ml)	0.85
QR	[f]$AREase_{HS}$ (U/ml) × 2.0 = $AREase_{LS}$ (U/ml)	0.66
RR	[f]$AREase_{HS}$ (U/ml) × 3.5 = $AREase_{LS}$ (U/ml)	0.83
QQ	$DZOase_{phys}$ (U/L) × 1.08 = $DZOase_{HS}$[g] (U/L)	0.90
QR	$DZOase_{phys}$ (U/L) × 1.01 = $DZOase_{HS}$ (U/L)	0.91
RR	$DZOase_{phys}$ (U/L) × 0.84 = $DZOase_{HS}$ (U/L)	0.87

[a]Correlation coefficient squared

[b]$AREase_{HS}$ = arylesterase activity measured in buffer and 2M NaCl

[c]$DZOase_{phys}$ = Diazoxonase activity measured under physiological conditions

[d]$CPOase_{phys}$ = Chlorpyrifos oxonase activity measured under physiological conditions

[e]$AREase_{LS}$ = arylesterase activity measured in buffer

[f]From Richter et al. (submitted to *Circulation: Cardiovascular Genetics*)

[g]$DZOase_{HS}$ = Diazoxonase activity measured at 2M NaCl, pH 8.5