The Relationship between PON1 Phenotype and PON1-192 Genotype in Detoxification of Three Oxons by Human Liver

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ABSTRACT:

Phosphorothioate pesticides (OP) such as diazinon, chlorpyrifos, and parathion are activated to highly toxic oxon metabolites by the cytochromes P450 (P450s), mainly in the liver. Simultaneously, the P450s catalyze detoxification of OP to nontoxic dearylated metabolites. The oxon is then detoxified to the dearylated metabolite by PON1, an A-esterase present in the liver and blood serum. The aims of this study were to define the influence of PON1-192 genotype and phenotype on the capacity of human liver microsomes (n = 27) to detoxify the oxons diazoxon, chlorpyrifos-oxon, and paraoxon. Near physiological assay conditions were used to reflect as closely as possible metabolicism in vivo and because the hydrolytic activity of the allelic variants of PON1-192 are differentially affected by a number of conditions. The rates of hydrolysis of diazoxon, chlorpyrifos-oxon, and paraoxon varied 5.7-, 16-, and 56-fold, respectively, regardless of PON1-192 genotype. Individuals with the PON1-192RR genotype preferentially hydrolyzed paraoxon (p < 0.01), and the R allele was associated with higher hydrolytic activity toward chlorpyrifos-oxon, but not diazoxon. There were strongly significant relationships between phenylacetate and paraoxon hydrolysis (p < 0.001) and phenylacetate and chlorpyrifos-oxon hydrolysis (p < 0.001), but not between phenylacetate and diazoxon hydrolysis. These data highlight the importance of PON1 phenotype for efficient hydrolysis of paraoxon and chlorpyrifos-oxon, but environmental and yet unknown genetic factors are more important than PON1-192 genotype in determining capacity to hydrolyze diazoxon.

The majority of organophosphate pesticides (OP) in current use are phosphorothioates (P = S), which are activated by the cytochromes P450 (P450s) to the oxon (P = O), that inhibit acetylcholinesterase in the nervous system and neuromuscular junctions to cause acute toxicity (Gallo and Lawryk, 1991). Occupationally and in the environment, humans are mainly exposed to the parent phosphorothioate pesticide and not the oxon, although variable levels of oxon have been detected in foliar residues following spraying with organophosphates (Yuknavage et al., 1997).

Our previous studies using human liver microsomes have reported that several P450s are involved in the simultaneous activation (to the oxon) and detoxification (to the dearylated metabolite) of OP (Mutch et al., 1999, 2003; Mutch and Williams, 2006). Hepatic metabolism of diazinon, chlorpyrifos, and parathion by the P450 isoforms results in formation of the oxons diazoxon, chlorpyrifos-oxon, and paraoxon, and the dearylated metabolites pyrimidinol (IHMP), 3,5,6-trichloro-2-pyridinol (TCP), and p-nitrophenol (PNP), respectively (Fig. 1). Detoxification of the oxon is mainly by hydrolysis via the A-esterase, PON1 (aryldiacylphosphatase, EC 3.1.8.1), present in the microsomal fraction of the liver and in the blood serum, where it is secreted. B-esterases, such as carboxylesterase (EC 3.1.1.1), remove the oxon by stoichiometric covalent binding (Chambers and Carr, 1993). The relative importance of the P450, PON1, and B-esterase metabolic pathways differs between OP, tissues, and individuals, and these differences will contribute to the differential toxicity of each OP because they greatly influence the level of oxon available to inhibit acetylcholinesterase.

The liver expresses the highest amount of P450s, PON1, and B-esterases; therefore, hepatic metabolism of OP is likely to primarily determine the concentration and profile of the parent OP, oxon, and dearylated metabolites entering the systemic circulation following exposure. The importance of the liver in P450- and esterase-mediated OP metabolism was clearly shown by Sultatos et al. (1984) and Sultatos and Minor (1986), who showed that little parathion or paraoxon and no chlorpyrifos or chlorpyrifos-oxon escaped mouse liver following perfusion in situ.

Human PON1 activity is subject to genetic polymorphism, and several studies have shown that individuals express widely different activities in serum because of their genetic makeup and various environmental factors (Davies et al., 1996; Jarvik et al., 2002; Sentil et al., 2003). Early studies showed that the amino acid substitution at position 192 (Arg->Glu) resulted in two isozymes, Q (Glu) and R (Arg), which differed in their hydrolytic activity toward OP oxons (Humbert et al., 1993). In human serum, the R isozyme hydrolyzed paraoxon more rapidly than the Q isozyme, whereas the Q isozyme hydrolyzed diazoxon, soman, and sarin more rapidly than the R isozyme in vitro (Davies et al., 1996). The R and Q isozymes hydrolyzed chlorpyrifos-oxon and phenylacetate, a “nonpolymorphic” sub-
strate, at about the same rate. Before the structural basis of the PON1-192 polymorphism became known, the three phenotypes were identified simply by observing the ratios obtained by dividing 1 M sodium chloride-stimulated paraoxonase activity by arylesterase activity (using phenylacetate as the substrate) (Eckerson et al., 1983). The ratios were later shown to correspond to the Q, QR, and R genotypes (Adkins et al., 1993).

The studies by Davies et al. (1996) had been carried out using supraphysiological (2 M) sodium chloride concentrations that differentially affected the efficiency of diazoxon hydrolysis by the Q and R isoforms. Later work by the same group showed that in human serum at physiological sodium chloride concentrations, the two isoforms have equivalent hydrolytic efficiency toward diazoxon (Li et al., 2000).

Another coding-region PON1 polymorphism, which results in a leucine (L) to methionine (M) (Leu-11022Met) substitution at position 55, has been described (Blatter Garin et al., 1997). Although O'Leary et al. (2005) showed that serum samples from individuals homozygous for the M variant had impaired hydrolysis of diazoxon, the frequency of the M allele of PON1-55MM is low (Blatter Garin et al., 1997), and only 3 of the 85 individuals investigated had the PON1-55MM genotype.

During the past decade, many researchers have studied the effect of the PON1-192 polymorphism on hydrolysis of OP oxons by using serum (e.g., Cherry et al., 2002), often with conflicting results (e.g., Mackness et al., 2003; O'Leary et al., 2005). Moreover, many studies that have genotyped for certain P450s, as well as PON1 and measured serum PON1 activity, have been unable to show whether genotype and/or phenotype for these enzymes predicted susceptibility to OP toxicity (e.g., Hernandez et al., 2003; Lee et al., 2003).

Our approach to investigate the role of the activation and detoxification metabolic pathways in OP metabolism has been to use a panel of human liver microsomes in which the major P450 isoforms and PON1 have been characterized. Our earlier studies (Mutch et al., 1999, 2003; Mutch and Williams, 2006) showed that P450-mediated metabolism of the OP determines the level and profile of oxon and dearylated metabolite produced. Here we report the relationship between PON1 phenotype and PON1-192 genotype in hydrolysis of diazoxon, chlorpyrifos-oxon, and parathion by the same livers.

Materials and Methods

Materials. Deoxynucleoside-5'-triphosphate was purchased from Promega (Southampton, UK), and Biotaq polymerase was from Bioline (London, UK). The sense and antisense primers were synthesized by VH Bio Ltd. (Gosforth, Newcastle upon Tyne, UK). Mutation detection enhancement gel was purchased from FMC (High Wycombe, Bucks, UK). Diazinon, chlorpyrifos, parathion, diazoxon, chlorpyrifos-oxon, paraaxon, IHMP, and TCP were from Greyhound Chromatography (Birkenhead, Merseyside, UK), and phenylacetate and PNP were supplied by Sigma-Aldrich (Poole, Dorset, UK).

Study Population. Liver samples were from 27 unrelated Caucasian males (n = 13) and females (n = 10), and 4 of unknown sex. Their ages ranged 21 to 66 years (median, 47 years). The recent smoking and alcohol intake histories of the liver donors have been presented previously (Mutch et al., 2003). Thirteen were smokers (or ex-smokers), five were nonsmokers, and smoking status was unknown for nine. The liver samples were obtained from patients in Newcastle and Aberdeen, with local ethical committee approval, and were flash-frozen before storage at −70°C. Samples were obtained following transplant surgery and were shown by histological investigation not to have liver disease.

Enzyme Assays. Human liver microsomes (n = 27) were prepared by
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Differential centrifugation as described previously (Mutch et al., 1999), and the protein content was determined by the method of Smith et al. (1985) using bovine serum albumin as standard.

Microsomes were incubated with saturating concentrations of diazoxon (500 μM), chlorpyrifos-oxon (500 μM), paraoxon (1 mM), and phenylacetate (3 mM) in 50 mM Tris-HCl/1 mM CaCl_2 buffer, pH 7.4. To investigate PON1-mediated hydrolysis of the oxons, nonphysiologically saturating concentrations were used to maximize PON1 hydrolytic activity compared with carboxylesterase- and cholinesterase-mediated covalent binding, which is relatively more important at lower substrate concentrations (Tang and Chambers, 1999; Tinnchall et al., 2002). Physiological conditions of pH (pH 7.4), temperature (37°C), and tissue concentration (approximately equivalent to 0.2 g liver/ml) were used to reflect, as closely as possible, enzyme concentrations in intact liver.

Predetermined conditions of linearity with respect to protein and time were used (data not shown). For incubations with diazoxon (500 μM), chlorpyrifos-oxon (500 μM), or paraoxon (1 mM), approximately 1 mg of protein (diazonon) or 1.5 mg of protein (chlorpyrifos-oxon or paraoxon) was incubated in 0.5 ml of 50 mM Tris-HCl/1 mM CaCl_2 buffer, pH 7.4, for 5, 2.5, or 10 min, respectively, at 37°C. The reaction was started by addition of the oxon contained in 2 μl of methanol and stopped with either 0.5 ml of methanol (diazoxon and chlorpyrifos oxon) or 0.5 ml of phosphoric acid (6%, v/v) (paraoxon). The denatured proteins were separated by centrifugation, and clear supernatant (80 μl) was injected onto the high-performance liquid chromatography system. Control incubations (without microsomes) were carried out in parallel to determine spontaneous hydrolysis of the oxons, which was less than 10% of enzyme-mediated hydrolysis using the conditions described.

The high-performance liquid chromatography system used a mobile phase of acetonitrile/water with gradient elution and detection at 245 nm (diazoxon hydrolysis), 60% methanol/40% water isocratic elution and detection at 230 nm (chlorpyrifos-oxon hydrolysis), or methanol/phosphoric acid (0.7% w/v) gradient elution and detection at 290 nm (paraoxon hydrolysis). Separation of the oxons and dearylated metabolites was on a Luna ODS1 5 μm reverse-phase column (250 × 4.6 mm, Phenomenex, Torrance, CA) with guard column. Determination of IHMP, TCP, or PNP formation from the oxon was made by extrapolation of peak areas from standard curves of authentic metabolites prepared in the presence of denatured microsomes. The limits of detection for IHMP, TCP, and PNP were 80, 10, and 15 pmol, respectively.

The method for hydrolysis of phenylacetate (3 mM) by human serum has been described previously (Mutch et al., 1992). Continuous monitoring of the hydrolysis product, phenol, was made at 272 nm using a Kontron spectrophotometer. Approximately 1 mg of protein was incubated in 3 ml of 50 mM Tris-HCl/1 mM CaCl_2 buffer, pH 8, at 37°C.

Enzyme activities were expressed as nanomole of IHMP, TCP, or PNP formed per minute per milligram protein (diazoxon hydrolysis), nanomole of TCP formed per minute per milligram protein (chlorpyrifos-oxon hydrolysis), nanomole of PNP formed per minute per milligram protein (paraoxon hydrolysis), and micromole of phenol formed per minute per milligram protein (phenylacetate hydrolysis).

Analysis of PON1-192 Genotype. Genomic DNA was extracted from about 500 mg of human liver (n = 27) from Caucasian individuals using the phenol chloroform method of Blin and Stafford (1976). The polymerase chain reaction (PCR) reaction for the PON1-192 genotype involved amplification using approximately 1 μg of DNA from either blood or liver in a total volume of 50 μl in a buffer consisting of 1.5 mM MgCl_2/50 mM KCl with 0.2 mM deoxyadenosine-5'-triphosphate and 1.5 units of Taq polymerase. The sense primer (0.25 μM) 5'-CTGCTT AATTGGAATGTAT-3' and antisense primer (0.25 μM) 5'-ATACCT GCCATCGGGTGAAA-3', which encompassed the codon 192 polymorphic region of the human PON1 gene, were used. The reaction mixture was subjected to 35 cycles comprising denaturing the DNA for 1 min at 95°C, 1.5 min annealing at 46°C, and 1 min extension at 72°C.

Single-stand conformational polymorphism analysis of the PCR product (approximately 200 base pair) was carried out using 1× mutation detection enhancement gels following the protocol described by Daly et al. (1996). Twenty microliters of PCR product, 25 μl of formamide, and 7 μl of STOP solution (0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA, and 95% formamide) were mixed and incubated at 95°C for 5 min before application to the gel. Electrophoresis was performed at 200 V overnight (15–17 h) at 4°C, and bands were visualized following staining with silver nitrate. Assignment of the three genotypes was confirmed by C.E. Furlong (personal communication) by genotyping representative samples using his published method (Hambert et al., 1993).

**Statistics.** Population distributions of diazoxonase, chlorpyrifos-oxonase, paraoxonase, and arylesterase (phenylacetate hydrolase) activity were analyzed by inspection of frequency distribution histograms, and deviation from normality was assessed by the Shapiro-Wilk W test (Shapiro and Wilk, 1965). Statistical comparisons between the three PON1-192 genotypes was determined by the nonparametric Mann-Whitney U test (two-tailed), and correlation analysis was by Spearman (two-tailed). Values of p < 0.05 were considered to be significant.

**Results.**

Diazoxon and chlorpyrifos-oxon were hydrolyzed by human liver microsomes more rapidly (about 55- and 65-fold, respectively) than paraoxon, regardless of PON1-192 genotype (Table 1). The higher hepatic hydrolytic activity toward diazoxon and chlorpyrifos-oxon compared with paraoxon is a major determinant of the higher toxicity of parathion to humans.

The rates of hydrolysis of diazoxon, chlorpyrifos-oxon, and paraoxon varied 5.7-, 16-, and 56-fold, respectively. There were wide variations in hydrolysis rates of all three oxons between individuals (Table 1). The QQ homozygotes had 4-, 13-, and 25-fold ranges in hydrolytic activity toward diazoxon, chlorpyrifos-oxon, and paraoxon, respectively, whereas the RR homozygotes had 5-, 6-, and 3-fold ranges in activity. Phenylacetate was hydrolyzed with nearly a 5-fold range in activity; the QQ, QR, and RR genotypes showed 4-, 3-, and 2-fold variations, respectively (Table 1).

There were strongly significant relationships between phenylacetate hydrolysis and diazoxon hydrolysis (n = 27, r = 0.835, p < 0.001) and phenylacetate hydrolysis and chlorpyrifos-oxon hydrolysis (n = 27, r = 0.834, p < 0.001) (Fig. 2). However, the relationship between phenylacetate hydrolysis and diazoxon hydrolysis was not significant (n = 19, r = 0.430, p = N.S.) (Fig. 2). Moreover, there was a strongly significant correlation between paraoxon hydrolysis and chlorpyrifos-oxon hydrolysis (n = 27, r = 0.735, p < 0.001) but not between diazoxon hydrolysis and paraoxon hydrolysis or chlorpyrifos-oxon hydrolysis (Fig. 3).

The frequency distribution of the PON1-192 genotype was as follows: QQ = 0.48 (13 individuals), QR = 0.30 (8 individuals), and RR = 0.22 (6 individuals) with allele frequencies of Q = 0.63 and R = 0.37. This is comparable with our observed distributions for a larger control population using the same genotyping assay, which showed a frequency of QQ = 0.41 (33 individuals), QR = 0.49 (39 individuals), and RR = 0.10 (8 individuals) with allele frequencies of 0.65 for Q and 0.35 for R (Mutch et al., unpublished observations). The distributions were also consistent with published reports of frequencies in Caucasians using a method based on PCR amplification and polymorphism-specific restriction enzyme digest (Leviev and James, 2000; Brophy et al., 2001).

As expected for hydrolysis of phenylacetate, a substrate that does not discriminate between the PON1-192 variants (Furlong et al., 1993), arylesterase activity (phenylacetate hydrolase) had a normal distribution (not shown), and the three genotypes hydrolyzed phenylacetate at similar rates (Fig. 4). Diazoxon hydrolysis (Fig. 4) and chlorpyrifos-oxon hydrolysis (Fig. 4) were both non-normally distributed (not shown, p < 0.05), and there was no significant difference between the activity of the three genotypes. The activity and genotype of the one outlier with very high ability to hydrolyze diazoxon (243.6 nmol/min/mg protein) were confirmed by repeat analysis (Fig. 4). Paraoxon hydrolysis also showed a non-normal distribution (not shown, p < 0.01), and the frequency plot suggested three populations. The RR homozygotes had significantly higher ability to hydrolyze...
paraoxon compared with the QQ homozygotes ($p < 0.01$), but not the QR variants (Fig. 4).

**Discussion**

This study reports for the first time the detoxification of diazoxon, chlorpyrifos-oxon, and paraoxon by human liver microsomes and shows wide interindividual variations in the hydrolysis of all three oxons. Near physiological assay conditions were used to reflect as closely as possible metabolism in vivo and because the hydrolytic activity of the allelic variants of PON1-192 are differentially affected by a number of conditions. For example, it has been reported that nonphysiological conditions of pH and temperature (discussed by O’Leary et al., 2005) and low tissue concentration (Tang and Chambers, 1999) influence oxon hydrolysis measurements that are relevant in vivo. More importantly, high sodium chloride levels markedly and differentially affect the activity of the PON1-192 variants (Li et al.,

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**TABLE 1**

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—, not determined.

**FIG. 2**. The relationship between phenylacacetate (3 mM) hydrolysis and paraoxon (1 mM) hydrolysis (A), phenylacacetate (3 mM) hydrolysis and chlorpyrifos-oxon (500 mM) hydrolysis (B), and phenylacacetate (3 mM) hydrolysis and diazoxon (500 mM) hydrolysis (C) by 27 (phenylacacetate, chlorpyrifos-oxon, and paraoxon) or 19 (diazoxon) human liver microsomal preparations. $R$ is the correlation coefficient. $***$, $p < 0.001$. 
2000). However, to investigate PON1-mediated hydrolysis of the oxons and to limit carboxylesterase- and cholinesterase-mediated covalent binding that is relatively more important at lower concentrations (Tang and Chambers, 1999; Timchalk et al., 2002), this study used nonphysiological, saturating oxon concentrations.

O’Leary et al. (2005) investigated the relationship between PON1 genotypes and hydrolysis of saturating concentrations of diazoxon using the sera of 47 Caucasian individuals and near-physiological assay conditions. They reported that individuals with the PON1-192RR genotype had significantly higher ability to hydrolyze diazoxon and that an earlier report to the contrary (Mackness et al., 2003) might have been as a result of artifacts of the assay conditions used, including measurement of activities using a nonphysiological (2 M) sodium chloride concentration. Our study found no difference in diazoxon hydrolysis rates between the PON1-192 genotypes, although the small sample size, having six RR homozygotes, made it difficult to define the importance of the one outlier with very high capacity to hydrolyze this oxon. It is possible that other polymorphisms, such as in the PON1-55 coding region or the \[\overline{108C/T}\] regulatory region, or environmental effects, such as consumption of dietary antioxidants (Aviram et al., 2000), contributed to the higher activity seen in the RR outlier.

In genetic analyses of more than 1406 individuals in 40 extended families, D. L. Rainwater (personal communication) observed that the PON1-192 polymorphism accounted for about 60% of the variation in...
paraoxon hydrolysis but only 30% of diazoxon hydrolysis, whereas the PON1-55 polymorphism accounted for about 17% of the variation in paraoxon hydrolysis but only 1% of diazoxon hydrolysis. These observations support the present data of poor correlations between phenylacetate and diazoxon hydrolysis, paraoxon and diazoxon hydrolysis, and chlorpyrifos-oxon and diazoxon hydrolysis and suggest environmental and yet unknown genetic factors are more important than PON1-192 genotype in determining individual capacity to hydrolyze diazoxon.

In our study, the RR homozygotes had a higher median chlorpyrifos-oxon activity, although this did not reach statistical significance. The QQ and QR individuals had intergenotypic ranges in activity that were similar to the intragenotype variation of the 27 individuals, suggesting that function is more important than genotype in determining the rate of hydrolysis of chlorpyrifos-oxon. The significant relationship between hydrolysis of phenylacetate and chlorpyrifos-oxon highlights the importance of function in the efficient hydrolysis of this oxon. Li et al. (2000) has shown that catalytic efficiency for chlorpyrifos-oxon hydrolysis, regardless of PON1-192 genotype, is far greater than for diazoxon or paraoxon, which were degraded about 3- and 55-fold less efficiently. This means that it is likely that low concentrations of chlorpyrifos-oxon will be hydrolyzed effectively by PON1, regardless of genotype. The RR homozygotes had higher hepatic activity toward paraoxon at saturating substrate concentrations, as previously reported for serum (Davies et al., 1996). However, it is unlikely that individuals expressing the RR protein would be less susceptible to paraoxon toxicity than QQ homozygotes because PON1’s overall catalytic efficiency toward this oxon was very poor (Li et al., 2000). It is more likely that carboxylesterases and cholinesterases, which are highly expressed in the liver, would mainly influence removal by covalent binding of the relatively low levels of paraoxon formed following exposure to parathion in vivo (Chambers et al., 1994). In support of this notion, a study by Karanth et al. (2001) highlighted the importance of both PON1 and carboxylesterases in the effective detoxification of chlorpyrifos-oxon in contrast to paraoxon that was degraded by carboxylesterases, but not PON1, at biologically relevant concentrations.

Although the number of livers was relatively small, this study extends current understanding of the importance of PON1 on detoxification of OP oxons, which so far has been based on studies with human sera. This study has clearly shown that the R allele is associated with higher hydrolytic activity toward paraoxon and chlorpyrifos-oxon but not diazoxon. It also complements studies with PON1 knockout mice because they cannot address the complication of variable PON1 expression and activity seen in human populations.

In conclusion, to predict more accurately those individuals at greatest risk of toxicity from phosphorothiates, the individual’s PON1 activity level should be determined in parallel with knowledge of the pesticide’s P450-mediated metabolism. Determination of PON1 genotype is also of importance, particularly, as reported here and by others (e.g., Leviev and James, 2000; Brophy et al., 2001), the allele frequency for PON1-192Q is about 70% in Caucasians.

References

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