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The relationship between PON1 phenotype and PON1-192 genotype in

detoxification of three oxons by human liver

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Running title

Hepatic detoxification of paraoxon, chlorpyrifos-oxon and diazoxon

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List of abbreviations

Paraoxon = O, O-diethyl-O-(p-nitrophenyl)-phosphate

Chlorpyrifos-oxon = O, O-diethyl-O-(3, 5, 6-trichloro-2-pyridinyl)-phosphate

Diazoxon = O, O-diethyl-O-(2-isopropyl-4-methyl-6-pyridimyl)-phosphate

Abstract

Phosphorothioate pesticides (OPs) such as diazinon, chlorpyrifos and parathion are activated to highly toxic oxon metabolites by the cytochromes P450 (CYPs), mainly in the liver. Simultaneously, the P450s catalyse detoxification of OPs to non-toxic 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 metabolism *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-fold, 16-fold and 56-fold, respectively, regardless of PON1-192 genotype. Individuals with the PON1-192RR genotype preferentially hydrolysed paraoxon ($p<0.01$) and the R allele was associated with higher hydrolytic activity towards 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 hydrolyse diazoxon.

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Introduction

The majority of organophosphate pesticides (OPs) in current use are phosphorothioates (P=S) which are activated by the cytochromes P450 (CYPs) 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, man is 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 cytochromes P450 are involved in the simultaneous activation (to the oxon) and detoxification (to the dearylated metabolite) of OPs (Mutch et al, 1999; 2003; 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 (**Figure 1**). Detoxification of the oxon is mainly by hydrolysis via the A-esterase, PON1 (aryldialkylphosphatase, 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 stochiometric covalent binding (Chambers and Carr, 1993). The relative importance of the P450, PON1 and B-esterase metabolic pathways differs between OPs, tissues and individuals, and these differences will contribute to the differential toxicity of each OP since they greatly influence the level of oxon available to inhibit acetylcholinesterase.

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The liver expresses the highest amount of P450s, PON1 and B-esterases and therefore hepatic metabolism of OPs 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 demonstrated by Sultatos et al (1984; 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 make-up and various environmental factors (Davies et al, 1996; Jarvic et al, 2002; Senti 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 towards OP oxons (Humbert *et al*, 1993). In human serum, the R isoform hydrolysed paraoxon more rapidly than the Q isoform, while the Q isoform hydrolysed diazoxon, soman and sarin more rapidly than the R isoform, in vitro (Davies et al, 1996). The R and Q isoforms hydrolysed chlorpyrifos-oxon and phenylacetate, a "non-polymorphic" substrate, 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 1M 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 supra-physiological (2 M) sodium chloride concentrations that differentially affected the

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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 towards diazoxon (Li et al, 2000).

Another coding-region PON1 polymorphism, which results in a leucine (L) to methionine (M) (Leu->Met) 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 PON1-55$_{MM}$ is low (Blatter-Garin et al, 1997) and only 3 out of the 85 individuals investigated had the PON1-55$_{MM}$ genotype.

Over the last decade, many researchers have studied the effect of the PON1-192 polymorphism on hydrolysis of OP oxons by using serum (eg Cherry et al, 2002), often with conflicting results (eg 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 (eg 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 characterised. Our earlier studies (Mutch et al, 1999; 2003; 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

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PON1-192 genotype in hydrolysis of diazoxon, chlorpyrifos-oxon and paraoxon by the same livers.

Materials and methods

Materials

dNTPs were purchased from Promega (Southampton, UK) and Biotaq polymerase from Bioline (London, UK). The sense and antisense primers were synthesised by VH Bio Ltd (Gosforth, Newcastle upon Tyne, NE3 4DB). Mutation detection enhancement gel was purchased from FMC (High Wycombe, Bucks, UK). Diazinon, chlorpyrifos, parathion, diazoxon, chlorpyrifos-oxon, paraoxon, pyridinol (IHMP), 3, 5, 6-trichloro-2-pyridinol (TCP) were from Greyhound Chromatography (Birkenhead, Merseyside, UK) while phenylacetate and p-nitrophenol (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 non-smokers and for nine smoking status was unknown. The liver samples were obtained from patients in Newcastle and Aberdeen, with local ethical committee approval, and were flash-frozen prior to 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 differential centrifugation, as described previously (Mutch et al, 1999) and the protein content determined by the method of Smith et al (1985) using bovine serum albumen as standard.

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

Pre-determined conditions of linearity with respect to protein and time were used (data not shown). For incubations with diazoxon (500µM), chlorpyrifos-oxon (500µM) or paraoxon (1mM), approximately 1mg protein (diazinon) or 1.5mg protein (chlorpyrifos-oxon or paraoxon) were incubated in 0.5ml of 50mM Tris-HCl/1mM $CaCl_2$ buffer (pH 7.4) for 5min, 2.5min or 10min, respectively, at 37°C. The reaction was started by addition of the oxon contained in 2µl methanol and stopped with either 0.5ml methanol (diazoxon and chlorpyrifos oxon) or 0.5ml phosphoric acid (6%, v/v) (paraoxon). The denatured proteins were separated by centrifugation and clear supernatant (80µl) was injected onto the HPLC 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 HPLC system employed a mobile phase of acetonitrile/water with gradient elution and detection at 245nm (diazoxon hydrolysis), 60% methanol/40%

water isocratic elution and detection at 230nm (chlopyrifos-oxon hydrolysis) or methanol/phosphoric acid (0.7% w/v) gradient elution and detection at 290nm (paraoxon hydrolysis). Separation of the oxons and dearylated metabolites was on a Luna ODS1 5µM reverse phase column (250 x 4.6 mm, Phenomenex) with guard column. Determination of IHMP, TCP or PNP formation from the oxon were 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 pmol, 10 pmol and 15 pmol, respectively.

The method for hydrolysis of phenylacetate (3mM) by human serum has been described previously (Mutch et al, 1992). Continuous monitoring of the hydrolysis product, phenol, was made at 272nm using a Kontron spectrophotometer. Approximately 1mg protein was incubated in 3ml of 50mM Tris-HCl/1mM CaCl$_2$ buffer (pH 8) at 37°C.

Enzyme activities were expressed as nmol pyrimidinol (IHMP) formed/min/mg protein (diazoxon hydrolysis), nmol 3,5,6-trichloro-2-pyridinol (TCP) formed/min/mg protein (chlorpyrifos-oxon hydrolysis), nmol p-nitrophenol formed/min/mg protein (paraoxon hydrolysis), and µmol phenol formed/min/mg protein (phenylacetate hydrolysis).

Analysis of PON1-192 genotype

Genomic DNA was extracted from about 500 mg human liver (n=27) from Caucasian individuals using the phenol chloroform method of Blin and Stafford (1976). The PCR reaction for the PON1-192 genotype involved amplification using approx. 1 µg of DNA from either blood or liver in a total volume of 50µl in a buffer consisting of

1.5mM MgCl$_2$/50mM KCl with 0.2mM dNTPs and 1.5 units Taq polymerase. The sense primer (0.25μM) 5'-CTGCCT AATTTGAATGATAT-3' and antisense primer (0.25μM) 5'-ATACTT 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 1min at 95°C, 1.5min annealing at 46°C and 1min extension at 72°C.

Single strand conformational polymorphism (SSCP) analysis of the PCR product (approx. 200bp) was carried out using 1X mutation detection enhancement gels following the protocol described by Daly et al (1996). Twenty μl PCR product, 25 μl formamide and 7 μl STOP solution (0.05% bromophenol blue, 0.05% xylene cyanol, 20mM EDTA, 95% formamide) were mixed and incubated at 95°C for 5min before application to the gel. Electrophoresis was performed at 200V overnight (15-17h) at 4°C and bands were visualised 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 (Humbert et al, 1993).

Statistics

Population distributions of diazoxonase, chlorpyrifos-oxonase, paraoxonase and arylesterase (phenylacetate hydrolase) activity were analysed by inspection of frequency distribution histograms and deviation from normality was assessed by the Shapiro Wilks W test (Shapiro and Wilk, 1965). Statistical comparison between the three PON1-192 genotypes was determined by the non-parametric Mann-Whitney U test (two-tailed) and correlation analysis was by Spearman (2 tailed).

Values of $p < 0.05$ were considered to be significant.

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Results

Diazoxon and chlorpyrifos-oxon were hydrolysed 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 towards diazoxon and chlorpyrifos-oxon compared to paraoxon is a major determinant of the higher toxicity of parathion to man.

The rates of hydrolysis of diazoxon, chlorpyrifos-oxon and paraoxon varied 5.7-fold, 16-fold and 56-fold, respectively. There were wide variations in hydrolysis rates of all three oxons between liver microsomes from individuals with the same PON1-192 genotype (**Table 1**). The QQ homozygotes had 4-fold, 13-fold and 25-fold ranges in hydrolytic activity towards diazoxon, chlorpyrifos-oxon and paraoxon, respectively, while the RR homozygotes had 5-fold, 6-fold and 3-fold ranges in activity. Phenylacetate was hydrolysed with nearly a 5-fold range in activity; the QQ, QR and RR genotypes showed 4-fold, 3-fold and 2-fold variations, respectively (**Table 1**).

There were strongly significant relationships between phenylacetate hydrolysis and paraoxon hydrolysis (n=27, r=0.835, p<0.001), and phenylacetate hydrolysis and chlorpyrifos-oxon hydrolysis (n=27, r=0.834, p<0.001) (**Figure 2**). However, the relationship between phenylacetate hydrolysis and diazoxon hydrolysis was not significant (n=19, r=0.430, p=n.s.) (**Figure 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 (**Figure 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); allele frequency Q=0.63 and R=0.37. This is comparable to 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, Daly and Williams, unpublished). 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 hydrolysed phenylacetate at similar rates (**Figure 4**). Diazoxon hydrolysis (**Figure 4**) and chlorpyrifos-oxon hydrolysis (**Figure 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 hydrolyse diazoxon (243.6 nmol/min/mg protein) were confirmed by repeat analysis (**Figure 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 hydrolyse paraoxon compared to the QQ homozygotes ($p<0.01$), but not the QR variants (**Figure 4**).

Discussion

This study reports for the first time the detoxification of diazoxon, chlorpyrifos-oxon and paraoxon by human liver microsomes and shows wide inter-individual 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 non-physiological 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 to in vivo. More importantly, high sodium chloride levels markedly and differentially affect the activity of the PON1-192 variants (Li et al, 2000). However, in order to investigate PON1-mediated hydrolysis of the oxons, and 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 non-physiological, saturating oxon concentrations.

O'Leary and colleagues (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 PON1-192$_{RR}$ genotype had significantly higher ability to hydrolyse diazoxon and that an earlier report to the contrary (Mackness et al, 2003) may have been due to artefacts of the assay conditions used, including measurement of activities using a non-physiological (2M) 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

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define the importance of the one outlier with very high capacity to hydrolyse this oxon. It is possible that other polymorphisms, such as in the PON1-55 coding region or the -108C/T regulatory region, or environmental effects, such as consumption of dietary anti-oxidants (Aviram et al, 2000), contributed to the higher activity seen in the RR outlier.

In genetic analyses of more than 1,406 individuals in 40 extended families, Rainwater et al 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 (D.L. Rainwater; personal communication, 2006). 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 hydrolyse diazoxon.

In our study, the RR homozygotes had a higher median chlorpyrifos-oxonase activity, although this did not reach statistical significance. The QQ and QR individuals had inter-genotypic ranges in activity that were similar to the intra-genotype 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

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(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-fold and 55-fold less efficiently. This means that it is likely that low concentrations of chlorpyrifos-oxon will be hydrolysed effectively by PON1, regardless of genotype.

The RR homozygotes had higher hepatic activity towards 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 since PON1's overall catalytic efficiency towards 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 influence 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 towards paraoxon and chlorpyrifos-oxon, but not diazoxon. It also compliments studies with PON1 knockout mice since they cannot address the complication of variable PON1 expression and activity seen in human populations.

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

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Legends

Figure 1

Activation and detoxification metabolic pathways for diazinon, chlorpyrifos and parathion.

Figure 2

The relationship between (**A**) phenylacetate (3mM) hydrolysis and paraoxon (1mM) hydrolysis, (**B**) phenylacetate (3mM) hydrolysis and chlorpyrifos-oxon (500μM) hydrolysis (**C**) phenylacetate (3mM) hydrolysis and diazoxon (500μM) hydrolysis by 27 (phenylacetate, chlorpyrifos-oxon and paraoxon) or 19 (diazoxon) human liver microsomal preparations.

R is the correlation coefficient

***$p < 0.001$

Figure 3

The relationship between (**A**) diazoxon (500μM) hydrolysis and paraoxon (1mM) hydrolysis, (**B**) diazoxon (500μM) hydrolysis and chlorpyrifos-oxon (500μM) hydrolysis (**C**) paraoxon (1mM) hydrolysis and chlorpyrifos-oxon (500μM) hydrolysis by 27 (phenylacetate, chlorpyrifos-oxon and paraoxon) or 19 (diazoxon) human liver microsomal preparations.

***$p < 0.001$

Figure 4

Hydrolysis of (**A**) phenylacetate (3mM), (**B**) diazoxon (500μM), (**C**) chlorpyrifos-oxon (500μM), (**D**) paraoxon (1mM) by 27 (phenylacetate, chlorpyrifos-oxon and paraoxon) or 19 (diazoxon) human liver microsomal preparations in relation to PON1-192 genotype.

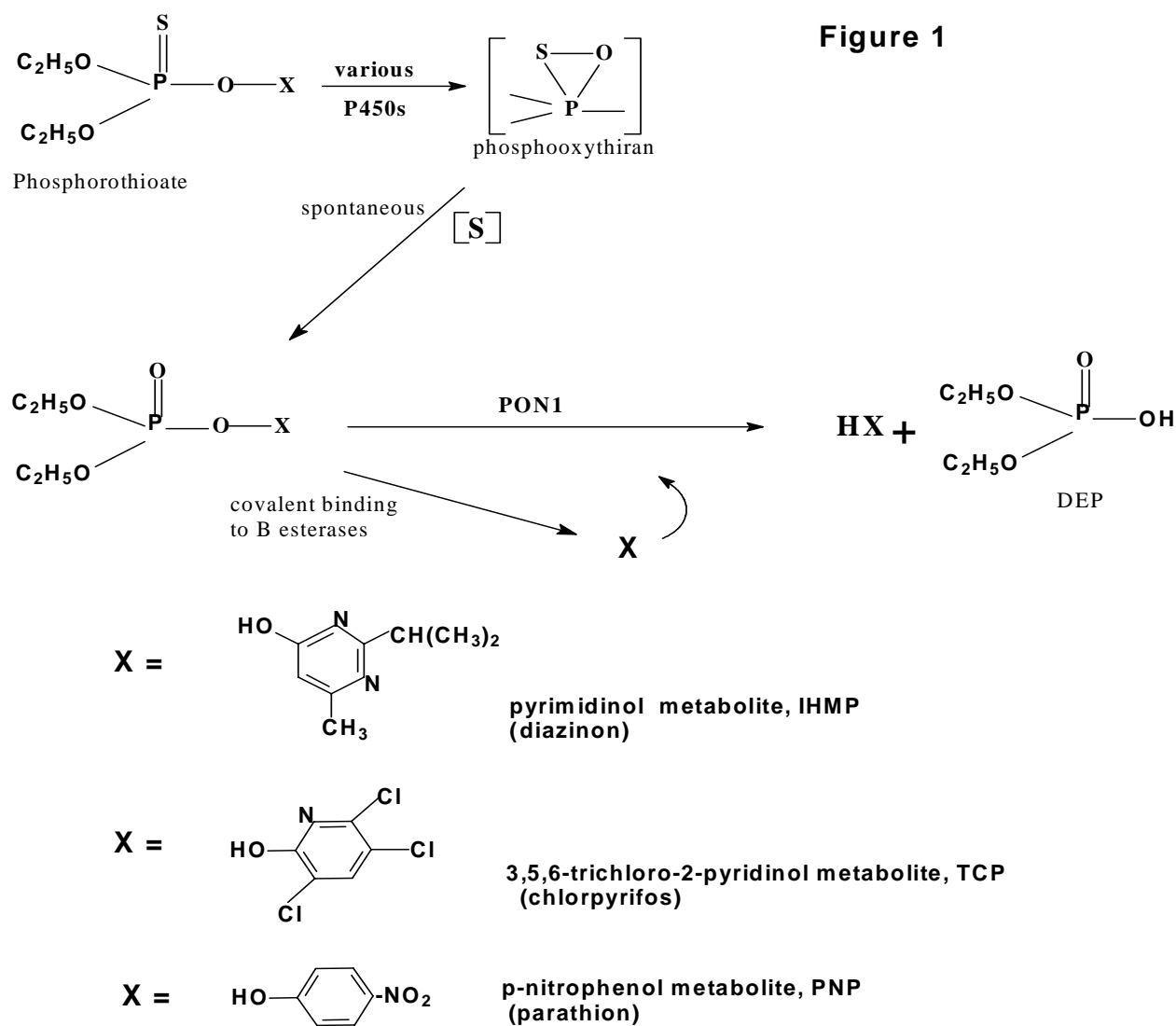**$p < 0.01$, compared to the QQ homozygotes

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Table 1

Enzyme activities for hydrolysis of the PON1 substrates diazoxon (DZO, 500µM), chlorpyrifos-oxon (CPO, 500µM), paraoxon (POX, 1mM) and phenylacetate (PA, 3mM) by 27 human liver microsomal (HLM) preparations in relation to PON1-192 genotype. Activities are expressed as nmol/min/mg protein, except PA hydrolysis (µmol/min/mg protein).

HLM	DZO hydrolysis	CPO hydrolysis	POX hydrolysis	PA hydrolysis	PON1-192 genotype
1	176.1	70.8	0.626	7.45	QQ
2	100.6	140.5	1.21	12.4	QQ
3	57.4	221.7	3.74	10.2	RR
4	88.2	182.1	1.43	10.6	QQ
5	42.7	92.5	0.665	8.44	QQ
6	76.1	151.6	1.04	9.49	QQ
7	59.5	123.0	2.53	8.47	RR
8	134.6	53.1	0.838	10.1	QQ
9	101.7	47.3	0.799	6.32	QR
10	--	39.0	2.63	9.65	QR
11	--	41.4	0.680	8.70	QR
12	64.7	20.7	0.243	6.72	QQ
13	49.5	40.5	2.20	10.1	RR
14	--	17.9	0.173	4.06	QQ
15	--	37.4	1.30	7.92	QR

16	--	43.0	0.633	6.96	QQ
17	--	34.4	0.450	8.03	QR
18	74.8	130.5	3.89	11.5	RR
19	66.6	160.4	4.00	12.5	RR
20	111.4	95.9	1.64	11.2	QQ
21	--	222.7	3.87	17.6	QQ
22	243.6	139.0	5.95	12.5	RR
23	47.4	70.3	1.09	8.43	QR
24	126.8	194.3	4.73	14.5	QR
25	--	228.1	4.42	15.7	QQ
26	185.6	288.1	9.60	19.9	QR
27	62.8	240.1	2.06	12.1	QQ

-- = not determined

27

Figure 1

Figure 2

Figure 3

Figure 4