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REVIEW

Paraoxonase (PON1) as a biomarker of susceptibility for organophosphate toxicity

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Paraoxonase (PON1) is an A-esterase capable of hydrolysing the active metabolites (oxons) of a number of organophosphorus (OP) insecticides such as parathion, diazinon and chlorpyrifos. PON1 activity is highest in liver and plasma, and among animal species significant differences exist, with birds and rabbits displaying very low and high activity, respectively. Human PON1 has two polymorphisms in the coding region (Q192R and L55M) and five polymorphisms in the promoter region. The Q192R polymorphism imparts different catalytic activity toward some OP substrates, while the polymorphism at position −108 (C/T) is the major contributor to differences in the level of PON1 expression. Animal studies have shown that PON1 is an important determinant of OP toxicity, with animal species with a low PON1 activity having an increased sensitivity to OPs. Administration of exogenous PON1 to rats or mice protects them from the toxicity of OPs. PON1 knockout mice display a high sensitivity to the toxicity of diazoxon and chlorpyrifos oxon, but not paraoxon. *In vitro* assayed catalytic efficiencies of purified PON_{192} isoforms for hydrolysis of specific oxon substrates accurately predict the degree of *in vivo* protection afforded by each isoform. Low PON1 activity may also contribute to the higher sensitivity of newborns to OP toxicity.

Keywords: paraoxonase (PON1), genetic polymorphisms, organophosphate toxicity, ecogenetics, transgenic mice

Introduction

There is increasing recognition that health risks from exposure to exogenous chemicals can be greatly influenced by genetically determined variations in the host. Such variations, which may involve biotransformation enzymes or target molecules, have been extensively studied in relation to adverse drug reactions and differences in drug effectiveness in the area of research known as pharmacogenetics. More recently, this field has evolved to include any kind of environmental xenobiotic agent, hence the term ecogenetics (Costa 2000). Genetic polymorphisms may lead to enzyme variants with higher or lower activity, or may cause partial or total absence of an enzyme protein. Such susceptibility genes are not

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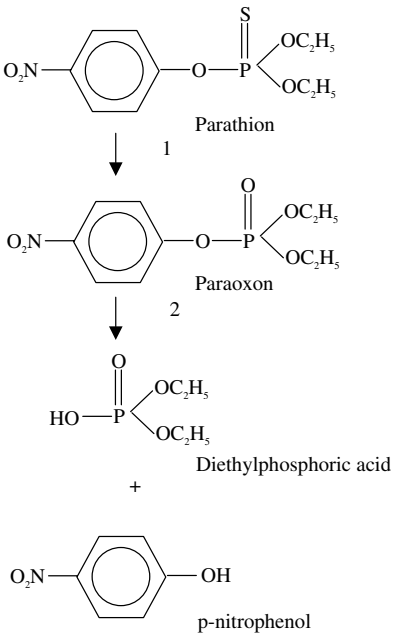

Figure 1. Activation and detoxication of the OP insecticide parathion. Reaction 1 is a CYP-mediated oxidative desulphuration to paraoxon, while reaction 2 is hydrolysis by PON1.

necessarily sufficient to cause disease, but may modify the risk when there is appropriate exposure (Eaton *et al*. 1998).

Pesticides represent a large and important class of environmental chemicals; among them, insecticides are of most concern because of their high acute toxicity, particularly toward the nervous system (Costa 1997). Insecticides undergo bioactivation and detoxication processes (figure 1) that can be affected by genetic polymorphisms in biotransformation enzymes. Genetic variations in such enzymes would, therefore, render an individual more or less susceptible to the adverse effects of these compounds. This review will focus on an important class of insecticides, the organophosphorus (OPs) insecticides, and on the detoxication enzyme paraoxonase (PON1). Of the estimated 3 million worldwide annual pesticide poisonings, many involve OPs (WHO 1990). Furthermore, there is the threat of exposure to nerve agent OPs, such as sarin (Ohbu *et al*. 1997). Several OPs can be detoxified by PON1, whose genetic polymorphisms and role in OP toxicity have been characterized over the past several years and are reviewed below.

OP insecticides: toxicity and metabolism

OP compounds are triesters of phosphoric acid, and their major use is as insecticides, though some of them have also been used as drugs (e.g. ecothiophate, trichlorfon) as well as nerve agents in chemical warfare (e.g. sarin, soman) (Lotti 2000). On acute exposure, OPs cause neurotoxicity, which can best be described as a cholinergic crisis due to overstimulation of the central cholinergic system and peripheral somatic and parasympathetic nervous systems. This is caused by

accumulation of acetylcholine in the synaptic cleft and by overstimulation of cholinergic receptors as a result of inhibition of acetylcholinesterase (AChE) by OPs. The main signs and symptoms of OP cholinergic toxicity include miosis, bronchoconstriction, diarrhoea, increased salivation, muscular weakness and fasciculation, confusion and respiratory depression (Lotti 2000). Certain OPs can also cause a central-peripheral distal sensory-motor axonopathy, known as OP-induced delayed polyneuropathy (OPIDP) (Lotti 2000). Symptoms of OPIDP begin 1 to 4 weeks after a single exposure, after the cholinergic symptoms have subsided, and include cramping muscle pain in the legs and progressive leg weakness, followed by wasting and weakness of the distal limb muscle (Lotti 2000). OPIDP is not related to inhibition of AChE, but rather is associated with phosphorylation of another esterase in the nervous system, neuropathy target esterase (NTE), whose physiological function is as yet unknown.

Most OP insecticides are organothiophosphates and require metabolic activation to the corresponding oxons by a process of oxidative desulphuration, by which the P═S moiety is changed to P═O (figure 1). Only OPs with a P═O moiety can interact with B-esterases, such as AChE. Thus, commonly used OPs, such as diazinon or chlorpyrifos, need to be bioactivated *in vivo* to their oxygen analogues (diazoxon and chlorpyrifos oxon). This activation is mediated by mainly hepatic isozymes of cytochrome P450 (CYP), which can also detoxify OPs. Variant forms of CYP genes have been identified, and these polymorphisms confer differences in catalytic activity or level of expression (Eaton 2000). However, the potential contribution of CYP polymorphisms to OP toxicity susceptibility has not been fully investigated (Costa 2001). A number of studies have suggested that certain OPs, particularly those with a methylester group (e.g. methylchlorpyrifos), may be metabolized by glutathione-S-transferases (GSTs), leading to glutathione conjugates. Though at least four human GSTs are polymorphic, no information exists on the potential significance of human GST polymorphisms as determinants of individual differences in human susceptibility to OPs (Costa 2001).

Several OPs can be hydrolysed, and hence detoxified, by the A-esterase PON1 (figure 1). Substrates of PON1 include the oxygen analogues of various commonly used OP insecticides (e.g. chlorpyrifos oxon, diazoxon, paraoxon) and nerve agents such as sarin or soman (Geldmacher-von Mallinckrodt and Diepgen 1988). High PON1 activity is found in serum and liver. Earlier studies measuring PON1 activity in serum from human subjects of Caucasian origin revealed a bimodal or trimodal distribution (Eckerson *et al.* 1983, Mueller *et al.* 1983). On the basis of enzymatic tests, humans could be divided into three serum PON1 phenotypes, with low, intermediate and high activity. The hypothesis that low metabolizers may be more sensitive to the toxicity of specific OP compounds has received confirmation in the past decade, which has seen tremendous progress in our knowledge about PON1 polymorphisms and their role in determining susceptibility to OP toxicity.

PON1 and its polymorphisms

Studies in the early nineties led to the purification of rabbit and human PON1 and subsequent cloning and sequencing of their respective cDNAs (Furlong *et al.* 1991, Gan *et al.* 1991, Hassett *et al.* 1991). The PON1 cDNA encodes a protein consisting of 355 amino acids, from which only the amino-terminal methionine

residue is removed during secretion and maturation. Physical mapping placed the PON1 gene on chromosome 7 q21–22 (Humbert *et al*. 1993).

Two related genes, PON2 and PON3, also exist. Both have been localized, like PON1, on chromosome 7 (Primo-Parmo *et al*. 1996). The human PON2 gene has two common polymorphisms at codons 148 and 311, which give rise to the amino acid substitutions Gl48A and C311S (Mochizuki *et al*. 1998). Neither PON2 or PON3 have catalytic activity toward OPs, but PON2 may have general antioxidant properties (Ng *et al*. 2001), while PON3 (from rabbit) has been shown to have lactonase activity (Draganov *et al*. 2000).

Two polymorphisms were observed in the PON1 coding sequence: a Gln (Q)/Arg (R) substitution at position 192, and a Leu (L)/Met (M) substitution at position 55 (Adkins *et al*. 1993, Humbert *et al*. 1993) (figure 2). Using a polymerase chain reaction (PCR) method described by Humbert *et al*. (1993), a large number of studies have been carried out to establish the PON1 192 and 55 genotypes for individuals in large populations. The polymorphism at position 192 has been the most studied, with gene frequencies for $PON1_{Q192}$ ranging from 0.75 for Caucasians of Northern European origin to 0.31 for some Asian populations (Brophy *et al*. 2002). On the other hand, gene frequency for $PON1_{L55}$ ranged from 0.7 in Caucasian populations to 0.9 in Asian populations (Brophy *et al*. 2002). Several studies have shown that the L_{55} and R_{192} alleles are in strong disequilibrium, with approximately 98% of the R_{192} alleles having L at position 55 (Brophy *et al*. 2002).

The coding region polymorphisms in the PON1 protein have been studied for effects on the catalytic efficiency of hydrolysis of specific substrates. The L/M polymorphism at position 55 has not been found to affect catalytic efficiency (Adkins *et al*. 1993, Humbert *et al*. 1993), but has been shown to be associated with variability in the plasma PON1 plasma levels, with $PON1_{M55}$ individuals on average having lower PON1 activity (Blatter Garin *et al*. 1997; Mackness *et al*. 1998, Brophy *et al*. 2001b). On the other hand, the Q/R polymorphism of position 192 significantly affects the catalytic efficiency of PON1 in a substrate-dependent manner. Initial studies indicated that the $PON1_{R192}$ isozyme could hydrolyse paraoxon more readily than $PON1_{Q192}$ (Adkins *et al*. 1993, Humbert *et al*. 1993). Further studies suggested that this polymorphism may be substrate-dependent, as the $PON1_{Q192}$ isoform was found to hydrolyse diazoxon, sarin and soman more rapidly than $PON1_{R192}$ in *in vitro* assays (Davies *et al*. 1996). The results of *in vivo* studies in transgenic mice (see below) led to a re-evaluation of such findings, and rather than studying the hydrolytic rate, the catalytic efficiency of substrate hydrolysis was measured under experimental conditions closer to physiological ones (Li *et al*. 2000). These studies indicated that, while the efficiency of $PON1_{R192}$ for hydrolysing paraoxon was eight times greater than that of $PON1_{Q192}$, the catalytic efficiency was significantly lower than those for hydrolysis of diazoxon and chlorpyrifos oxon (table 1; Li *et al*. 2000). Furthermore, the catalytic efficiency of $PON1_{Q192}$ and $PON1_{R192}$ for diazoxon was almost identical, while the $PON1_{R192}$ isoform had a less than two-fold higher catalytic efficiency for chlorpyrifos oxon than $PON1_{Q192}$ (Li *et al*. 2000). These *in vitro* results provide explanations for the initially unexpected results provided by the *in vivo* studies (see below).

Additional polymorphisms have been found in the non-coding region of the PON1 gene (Levier and James 2000, Suehiro *et al*. 2000, Brophy *et al*. 2001a).

Table 1. Catalytic efficiency determines the *in vivo* efficacy of PON1 for detoxifying OP compounds.

	Paraoxon		Diazoxon		Chlorpyrifos oxon	
	$PON1_{Q192}$	$PON1_{R192}$	$PON1_{Q192}$	$PON1_{R192}$	$PON1_{Q192}$	$PON1_{R192}$
K_m (mM)[1]	0.81	0.52	2.98	1.02	0.54	0.25
V_{max} (units mg^{-1})[1]	0.57	3.26	222	79	82	64
Catalytic efficiency (V_{max}/K_m)[1]	0.71	6.27	75	77	152	256
Provides protection *in vivo*[2]	−	−	+	+	+	+ +

[1] Paraoxonase, diazoxonase and chlorpyrifos oxonase activities were determined *in vitro* at physiologically relevant salt concentrations, using purified human plasma $PON1_{Q192}$ or $PON1_{R192}$ isoforms (Li *et al*. 2000).

[2] PON1 knockout mice were injected with purified human plasma $PON1_{Q192}$ or $PON1_{R192}$ 4 h prior to OP exposure (0.3 mg kg^{-1} paraoxon, 1 mg kg^{-1} diazoxon; 2 mg kg^{-1} chlorpyrifos oxon), and cholinesterase inhibition was measured in the brain and diaphragm two hours following OP exposure. Neither PON1 isoform protected against paraoxon toxicity. The two isoforms provided equivalent protection against diazoxon toxicity. $PON1_{R192}$ afforded twice the protection of $PON1_{Q192}$ against chlorpyrifos oxon toxicity (Li *et al*. 2000)

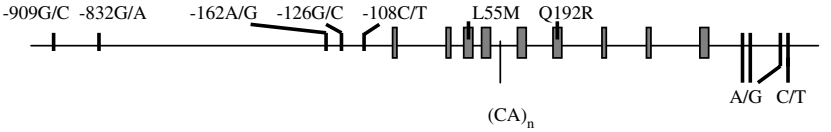

Figure 2. Polymorphisms of PON1. The L55M and Q192R polymorphisms in the coding region, and the five polymorphisms in the promoter region (−108, −126, −162, −832, −909) are indicated.

These occur at positions −108 (C/T), −126 (G/C), −162 (A/G), −832 (G/A) and –909 (C/G) (Brophy *et al*. 2001a) (figure 2). The most significant of these promoter region polymorphisms turned out to be that at position −108, which contributes 22.4% of the variation in PON1 expression, while the polymorphism at position −162 contributes only a small (2.4%) amount (Brophy *et al*. 2001b).

The existence of PON1 coding region polymorphisms that affects catalytic activity toward OPs and of a promoter region polymorphism that affects the levels of PON1 expression led to the proposal to determine the 'PON1 status' of an individual (Li *et al*. 1993, Richter and Furlong, 1999). By plotting rates of diazoxon hydrolysis against paraoxon hydrolysis at high salt concentrations (2 M NaCl), an accurate inference of $PON1_{192}$ genotype as well as PON1 activity levels for individuals can indeed be made (figure 3; Richter and Furlong, 1999, Jarvik *et al*. 2000).

Role of PON1 in OP toxicity

The existence of polymorphisms in PON1 that infer different hydrolysing ability toward OPs, as well as different levels of expression, has long led to the hypothesis that certain individuals may be more sensitive to OP toxicity. This hypothesis has received confirmation only in the past decade, primarily from animal studies. Evidence for the role of PON1 in OP toxicity has been derived initially from cross-species comparisons, from animal experiments in which

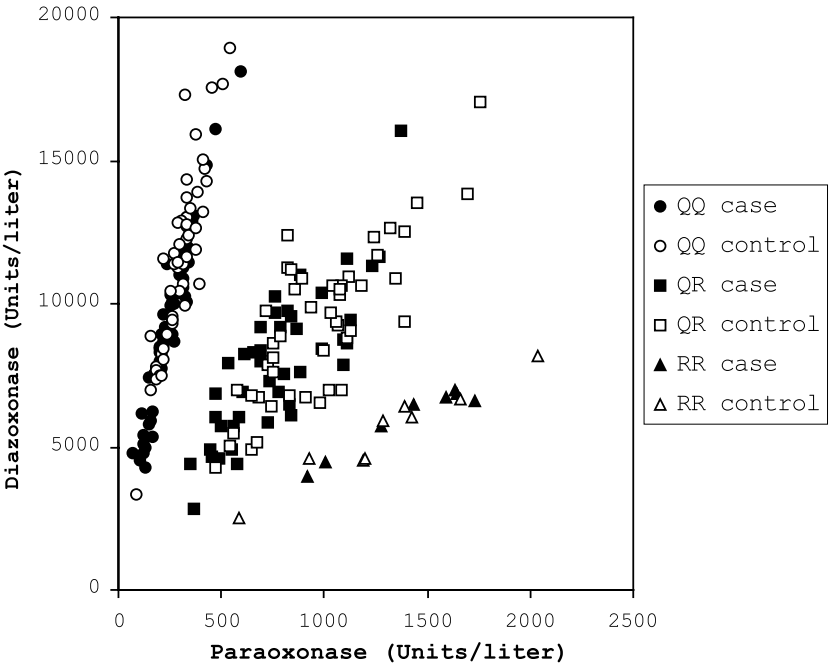

Figure 3. Plot of diazoxonase versus paraoxonase activities for controls and carotid artery disease cases, coded for $PON1_{192}$ genotype (determined by PCR). From Jarvik *et al*. 2000, with permission.

purified PON1 was utilized, and more recently from studies with PON1 knockout mice. Earlier findings indicated that birds, which have no to very low plasma PON1 activity, were more sensitive than rats to the toxicity of various OPs (Brealey *et al*. 1980). In turn, rats were found to be more sensitive to the toxicity of OPs than rabbits, which have a seven-fold higher plasma PON1 activity (Costa *et al*. 1987). Though several other factors may contribute to the species differences in OP toxicity, these earlier findings provided some supporting evidence to the hypothesis that low plasma PON1 activity would lead to an increased sensitivity to the acute effects of OPs.

A more direct approach was provided by studies in which exogenous PON1 was injected into rats or mice. A pioneering experiment was carried out by Main (1956), who injected partially purified PON1 obtained from rabbit serum intravenously in rats, and noted that the acute toxicity of paraoxon was decreased. More recent studies in rats and mice have confirmed and expanded this early finding. Initially, PON1, purified to homogeneity from rabbit serum (Furlong *et al*. 1991), was given by intravenous injection to rats (Costa *et al*. 1990). Administration of the enzyme raised rat serum PON1 activity toward paraoxon and chlorpyrifos oxon by nine- and fifty-fold, respectively. When rats were challenged with either OP, significant protection (assessed by measuring inhibition of AChE in different tissues) was observed. Protection was more evident in the case of chlorpyrifos oxon, was more prominent in two of the target tissues (brain and diaphragm), and was also present when OP exposure occurred by the dermal route, which represents an important route of exposure for occupationally exposed workers

(Costa *et al*. 1990). Further experiments following a similar protocol extended these findings to mice (Li *et al*. 1993). Furthermore, it was found that serum PON1 levels could be increased for extended periods ($t_{1/2} > 30$ h) by administering purified PON1 both intraveneously and intramuscularly (Li *et al*. 1993). Additional studies indicated that exogenous PON1 could also afford protection against the toxicity of chlorpyrifos, the parent compound used as an insecticide, when given before or even after (up to 3 h) OP exposure (Li *et al*. 1995). Recently, recombinant human PON1 (either LR or LQ) expressed from an adenoviral vector was shown to increase serum PON1 activity by about 60%, and to protect mice against the toxicity of chlorpyrifos (Cowan *et al*. 2001). Overall, these studies indicated that by artificially increasing serum levels of PON1 it is possible to decrease the toxicity of certain OPs.

More recent experiments have investigated the toxicity of OPs in PON1 knockout ($PON1^{-/-}$) mice, which were produced by targeted disruption of exon 1 of the PON1 gene (Shih *et al*. 1998). $PON1^{-/-}$ mice have no plasma or liver hydrolytic activity toward paraoxon and diazoxon, and a very low level of activity toward chlorpyrifos oxon (Li *et al*. 2000). PON1 knockout mice have dramatically increased sensitivity to chlorpyrifos oxon and diazoxon, and a slightly increased sensitivity to the respective parent compounds chlorpyrifos and diazinon (Shih *et al*. 1998, Li *et al*. 2000). A surprising finding came from the experiments with paraoxon, the OP after which PON1 was named. PON1 knockout mice did not show increased sensitivity to paraoxon, despite the total lack of PON1 activity in plasma and liver (Li *et al*. 2000).

Further experiments have investigated whether administration of purified PON1 would restore plasma (but not liver) PON1, and thereby OP resistance, in $PON1^{-/-}$ mice. Either human $PON1_{Q192}$ or $PON1_{R192}$ were injected, by the intravenous route, into $PON1^{-/-}$ mice, and the effects of OPs on brain and diaphragm AChE were determined. In the case of chlorpyrifos oxon, both isoforms were protective, and $PON1_{R192}$ offered about 50% better protection than $PON1_{Q192}$, in accordance with the previously discussed *in vitro* findings (Davies *et al*. 1996, Li *et al*. 2000). Both $PON1_{R192}$ and $PON1_{Q192}$ offered equal protection against diazoxon; this appeared to be in contrast to earlier findings (Davies *et al*. 1996), but is in agreement with the *in vitro* experiments on catalytic efficiency (Li *et al*. 2000). Neither human PON1 isoform afforded protection against the toxicity of paraoxon, substantiating the surprising results described above. However, the results from the kinetic analysis of substrate hydrolysis by purified human $PON1_{192}$ isoforms provide an explanation for such findings. Though the efficiency of the $PON1_{R192}$ in hydrolysing paraoxon is eight times greater than that of the $PON1_{Q192}$ isoform, the catalytic efficiency of either isoform is significantly lower than those for hydrolysis of diazoxon or chlorpyrifos oxon (table 1). This confirms the hypothesis (Pond *et al*. 1995) that PON1 is not efficient at hydrolysing paraoxon at low concentrations, suggesting that PON1 may not degrade paraoxon efficiently *in vivo*, and that other pathways (e.g. CYP, carboxylesterase) are primarily responsible for detoxifying paraoxon *in vivo* (Chambers *et al*. 1994, Pond *et al*. 1995).

In summary, this series of studies have provided evidence that PON1, whose catalytic activity and level of expression are determined by polymorphisms in the coding and promoter regions, respectively, plays an important role in modulating the toxicity of some, but not all OPs, which are *in vitro* substrates for this enzyme.

The catalytic efficiency of hydrolysis of oxons is the determining factor as to whether PON1 provides protection against exposure to a specific OP.

PON1 and the developmental toxicity of OPs

There is an increasing concern that young children may be more sensitive than adults to the toxic effects of certain pesticides (Eskenazi *et al*. 1999). The toxicity of OPs appears to be influenced by age, with young animals being more sensitive to the effects of acute exposure (Berke and Murphy 1975, Pope and Liu 1997), though whether such enhanced sensitivity extends to situations involving repeated sublethal exposure is still unclear (Sheets 2000). Furthermore, young animals have been suggested to be especially sensitive to the central neurotoxicity of certain OPs (Dam *et al*. 2000), while they appear to be resistant to OPIDP (Moretto *et al*. 1991). There is an increasing consensus that the lower metabolic abilities of young animals are a major determinant of their increased sensitivity to OP toxicity, as suggested a few decades ago (Benke and Murphy 1975). In particular, studies with chlorpyrifos have concluded that a lower hydrolytic detoxication by PON1, and perhaps carboxylesterase, accounts for the differential age-related sensitivity in its acute toxicity (Mortensen *et al*. 1996, Padilla *et al*. 2000). Indeed, in both rats and mice, liver and serum PON1 activity toward different substrates, as well as liver mRNA, increase from birth to postnatal day 21 (Li *et al*. 1997). There is also evidence of low serum PON1 activity, measured with phenylacetate as the substrate, in human infants and children (Augustinsson and Barr 1963, Mueller *et al*. 1983), which seems to reach a plateau (whose level is determined by the genetic background of the individual) at 15–25 months of age (Costa *et al*. unpublished data).

PON1 as a biomarker of other diseases

Though this review focuses on the role of PON1 and its polymorphisms in modulating the toxicity of OPs, it should be remembered that PON1 has been reported to play important roles in various diseases. First and foremost, the PON1 status of an individual appears to be relevant with regard to cardiovascular disease and atherosclerosis (Durrington *et al*. 2001, Shih *et al*. 2002). Plasma PON1 is closely associated with high density lipoprotein (HDL) particles, which has long suggested its potential role in lipid metabolism. PON1 can prevent lipid peroxide accumulation on low density lipoproteins (LDLs) both *in vitro* and *in vivo* (Mackness *et al*. 1993, Watson *et al*. 1994) and the R_{192} allozyme appears to be less efficient in this regard (Mackness *et al*. 1998b). Accordingly, several epidemiological studies have shown that R_{192} may represent a risk factor for coronary heart disease; however, many other studies have failed to observe such an association (see references in Mackness *et al*. 2001, Shih *et al*. 2002). A possible reason for these discrepancies lies in the fact that most of the epidemiological studies were exclusively genetic, and failed to take into account the PON1 status, i.e. the levels of enzyme expression, whose relevance has been clearly shown (Jarvik *et al*. 2000, Mackness *et al*. 2001). Thus, the R_{192} genotype and in particular low levels of PON1, independently of the 192 genotype, appear to be a major risk factor in coronary artery disease. PON1 promoter polymorphism at position −108 and

relative PON1 deficiency have also been reported as determinants of the risk of coronary artery disease (Leviev *et al*. 2001).

Reduced serum PON1 levels are also found in patients with type 2 diabetes, though no significant differences were found in the allelic frequencies of the 192 Q/R, 55 L/M or 108 C/T polymorphisms between patients and controls (Abbott *et al*. 1995, Inoue *et al*. 2000). However, it was found that the −108 C/T polymorphism and low PON1 levels are associated with increased serum glucose concentrations in non-diabetic patients (Leviev *et al*. 2001), suggesting that it may also have a role in promoting higher glucose concentrations in diabetes, or may be linked to other genes involved in this process.

The R_{192} and L_{55} alleles have been shown to be risk factors for age-related macular degeneration, and were associated with an increase in plasma oxidized LDLs (Ikedo *et al*. 2001). Additionally, it has been recently shown that PON1 may play a significant role within the antioxidant systems in the liver (Ferrè *et al*. 2001), suggesting that the PON1 status of an individual may be relevant in the pathophysiology of chronic liver disease.

Finally, a few studies have also examined the role of PON1 polymorphisms as risk factors for Parkinson's disease (PD). The rationale behind these studies lies in the fact that exposure to pesticides, often associated with living in rural areas, has been shown to be a potential risk factor for PD (Priyadarshi *et al*. 2001). Furthermore, as PON1 appears to be a protein with antioxidant capacity (see above), it may be involved in PD; as an increased rate of oxidative stress has been implicated as a pathogenetic factor for this disease (Fahn and Cohen 1992). Kondo and Yamamoto (1998) reported that the R_{192} allele might confer genetic susceptibility to PD; however, this was not confirmed by other investigators (Akhmedova *et al*. 1999, Wang and Liu 2000). Recently, the Met_{55} allele has been found to be a risk factor for PD (Akhmedova *et al*. 2001). No studies have assessed the PON1 status (genotype/phenotype) of PD patients and controls.

Conclusions

The studies summarized in this review provide evidence that PON1 plays a relevant role in the *in vivo* metabolism of certain OPs and in their acute toxicity. Of interest is that the toxicity of the oxygen analogues of chlorpyrifos and diazinon, but not of parathion, are influenced by PON1. Furthermore, PON1 appears to affect sensitivity to the oxon forms of diazinon and chlorpyrifos much more than their parent compounds, which are used as insecticides. As the oxon forms are many times more toxic, even limited exposures may be significant. In this regard, the finding that residues of oxons for various OPs can be found following OP application is particularly noteworthy (Yukvanage *et al*. 1997, California Environmental Protection Agency 1998). Since the genetic polymorphisms of PON1 (at positions 192 and −108) infer different catalytic activity and levels of expression, it is reasonable to assume that some individuals in the population will exhibit a significantly increased sensitivity to OP exposure.

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