

Paraoxonase (PON1): from toxicology to cardiovascular medicine

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Abstract. Paraoxonase (PON1) is a liver and plasma enzyme most studied because of its ability to hydrolyze the active metabolites of several organophosphorus insecticides. The discovery that PON1 can also metabolize oxidized phospholipids has spurred research on its possible role in coronary heart disease and atherosclerosis. Additionally, its potential roles in metabolizing pharmaceutical drugs and microbial quorum sensing factors are also being explored. PON1 displays several polymorphisms that influence both its level of expression and its catalytic activity, thus determining the rates at which a given individual will detoxify a specific insecticide, metabolize harmful oxidized lipids, and activate or inactivate specific drugs and quorum sensing factors.

Key words: Paraoxonase (PON1) polymorphisms, organophosphates, PON1 status, coronary artery disease, drug metabolism, quorum sensing factors

Introduction

Paraoxonase (PON1) received its name from paraoxon, the active metabolite of the organophosphorus (OP) insecticide parathion, which is its first and one of its most studied substrates. As PON1 hydrolyzes the active metabolites of several other OPs (e.g. chlorpyrifos oxon, diazoxon), as well as nerve agents such as soman, sarin or VX, it has been studied mostly by toxicologists, interested in its role in modulating OP toxicity. Yet the discovery that PON1 can metabolize oxidized lipids (LDL and HDL) has spurred an extensive series of investigations on the potential role of PON1 in cardiovascular disease, particularly in atherosclerosis. Furthermore, the finding that PON1 and the related proteins PON2 and PON3 display lactonase activity, has provided evidence that they may be involved in drug metabolism and inactivation of quorum sensing factors as well. As a result, research in these fields has blended, with the goal of further elucidating the role of this polymorphic enzyme in disease and in an individual's response to exogenous agents.

PON1 and its human polymorphisms

PON1 is a member of a family of proteins that also includes PON2 and PON3, the genes of which are clustered in tandem on the long arm of human chromosome 7 (q21.22). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high density lipoprotein (HDL) particles (1-3).

Earlier studies had indicated that the plasma paraoxonase activity in human populations exhibited a polymorphic distribution, and individuals with low, intermediate or high paraoxonase activity could be identified (4, 5). Studies in the early 1990s led to the purification, cloning and sequencing of human PON1 (6, 7), and to the molecular characterization of its polymorphisms (8, 9). More recently, the molecular structure of an engineered recombinant PON1 protein has also been elucidated (10). Two polymorphisms are present in the PON1 coding sequence: a Gln(Q)/Arg(R) substitution at position 192, and a Leu(L)/Met(M) substitution at position 55 (8, 9).

The Q/R polymorphism at position 192 significantly affects the catalytic efficiency of PON1. Initial studies demonstrated that the PON1_{R192} allozyme hydrolyzes paraoxon more readily than PON1_{Q192} (8, 9). Further studies showed that this polymorphism was substrate-dependent, as the PON1_{Q192} alloform was found to hydrolyze diazoxon, sarin and soman more rapidly than PON1_{R192} *in vitro* (11). More recent studies, however, have shown that under physiological conditions, both PON1 alloforms hydrolyze diazoxon with nearly equivalent catalytic efficiencies (12). Gene frequencies of PON1_{Q192} range from 0.75 for Caucasians of Northern European origin, to 0.31 for some Asian populations (13).

The L/M polymorphism at position 55 does not affect catalytic activity, but has been associated with plasma PON1 protein levels, with PON1_{M55} being associated with low plasma PON1 (14, 15). However, this appears to result primarily from linkage disequilibrium with the low efficiency -108T allele of the T-108C promoter region polymorphism (13). Four additional polymorphisms have been found in the 5'-regulatory region of PON1 (16), but they have a lesser effect on PON1 protein level (17). Recent complete resequencing of PON1 from several individuals has led to the identification of nearly 200 new single nucleotide polymorphisms, some in the coding regions and others in introns and regulatory regions of the gene (18; <http://pga.gs.washington.edu>). Though most of the new polymorphisms have not yet been characterized, a few have already explained discrepancies found when comparing PON1 status and PCR analysis of codon 192 [see below; (18)].

PON1 and its role in organophosphate toxicity

PON1 hydrolyzes the oxygen analogs of several OPs; however, it does not hydrolyze directly the parent compound of such insecticides (i.e. parathion, chlorpyrifos, diazinon), nor several other OPs (e.g. malaxon, guthion, dichlorvos) (3). Nevertheless, the ability of PON1 to hydrolyze several OPs *in vitro* has long been taken as an indication that it may modulate OP toxicity *in vivo*. Furthermore, the presence of polymorphisms in PON1, which confer different en-

zyme levels or catalytic efficiencies, has suggested that certain individuals may be more susceptible to the toxic effects of OP exposure. Over the past 15 years, a series of studies in rodents has provided important evidence to ascertain the role of PON1 in modulating OP toxicity. In earlier studies, PON1 purified from rabbit serum was injected into rats or mice to increase plasma hydrolytic activity toward OP substrates. When challenged with an OP (chlorpyrifos oxon was used in most instances), animals which had received exogenous PON1 were significantly more resistant than controls to the acute cholinergic toxicity (19–21). Of interest is that PON1 provided some protection against chlorpyrifos, the parent compound that is used as insecticide, when given before or even after this OP (21).

More recently, transgenic and knockout mice have provided important new tools to investigate the role of PON1 in modulating OP toxicity. PON1 knockout mice, which have no detectable plasma or liver hydrolytic activity toward paraoxon and diazoxon and very limited chlorpyrifos-oxonase activity, have dramatically increased sensitivity to the toxicity of chlorpyrifos oxon and diazoxon, and a small increase in sensitivity to their respective parent compounds (12, 22). Surprisingly, they did not show an increased sensitivity to paraoxon (12).

Administration of pure human PON1_{Q192} or PON1_{R192} to PON1 knockout mice to restore plasma PON1 provided additional important information. PON1_{R192} provided better protection than PON1_{Q192} toward chlorpyrifos oxon, while both alloforms were equally effective in protecting against the toxicity of diazoxon (12). However, neither PON_{R192} nor PON1_{Q192} afforded protection against paraoxon toxicity (12). A kinetic analysis of substrate hydrolysis by purified human alloforms carried out under physiological conditions (low NaCl concentration) provided an explanation for the *in vivo* findings. In the case of chlorpyrifos oxon, the PON1_{R192} alloform had significantly higher catalytic efficiency (250 *vs.* 150) than the PON1_{Q192} alloform and provided better protection against CPO exposure. In the case of diazoxon, both PON1_{Q192R} alloforms had equivalent catalytic efficiencies (~76) and provided equivalent protection. With paraoxon, the PON1_{R192} alloform was much more effi-

cient than the PON1_{Q192} alloform (6 vs. 0.7) with *in vitro* assays, but its overall catalytic efficiency was very low and unable to confer protection *in vivo* (12). This confirmed the hypothesis that PON1 is not efficient at hydrolyzing paraoxon at low concentrations (23), indicating that human and mouse PON1s do not degrade paraoxon efficiently *in vivo*. Additional experiments carried out in PON1 transgenic mice (mice expressing either human PON1_{Q192} or PON1_{R192} on a knockout background) provided further evidence for the conclusions made from experiments administering PON1 to PON1 knockout mice. For example, hPON1_{R192}-TG mice were significantly less sensitive to the toxicity of chlorpyrifos oxon than hPON1_{Q192}-TG mice, despite having the same level of PON1 protein in liver and plasma (24).

Altogether, these animal experiments indicate that the role of PON1 in modulating the toxicity of OPs varies depending on the specific compound. In case of paraoxon, no apparent *in vivo* effect of PON1 is seen; with diazoxon, protection or susceptibility is dictated primarily by the level of expression of PON1, independently of the Q192R genotype; in the case of chlorpyrifos oxon, both the level of expression and the Q192R genotype are important determinants of susceptibility.

Genotype/Phenotype: the importance of determining an individual's PON1 status

Most studies investigating the association of PON1 polymorphisms with sensitivity to OP toxicity or with diseases (particularly cardiovascular diseases) have examined only one or more of the nucleotide polymorphisms Q192R, L55M, C-108T with polymerase chain reaction (PCR)-based assays that provide no information on plasma PON1 levels. A functional genomic analysis, however, provides a much more informative approach, as direct measurement of an individual's PON1 function (plasma activity) takes into account all polymorphisms that might affect activity. This is accomplished through the use of a high-throughput enzyme assay involving two PON1 substrates, paraoxon and diazoxon (25, 26). This approach, in addition to providing a functional assessment of the plasma PON1₁₉₂ alloforms, also provides

the plasma level of PON1 for each individual, thus encompassing the two factors that affect PON1 levels or activity. For example, sensitivity to diazoxon exposure (12) or risk of carotid artery disease (27) is influenced primarily by PON1 levels, which are not assessed by PCR-based assays. The catalytic efficiency with which PON1 degrades toxic OPs determines the degree of protection provided by PON1; in addition, higher concentrations of PON1 provide better protection. Thus, it is important to know PON1 levels and activity. This approach has been referred to as the determination of PON1 "status" for an individual (25). In a given population, plasma PON1 activity can vary up to 40-fold (4, 5, 11, 25), and differences in PON1 protein levels up to 13-fold are also present within a single PON1₁₉₂ genotype (3, 11). Recent studies investigating the role of PON1 in cardiovascular disease (see below) have indeed provided evidence that PON1 status (encompassing genotype and activity levels) is a much better predictor of disease than PON1 genotype alone (27).

Although it might be possible to determine all of the PON1 single nucleotide polymorphisms for a given individual, doing so would not provide an accurate assessment or prediction of the individual's PON1 status. On the other hand, a two-substrate enzymatic analysis provides an accurate assessment of an individual's PON1 level and position 192 functional status (i.e. the individual's PON1 functional genomics). These assays are described in detail by Richter et al. (26). Briefly, plasma samples are isolated from heparin-collected blood. Citrate plasma or serum can also be used for determination of PON1 status (though consistency of sample preparation is recommended). Plasma samples from blood collected in EDTA are not useful for these analyses as PON1 enzyme activity is calcium-dependent, and EDTA irreversibly inhibits PON1. The two-substrate assays are carried out using a Molecular Devices SPECTRAMax PLUS Microplate Spectrophotometer or equivalent instrument. Initial rates of substrate hydrolysis are determined, and rates of diazoxon hydrolysis (y axis) are plotted against the rates of paraoxon hydrolysis (x axis). Hydrolysis of paraoxon produces *p*-nitrophenol, monitored at 405 nm, while hydrolysis of diazoxon produces 2-isopropyl-4-methyl-6-hydroxypyrimidine,

monitored at 270 nm. This method separates individuals into the three phenotypes of PON1₁₉₂ activity, (PON1_{192Q/Q}, PON1_{192Q/R} and PON1_{192R/R}), allowing the functional PON1₁₉₂ genotype to be accurately inferred. In addition, PON1 activities within a genotype provide information on the levels of PON1 in the plasma of a given individual within that genotype group, which is most relevant to assess an individual's sensitivity to a specific OP. The PON1 phenotype at position 192 can be verified by genotyping the individual's DNA (26). However, because very few discordant samples are observed where PCR data and functional enzyme analysis are in disagreement (18), this functional genomic analysis is very accurate, and does not require verification (27). Examples of discordance have been observed where an individual genotypes as a PON1_{Q/R} heterozygote but has a PON1 status functional analysis indicating that they are a PON1_{192Q} or PON1_{192R} homozygote. This would indicate that one of the alleles is nonfunctional, underlining the advantage of the PON1 status analysis over straight genotyping. Several examples of this were reported recently, where the specific mutations inactivating one of the PON1 alleles were identified (28).

Though some of the variation in serum PON1 activity can be explained by its polymorphisms in the coding region (Q192R) and the 5'-regulatory region (T-108C), modulation of PON1 by a variety of other factors should also be considered (29). Alcohol, smoking, certain drugs, diet and certain physiological and pathological conditions can increase or decrease PON1 activity, and their contribution to measured PON1 levels should be not overlooked (29). It is also clear that as yet uncharacterized factors contribute significantly to the individual differences observed in plasma PON1 levels. Possible candidates include trans-acting factors, polymorphisms of intronic and exonic splice enhancer sequences, and other uncharacterized polymorphisms in 5' or 3' untranslated sequences.

Clinical evidence for a role of PON1 in OP toxicity

The studies summarized in the previous sections have characterized the PON1 polymorphisms responsible for different catalytic activities and for influenc-

ing levels of expression, have demonstrated the relevance of PON1 in modulating OP toxicity in various animal models, and have indicated the importance of an individual's PON1 status. However, direct confirmation in humans of the relevance of PON1 status in determining relative sensitivity to OP toxicity is still elusive, though it can be inferred from all previous findings. In some instances, only PON1 genotyping was carried out in humans, thus precluding an evaluation of PON1 status. For example, in the 1995 terrorist attack in the Tokyo subway, several thousand people were exposed to the nerve agent sarin, an OP which is metabolized preferentially by PON1_{192Q} (11). However, among 10 of the 12 victims, 7 expressed the PON1_{192Q} genotype, including 6 Q/R heterozygotes and 1 Q/Q homozygote (30). Thus, the genotype which confers higher sarin hydrolyzing activity (PON1_{192Q}) did not appear to provide protection from acute poisoning. In Caucasian populations the range of sarinase activity among individuals with the QQ or QR genotype ranged from 0 to 758 U/L (11). In addition, the catalytic efficiency of sarin hydrolysis by PON1 is low. Thus, as in the case of paraoxon, little protection by PON1 would be expected (31, 32). Indeed, for PON1 to be used for treating sarin or other nerve agent exposures, recombinant PON1 variants with increased catalytic efficiency will need to be developed and tested.

Other studies have been carried out in sheep dippers exposed to diazinon experiencing chronic central and/or peripheral nervous system abnormalities (33, 34), and in cohorts of veterans from the 1990-91 Persian Gulf War in the US and the UK, who were potentially exposed to OP insecticides and to sarin and were diagnosed with Gulf War Syndrome (35, 36). Such studies are suggestive of a role of PON1 polymorphisms in modulating sensitivity to OPs in humans, but do not allow any firm conclusion (31, 32).

Of interest is the finding that PON1 activity is low in neonates and young children (as well as young animals), suggesting that PON1 may modulate the age-dependent sensitivity to OPs (37). The findings also suggest that PON1 levels may be even lower before birth. Indeed, early studies on A-esterase activities in premature infants showed even lower levels

than in newborns (57). In addition, an expectant mother with low PON1 status would be predicted not to be able to provide protection for her fetus against exposure to some OPs. Recently, offspring of mothers with low PON1 status exposed *in utero* to chlorpyrifos were reported to have significant smaller head circumference compared to those born to mothers with high PON1 activity or those not exposed to chlorpyrifos (38). Since small head size has been found to be predictive of subsequent cognitive ability, these findings suggest that prenatal exposure to chlorpyrifos may have even more detrimental long-lasting effects in offspring of mothers with low PON1 activity.

It is clear that more and better-designed studies are needed in human populations to address directly the issue of whether an individual's PON1 status may confer protection or increased sensitivity to the toxicity of specific OPs. In particular, better indications of the level and nature of exposure and of the consequences of the exposure need to be documented, as well as proper determination of PON1 status (25, 31, 32).

PON1 and coronary heart disease

In recent years, PON1 has taken center stage in research endeavors aimed at identifying possible risk factors for coronary heart disease (CHD) (3, 39–43). Among such risk factors, one of the most significant is low levels of HDL (44). In addition to its role in the reverse cholesterol transport pathway, HDL is believed to protect against atherosclerosis by inhibiting or metabolizing the oxidative modifications of LDL that play an integral role in the initiation and progression of atherosclerosis (43). The antioxidant properties of HDL are dependent on its containing PON1 which has been shown to metabolize active oxidized phospholipids and to destroy lipid hydroperoxides (45). PON1 can modulate oxidation in both LDL and HDL. Overall, *in vitro* studies have shown that PON1 has esterase, peroxidase-like and phospholipase-like activities that either inhibit the formation of proinflammatory oxidized phospholipids or degrade them once they are formed (3). This antioxidant capacity of PON1_{192Q} has been reported to be more effi-

cient than that of PON1_{192R} toward oxidized lipids (47, 49).

Studies in genetically modified mice have added strong evidence for a role of PON1 in development of arteriosclerotic lesions. PON1 knockout mice fed a high-fat diet exhibited larger aortic arteriosclerotic lesions than wild-type mice (22). In contrast, transgenic mice expressing human PON1 (L55, Q192) had significantly decreased arteriosclerotic lesions (46). Consistent with these *in vivo* findings, HDL isolated from PON1 knockout mice and human hPON1 transgenic mice had no ability, or an increased ability, respectively, to prevent LDL oxidation *in vitro*. Additional studies have shown that PON1_{Q192} is twice as efficient as PON1_{R192} in reducing total lipid peroxide content of human arteriosclerotic lesion homogenates (47).

Prompted by these *in vitro* and *in vivo* experimental findings, a large number of population studies have tested the association of the PON1_{Q192R} and PON1_{L55M} polymorphisms with CHD. Of these studies, about half yielded significant results, where the R192 allele was consistently associated with CHD (3, 40). A recent meta-analysis of 43 studies indicated a relative risk of the R192 allele of 1.12 (95% CI = 1.07 – 1.16), with no significant overall associations for other polymorphisms (48). The PON1_{R192} and PON1_{55L} alleles are in strong linkage disequilibrium, and the PON1_{L55} genotype predicted CHD in some studies, including one in which the PON1₁₉₂ genotype did not predict disease. However, the PON1_{L55} polymorphism is in linkage disequilibrium with the C-108 promoter polymorphism, which is correlated with higher plasma PON1 levels (13). Hence the importance, as pointed out earlier, of determining each individual's PON1 status, which encompasses both plasma PON1 levels as well as PON1₁₉₂ genotypes (25). Indeed, PON1 phenotype has been shown to be a better predictor of vascular disease than the PON1₁₉₂ or PON1₅₅ genotype (27), a finding that has been confirmed in other studies (18, 28, 50). Fundamental biochemical principles dictate that higher levels of enzyme will metabolize oxidized lipids at higher rates. Thus, it is inappropriate to ignore PON1 levels in studies examining associations of PON1 genetic variability with diseases.

PON1 as a lactonase: role in drug metabolism

While PON1 has been characterized as an esterase, PON2 and PON3 lack any significant paraoxonase activity (51). All three PONs share, however, the ability to hydrolyze aromatic and long chain aliphatic lactones, suggesting that their primary activity may be that of lactonases (51–53). Lactone derivatives of arachidonate, such as 5-HETE lactone and 5-iodolactone, are hydrolyzed by PON1 and have been suggested as potential endogenous substrates (52). PON1's lactonase activity has been utilized in the development of locally acting glucocorticoid drugs (e.g. glucocorticoid γ -lactones) which undergo rapid hydrolysis and inactivation when they reach the circulation (51, 54). Statin lactones (simvastatin, lovastatin) and the diuretic spironolactone, previously reported to be hydrolyzed by PON1, are instead metabolized by PON3 (51). PON1, however, hydrolyzes and activates the antibacterial drug prulifloxacin, with PON1_{R192} displaying higher activity (55). Though evidence is still limited, novel classes of so-called pro-drugs may be developed by incorporating a lactone or cyclic carbonate variety into their molecules, which can be inactivated or activated *in vivo* by PON1. Recently, PON1 and PON2 have been shown to hydrolyze the N-acylhomoserine lactones used by *Pseudomonas* as quorum signaling molecules that control pathogenesis and biofilm formation (56). Other lactonase activities are associated with the metabolism of lipid mediators generated from the oxidation of polyunsaturated fatty acids (56).

Conclusions

This brief overview highlights how the study of PON1 has evolved in the past two decades from the field of insecticide toxicology to those of cardiovascular medicine and of drug metabolism. Polymorphisms in the PON1 gene influence both the quantity and the quality of PON1 (i.e. PON1 status). Available evidence indicates that PON1 levels in all cases, and the Q192R polymorphisms in some cases, determine the rate at which a given individual will detoxify a specific insecticide, metabolize harmful oxidized lipids and

quorum signaling molecules, and activate or inactivate specific drugs (3). Given the high occurrence of OP poisonings, the widespread incidence of CHD, and the robust market of pharmaceuticals, research on PON1 is undoubtedly destined to continue and to expand.

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