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Paraoxonases-1, -2 and -3: What are their functions?

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ABSTRACT

Paraoxonase-1 (PON1), an esterase/lactonase primarily associated with plasma high-density lipoprotein (HDL), was the first member of this family of enzymes to be characterized. Its name was derived from its ability to hydrolyze paraoxon, the toxic metabolite of the insecticide parathion. Related enzymes PON2 and PON3 were named from their evolutionary relationship with PON1. Mice with each *PON* gene knocked out were generated at UCLA and have been key for elucidating their roles in organophosphorus (OP) metabolism, cardiovascular disease, innate immunity, obesity, and cancer. PON1 status, determined with two-substrate analyses, reveals an individual's functional Q192R genotype and activity levels. The three-dimensional structure for a chimeric PON1 has been useful for understanding the structural properties of PON1 and for engineering PON1 as a catalytic scavenger of OP compounds. All three PONs hydrolyze microbial N-acyl homoserine lactone quorum sensing factors, quenching *Pseudomonas aeruginosa*'s pathogenesis. All three PONs modulate oxidative stress and inflammation. PON2 is localized in the mitochondria and endoplasmic reticulum. PON2 has potent antioxidant properties and is found at 3- to 4-fold higher levels in females than males, providing increased protection against oxidative stress, as observed in primary cultures of neurons and astrocytes from female mice compared with male mice. The higher levels of PON2 in females may explain the lower frequency of neurological and cardiovascular diseases in females and the ability to identify males but not females with Parkinson's disease using a special PON1 status assay. Less is known about PON3; however, recent experiments with PON3 knockout mice show them to be susceptible to obesity, gallstone formation and atherosclerosis. Like PONs 1 and 2, PON3 also appears to modulate oxidative stress. It is localized in the endoplasmic reticulum, mitochondria and on HDL. Both PON2 and PON3 are upregulated in cancer, favoring tumor progression through mitochondrial protection against oxidative stress and apoptosis.

1. Introduction

This brief overview will discuss the paraoxonase family of enzymes which includes paraoxonases 1, 2 and 3 (PON1, PON2 and PON3, respectively). The three genes encoding the PONs are located in tandem on the long arm of human chromosome 7 (7q21-22). This family of enzymes derives its nomenclature from early studies on PON1 where it was shown to hydrolyze the toxic metabolite of parathion (PS), paraoxon (PO) using *in vitro* assays. Aldridge had classified organophosphate (OP) hydrolases as either A- or B-

Abbreviations: AD, Alzheimer's disease; *apoE*$^{-/-}$, *apolipoprotein E* knockout; AREase, arylesterase; Blmh, bleomycin hydrolase; BPHL, biphenyl hydrolase-like protein; CAAD, carotid artery disease; CNS, central nervous system; CVD, cardiovascular disease; CPO, chlorpyrifos oxon; CPS, chlorpyrifos; DHC, dihydrocoumarin; DMNQ, 2,3-dimethoxy-1,4-naphtoquinone; DZO, diazoxon; DZS, diazinon; E600, diethyl *p*-nitrophenyl phosphate or paraoxon; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; GSH, glutathione; H_2O_2, hydrogen peroxide; HCTL, homocysteine thiolactone; HCTLase, homocysteine thiolactonase; HDL, high-density lipoprotein; HIV, human immunodeficiency virus; kDa, kilodalton; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LDL, low-density lipoprotein; *LDLR*$^{-/-}$, *low-density lipoprotein receptor* knockout; MW, molecular weight; NADPH, nicotinamide adenine dinucleotide phosphate; OP, organophosphate; PD, Parkinson's disease; PO, paraoxon; POase, paraoxonase; PON1, PON2, PON3, paraoxonase-1, -2, -3; *PON1*$^{+/+}$, *PON2*$^{+/+}$, *PON1* and *PON2* wild-type; *PON1*$^{-/-}$, *PON2*$^{-/-}$, *PON3*$^{-/-}$, *PON1*, *PON2* and *PON3* knockout; PS, parathion; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SNV, single nucleotide variant; UCLA, University of California Los Angeles; UTR, untranslated region.

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esterases, based on whether they catalytically hydrolyzed OP compounds (A esterases) or were irreversibly inhibited by binding an OP compound (B esterases) [1]. PON1 was shown to be an A-esterase, catalytically hydrolyzing PO. Following the discovery of the linked genes PON2 and PON3, they were also termed paraoxonases although neither one hydrolyzed PO [2]. Following discussions on the appropriate nomenclature for the PONs, it was agreed that naming of the three PONs should be delayed until the natural physiological substrates were identified [3]. However, the range of physiologically relevant substrates remains an open question.

2. PON1

PON1, the most studied of the PONs, is a 45-kDa glycoprotein synthesized in the liver and found mainly in high-density lipoproteins (HDLs). Earlier results showing the transfer of PON1 between membranes [4] and the localization of PON1 in multiple mouse tissues [5] suggested that PON1 is transferred via HDLs from the liver to tissues where its activity is needed. PON1 is a highly promiscuous calcium-dependent enzyme, capable of hydrolyzing a wide range of substrates, from OPs to aromatic carboxylic acid esters, quorum sensing signal molecules (N-acyl homoserine lactone) and lipo-lactones.

2.1. Early studies

Detailed studies by Aldridge reported in 1953 categorized serum esterases into those inhibited by diethyl *p*-nitrophenyl phosphate (E600 or PO) as B-esterases and those not inhibited by PO as A-esterases since they preferred the substrate *p*-nitrophenyl acetate to the longer chain esters [1]. The B-type esterases were inhibited by 10^{-7} to 10^{-8} M E600 and hydrolyzed the *p*-nitrophenyl butyrate at the same or higher rate than *p*-nitrophenyl acetate. In a second publication, Aldridge noted that the rabbit had the highest serum A-esterase of the species examined and the ratio of the serum to liver activity was very high while the opposite was true in rat [6]. The high activity of the rabbit plasma A-esterase facilitated the development of purification protocols for the human serum A-esterase as well as the cloning of both the rabbit and human cDNAs discussed below. Aldridge's studies also indicated that PO and *p*-nitrophenyl acetate were hydrolyzed by the same enzyme. Later, there had been some controversy as to whether phenyl acetate and PO were hydrolyzed by one or two enzymes resulting in a reclassification of PON1 [3]. It has since been clearly shown that both substrates are hydrolyzed by PON1 as originally proposed by Aldridge [7,8].

Studies on human A-esterase in the 1960s and 1970s revealed that plasma paraoxonase (POase) activity showed a large inter-individual variability in activity and that the activity was polymorphically distributed in human populations with differences among populations in the frequency of the "low activity allele" *vs.* the "high activity allele" (reviewed in Ref. [9]). In Northern European populations, approximately 50% of individuals are homozygous for the low activity allele, while populations of African and Asian origin had a higher frequency of individuals homozygous for the high activity allele with other populations having even higher frequencies of the high activity allele (reviewed in Refs. [9,10]). The early studies made use of a single substrate, PO and presented data as histograms of activity (reviewed in Ref. [9]). In the early years, many different assays were developed for characterizing the A-esterase activity in individuals. These assays used variable conditions of pH, salt and inhibitor (EDTA) to characterize the A-esterase activity (reviewed in Ref. [11]). Based on the high variability of serum POase activity among individuals, it was proposed that individuals with high activity levels would be resistant to exposures of PS/PO. The oxon form of the insecticide is also included in exposures as most, if not all, exposures contain a variable percentage of the highly toxic oxon [12–14]. As noted below, human plasma POase does not appear to provide protection against PS/PO exposures as the catalytic efficiency of PO hydrolysis is too low.

Efforts aimed at purifying PON1 first revealed that gel filtration chromatography resolved two peaks of activity, a higher molecular weight peak and a lower molecular weight peak. The low molecular weight activity peak co-fractionated with albumin [11]. The availability of plasma from an analbuminemic individual provided solid evidence that the lower molecular weight activity was indeed associated with albumin. The activity associated with the albumin peak was completely absent in the plasma from the analbuminemic individual. Resolution of the two activity peaks allowed for the characterization of the pH optimum of each peak. The albumin-associated activity was active at only high pH values whereas the POase activity fractionating at higher molecular weight retained significant activity at pH 8.5. This study provided three key observations that facilitated purification and ultimately cloning of rabbit and human cDNAs; 1) the POase activity of the albumin peak had little activity below pH 8.5; 2) the POase activity associated with albumin was resistant to inhibition by EDTA; and 3) the albumin peak did not hydrolyze phenyl acetate. Unfortunately, some studies are still carried out that measure PON1 activity at high pH values. In an individual with low PON1 levels, at the high pH value, albumin hydrolyzes more PO than their PON1 [11].

2.2. Post cloning studies

Serum rabbit PON1 is significantly more stable than human PON1 and was purified and sequenced to allow the design of probes for isolating and sequencing rabbit *PON1* cDNA from a rabbit liver cDNA library [15]. The property of being capable of activity staining rabbit PON1 following SDS gel electrophoresis provided assurance that the protein sequenced was indeed PON1 [15]. Probe design based on the sequence of rabbit PON1 allowed for the isolation of the rabbit *PON1* cDNA which in turn allowed for the isolation and characterization of human *PON1* cDNA [16]. The human cDNA clones revealed the two common coding polymorphisms occurring in human PON1 (L55 M and Q192R). The latter was subsequently shown to determine the catalytic efficiency of hydrolysis of PO [17,18]. The Q192R polymorphism did not significantly affect the catalytic efficiency for hydrolysis of phenyl acetate (arylesterase activity – AREase) or diazoxon (DZO) but did affect the catalytic efficiency of PO, chlorpyrifos oxon (CPO) [19] and the nerve agents sarin and soman [20]. In addition to revealing the two common coding region polymorphisms, sequencing revealed single nucleotide variants (SNVs) in the 5′ and 3′ UTRs. Examination of the effects of the 5′ SNVs on expression of PON1 revealed that the C-108T 5′ UTR promoter region polymorphism in an Sp1 transcription factor binding site had a significant effect on PON1 expression with the C-108 allele expressing on average twice the level of PON1 compared with the T-108 allele [21–23]. An interesting report noted that a 3′ UTR SNV (CT at rs3735590) affected binding of a miRNA (miR-616) to the 3′ UTR of PON1 mRNA with the T allele having lower binding affinity, causing higher levels of expression of PON1 and reducing the risk of ischemic stroke and carotid atherosclerosis in individuals with the CT or TT genotype [24]. The effects of SNVs in the 3′ UTR regions on gene expression plus the competing/stabilizing effects of RNA binding proteins (e.g. Ref. [25]) warrant further studies.

Sequencing studies in which the *PON1* genes of 47 individuals (24 African-Americans and 23 Europeans) revealed 108 new PON1 polymorphisms, including 8 new promoter region SNVs, 9 additional 3′ SNVs and a new coding region SNV (W194X) [26].

Discovery of the latter SNV prompted the sequencing of *PON1* genes where we had observed a discrepancy between the characterization of DNA SNVs and the determination of the functional PON1 status (Q192R polymorphism and PON1 activity levels – see below). Sequencing of the *PON1* genes from individuals discrepant for the functional analyses and SNV analysis of the Q192R polymorphism revealed a P90L SNV, an Asp124missplice mutation and a partial deletion of the *PON1* allele bearing the Q192 genotype [27]. Recently, a PON1 V109I SNV has been associated with ischemic stroke in African-Americans [28].

2.3. PON1 and OP insecticide sensitivity

While the early measurements of variability in inter-individual activity levels of human PON1 suggested that high levels would protect against exposure to PS, it took the development of an animal model system to understand the ability of PON1 to protect against specific OP exposures. The earliest direct test of PON1 protecting against OP exposure was carried out by Main in 1956 [29] where he injected partially purified rabbit PON1 intravenously into rats raised the plasma activity of the A-esterase and decreased the toxicity of PO. Experiments with rats [30] and mice [31,32] extended Main's initial observations to include both PO and CPO. CPO was a much better human PON1 substrate than PO. Taken together, these experiments clearly demonstrated that high levels of injected rabbit PON1 provided some protection against PO, but significantly better protection against CPO. The question of the consequences of low PON1 levels was addressed with *PON1* knockout mice (*PON1*$^{-/-}$) mice generated by Shih, Lusis and colleagues at UCLA [33]. The *PON1*$^{-/-}$ mice were dramatically more sensitive to CPO and DZO than wild type mice, but surprisingly did not exhibit increased sensitivity to PO compared with *PON1*$^{+/+}$ mice [19]. The *PON1*$^{-/-}$ mice also provided a model system for testing under physiological conditions the efficacy of purified [19] and recombinant engineered human PON1 [8] to protect against OP exposure when injected into the *PON1*$^{-/-}$ mice. Experiments with the two purified human PON1$_{192}$ alloforms demonstrated that protection against specific OPs was determined by the catalytic efficiency with which PON1 hydrolyzed the specific OP. Again, these experiments demonstrated that human PON1 was not effective in protecting against PO exposures. The PON1$_{R192}$ alloform protected better than the PON1$_{Q192}$ alloform against CPO exposures while either alloform protected equally well against DZO exposure [19].

Since the *PON1*$^{-/-}$ mice have no measurable activity against DZO, it was straightforward to follow the half-life of injected engineered recombinant human PON1. Experiments with PON1$_{K192}$ showed that this engineered PON1 variant provided good protection against DZO exposure when injected pre- or post-exposure and was able to provide protection for at least 48 h post-injection [8]. The R/Q192K substitution was based on the high rate of CPO hydrolysis by rabbit PON1$_{K192}$ [34].

Examination of the developmental time course of appearance of PON1 in newborn humans showed that at birth, babies had only one fourth to one third adult levels of PON1 and that they required 6 months to 2 years to reach adult levels of PON1, indicating increased sensitivity of the very young to exposures of chlorpyrifos (CPS)/CPO or diazinon (DZS)/DZO. The oxon metabolites are listed in the exposures since levels of oxon are detected in most if not all exposures as noted above [12–14].

In addition to the *PON1*$^{-/-}$ mice, Shih, Lusis and Tward also generated transgenic mice that expressed human PON1$_{Q192}$ (tgHuPON1$_{Q192}$) and HuPON1$_{R192}$ (tgHuPON1$_{R192}$) on the mouse *PON1*$^{-/-}$ background which provided an animal model in which to characterize the two human PON1$_{192}$ alloforms under physiological conditions [35]. The experiments with the transgenic mice showed that the efficacy of protection by each human PON1$_{192}$ alloform was consistent with the experiments where purified human PON1 was injected into the *PON1*$^{-/-}$ mice. Two other important observations came from experiments with the genetically modified mice. When the entire human *PON1* genes (*PON1*$_{Q192}$ and *PON1*$_{R192}$) were introduced into the *PON1*$^{-/-}$ mice complete with the 5′ promoter regulatory sequences, rather than following the human time course for developmental expression (6 months–2 years), the expression followed the developmental time course of PON1 appearance in mice, peaking at 3 weeks of age indicating a high degree of conservation of the regulatory components of the *PON1* genes between man and mouse. Importantly, a comparison between the OP sensitivity of wild type mice (*PON1*$^{+/+}$) and *PON1*$^{-/-}$ mice showed that by 4 days of age, the *PON1*$^{+/+}$ mice were already 2.5 times more resistant to CPO exposure than the *PON1*$^{-/-}$ mice [36].

Taken together, all of the experiments on PON1 and resistance to OP exposure indicated that PON1 is important in modulating exposures to CPS/CPO and DZS/DZO, but not to PS/PO or the nerve agents sarin and soman. Engineering more catalytically efficient variants of PON1 will be required for treating exposures to OPs that are hydrolyzed at low catalytic efficiency by PON1. Work by Harel and colleagues provided a variant of PON1 that could be crystallized to provide a tentative 3D structure of PON1, a six-bladed beta-sheet propeller structure [37]. This structure has provided the basis for further engineering of PON1 for higher catalytic efficiency of hydrolysis of nerve agents (e.g. Refs. [38,39]).

2.4. Physiological functions of PON1

2.4.1. Lipid metabolism

The demonstration by Mackness and colleagues in 1991 that PON1 could prevent the accumulation of lipid peroxides in low-density lipoproteins (LDL) [40] generated an avalanche of papers examining the relationship of the genetic variability in PON1 to disease. We developed two approaches for examining PON1 genetic variability; 1) protocols for characterizing PON1 SNVs [17,21] and 2) activity measurements with two substrates when plotted as two-dimensional plots provided both the functional position 192 genotype as well as the activity levels of PON1. The development of the protocols characterizing *PON1* SNVs provided convenient protocols for epidemiologists to look for associations between the genetic variability of PON1 and disease. However, SNV analysis alone provided no information on the activity levels of PON1 that vary by at least 13-fold among individuals (Fig. 1). Since it is the PON1 activity that determines rates of detoxification/hydrolysis of both endogenous substrates as well as xenobiotics, the activity measurements are the most important factor to characterize in examining risk of exposure or disease. We termed the two-substrate analysis PON1 status (position 192 functional genotype and activity level) [31]. The two substrate analysis initially involved plotting rates of DZO hydrolysis *vs.* PO hydrolysis [41]. Further development of the PON1 status analysis protocol provided assays that could be carried out without the use of the highly toxic OP substrates. A comparison of the two PON1 status analyses is shown in Fig. 1 [42].

For epidemiological studies, since the two PON1$_{192}$ alloforms have quite different rates of hydrolysis of substrates, it is important to analyze the functional genotypes separately or adjust for them in analyses. An example of this analysis applied to a study of PON1 variability and carotid artery disease (CAAD) is shown in Fig. 2 [43]. Note that individuals with carotid artery disease had lower activity of PON1 than control subjects without CAAD. This study was carried out in a population of primarily Northern European origin where only approximately 10% of the population is homozygous for

PON1$_{R192}$. Cohorts of African and Asian origin with much higher frequencies of *PON1*$_{R192}$ homozygotes should be characterized to better understand the contribution of variability in levels of *PON1*$_{R192}$ to CAAD. Two reviews have addressed the issue of SNV analysis *vs.* activity measurements in epidemiological studies that examine the relationship of PON1 genetic variability to risk of disease or exposure [26,44]. Unfortunately, status assays (activity levels) have not been well-developed for PONs 2 and 3, and assays for PON1 activity cannot be performed on plasma from the EDTA tubes used for most epidemiological studies, due to PON1 inactivation secondary to calcium depletion (sodium lithium tubes are optimal).

In addition to PON1 status, PON1 concentration should also be measured when possible. In this regard, it is worth examining Figure 8 from the publication by Besler et al. [45] which shows high PON1 activity in healthy individuals, but low activity in individuals with CVD, data consistent with other studies by Camps, Joven and colleagues [46–49] [50]. Measurement of PON1 protein levels, however show that there is apparently much higher levels of inactive or less active PON1 in the diseased individuals, suggesting that an additional measurement of PON1 protein levels (by mass spectrometry, quantitative Western blot or ELISA) will provide additional information on PON1 status and risk of disease. These data indicate that PON1 is most likely being damaged by oxidative stress.

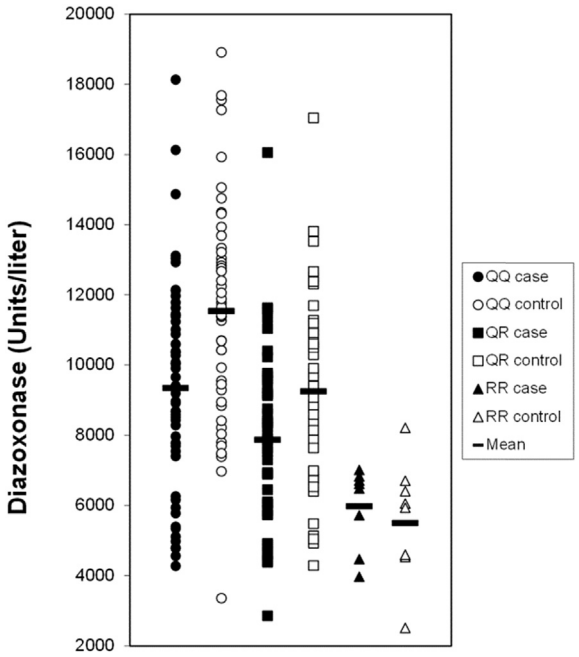

Fig. 2. Hydrolysis activity phenotype distributions in cases and controls stratified by *PON1*$_{192}$ genotype. Reproduced with permission from Jarvik et al. [43].

2.4.2. Quorum sensing

All three PONs inactivate the *Pseudomonas* quorum sensing factor N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) [51]. An elegant experiment carried out by Stoltz and colleagues at the University of Iowa demonstrated that a transgenic *Drosophila melanogaster* expressing human tgHuPON1 was resistant to the lethality of infection by *Pseudomonas aeruginosa* as well as resistant to CPS exposure [52].

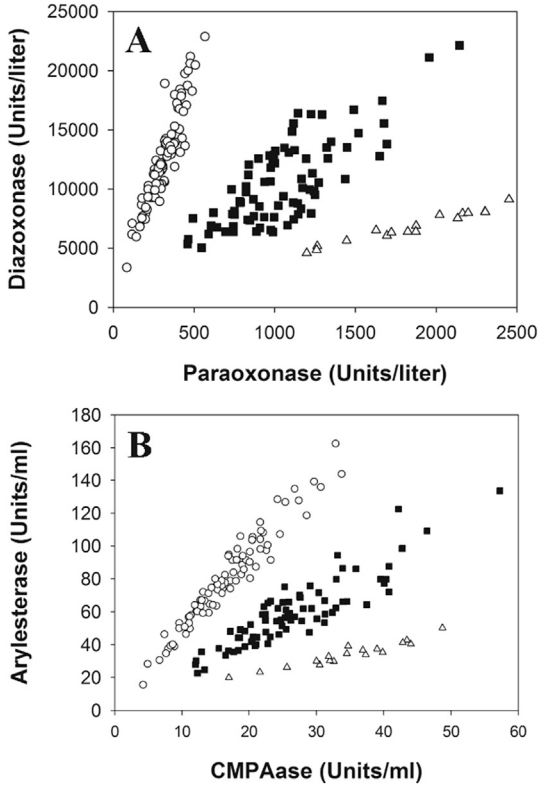

Fig. 1. Comparison of the two protocols for determining PON1 status. A, Assays using the highly toxic OP substrates DZO and PO. B, Assays using the non-OP substrates phenyl acetate and 4-(chloromethyl)phenyl acetate (CMPA). (O) indicates *PON1*$_{Q/Q192}$; (■), *PON1*$_{Q/R192}$; (Δ), *PON1*$_{R/R192}$. Reproduced with permission from Richter et al. [42].

2.4.3. Homocysteine thiolactonase

High plasma levels of the amino acid homocysteine lead to the synthesis of homocysteine thiolactone (HCTL), a toxic metabolite that results from an error-editing process catalyzed by methionyl tRNA-synthetase. HCTL has the ability to interact with and modify proteins, resulting in protein inactivation and loss of function. In fact, hyperhomocysteinemia is considered a risk factor for the development of a wide range of diseases associated with cardiovascular, neurological and autoimmune diseases [53–56]. HCTL is hydrolyzed *in vitro* by enzymes with homocysteine thiolactonase (HCTLase) activity, such as serum PON1 [57] and the intracellular bleomycin hydrolase (Blmh) [58]. However, the reported low substrate affinity and very low specific activity for hydrolysis of HCTL by PON1 [57,59] have raised doubts about the physiological relevance of this activity [10,60]. Recently, a more physiologically relevant HCTLase has been identified in the enzyme biphenyl hydrolase-like protein (BPHL) *in vitro* [61]. BPHL, also called valacyclovir (VC) hydrolase or valacyclovirase, is highly expressed in human liver and kidney and at lower levels in heart, intestine and skeletal muscle [62]. BPHL hydrolyzes and activates the antiviral prodrug esters VC and valganciclovir [63]. We found that BPHL has a catalytic efficiency 7700-fold higher than PON1 and 77-fold higher than Blmh. Thus, an important physiological function of BPHL, which had not been previously described, appears to be detoxification of HCTL. Understanding the mechanism of HCTL detoxification by BPHL *in vivo* is an essential issue that has not yet been addressed.

3. PON2

PON2 is a ≈43-kDa ubiquitously expressed intracellular enzyme, but unlike PON1 and PON3, it is not present in plasma

[5,64–66]. PON2 mRNA and/or protein have been detected in several tissues including liver, lung, kidney, heart, pancreas, small intestine, muscle, testis, endothelial cells, tracheal epithelial cells, and macrophages [64,65,67–69]. In mice, the highest levels were found in lung and small intestine, followed by heart and liver, with lower levels in testis, kidney and brain [66]. Of particular interest from the latter study was the novel observation that in all tissues PON2 expression was always significantly higher in female than in male mice [66]. Sub-cellular distribution studies have shown that PON2 is localized primarily in the mitochondria and the endoplasmic reticulum [2,65,66,70,71].

3.1. PON2 activity and SNVs

Phylogenetic analysis suggests that PON2 is the oldest PON family member, from which PON1 and PON3 have evolved [72]. PON2 does not have the OP-hydrolyzing activities of PON1, but as the other two PONs, it is a lactonase, displaying overlapping but distinct substrate specificities for lactone hydrolysis [73]. In particular, PON2 has the highest hydrolytic activity of the PONs toward a number of acyl-homoserine lactones (acyl-HCL), molecules which mediate bacterial quorum-sensing signals, important in regulating expression of virulence factors and in inducing a host inflammatory response [73–76]. Two common SNVs have been found in human *PON2*, an Ala/Gly substitution at position 147, and a Ser/Cys substitution at position 311 [2,64]. The PON2 S311C SNV has been shown to affect lactonase activity [77]. Carriers of the Cys311 allele have been found to be at risk for myocardial infarction and other cardiovascular diseases (CVD), as well as for Alzheimer's disease (AD) in several studies [78–82].

3.2. PON2 physiological function

In several tissues, PON2 has been shown to exhibit antioxidant properties [65]. PON2 antagonizes oxidative stress generated by various sources in the intestine of humans and rats [68], in human vascular endothelial cells [71], in lung epithelial carcinoma cells [76], in Caco-2/15 intestinal epithelial cells [83], and in mouse macrophages [67]. These antioxidant effects of PON2 are believed to play a major role in preventing the atherosclerotic process, as shown by studies indicating that PON2 over-expression decreases atherosclerotic lesions, while the opposite is true in *PON2*-null ($PON2^{-/-}$) mice [84,85]. In macrophages, PON2 has been suggested to protect against accumulation of triglycerides and oxidative stress, thereby attenuating the development of vascular complications in diabetes [86,87]. Mitochondria are a major source of free radical-related oxidative stress [88], and the preponderant localization of PON2 in mitochondria would support a role for this enzyme in protecting cells from oxidative damage. In HeLa cells, PON2 has been shown to bind to coenzyme Q10 that associates with mitochondrial complex III, and PON2 deficiency causes mitochondrial dysfunction [70]. In human endothelial cells PON2 has been shown to reduce, indirectly but specifically, the release of superoxide from the inner mitochondrial membrane, without affecting levels of other radicals such as hydrogen peroxide (H_2O_2) and peroxynitrite [89]. Also of interest is that the C311S SNV, which influences lactonase activity [77], does not appear to affect PON2's antioxidant properties, suggesting independent hydrolytic and antioxidant functions [89]. PON2 also appears to exert anti-inflammatory effects. In the gastrointestinal tract, PON2 antagonizes oxidative and inflammatory processes that may affect mucosal integrity [68]. The absence of PON2 in $PON2^{-/-}$ mice exacerbates the macrophage inflammatory response [90]. Furthermore, PON2 acts as a potent anti-inflammatory agent against the inflammatory response caused by administration of pyocyanin (a quorum sensing signal factor) [91].

3.3. PON2 in brain

PON2 mRNA has also been found in mouse and human brain [2,64,65,92], and PON2 protein has been detected in mouse [5,85] and monkey brain [93]. In a series of recent studies, the protein expression of PON2 has been characterized in mouse brain [66,94,95]. The highest levels of PON2 protein were found in three dopaminergic regions, the substantia nigra, the striatum, and the nucleus accumbens, with lower levels in cerebral cortex, cerebellum, hippocampus and brainstem. In every brain region, PON2 levels were higher (by ~2–3-fold) in female mice than in male mice. The higher levels of PON2 in dopaminergic areas are of interest, as they may be related to the higher levels of oxidative stress, due to dopamine metabolism, present in these regions. The regional distribution and gender difference of PON2 was confirmed by measurements of its lactonase activity [measured by dihydrocoumarin (DHC) hydrolysis] and of PON2 mRNA levels [66]. In brain, and to a lesser extent in kidney and testis, but not in all tissues, the PON2 antibody recognized two bands, the lower at MW ~43 kDa, which corresponds to the reported MW of PON2, and an upper band at MW ~55 kDa. This upper band had been found at times by some investigators [71,84,96–98], but not by others [65,68,83,99], and may represent a PON2 alloform, in accordance with the two mRNA splice variants [64,71,76]. However, its exact nature and function have not yet been defined. Though PON2 is known to have four putative N-linked glycosylation sites at asparagine residues [77], deglycosylation experiments indicated that both putative alloforms are glycosylated [66]. Thus, purification of the upper band, and its analysis (e.g. by mass spectrometry) are needed to identify its structural features and other potential post-translational modifications. Nevertheless, neither band was detected in brain from PON2-deficient mice [66]. Also of interest, PON1 was detected at very low levels in all brain areas and did not show any regional brain or gender differences [66]. Such low levels in tissue homogenates may be due to residual blood, as no PON1 could be detected in striatal astrocytes or neurons [95]. PON3 was not detected in any brain region (either homogenate or cells).

PON2 protein levels (by Western blot), mRNA (by qRT-PCR) and activity (by DHC hydrolysis) were also examined in astrocytes and in neurons isolated from several brain regions. PON2 was significantly higher in astrocytes than in neurons in all brain regions, with the highest levels in cells isolated from the striatum. Striatal neurons and astrocytes isolated from female mice expressed higher levels of PON2 than the same cells from male animals. PON2 was also present in cortical microglia, at levels similar to those found in neurons [66]. The sub-cellular distribution of the PON2 protein was assessed in cerebellar granule neurons and cerebellar astrocytes, and found to be similar in astrocytes and neurons. Differences were found in the localization of the 43 kDa lower band (the putative PON2) and the upper 55 kDa band (the putative PON2 alternate alloform). In both cell types, the highest levels of 43 kDa PON2 were found in mitochondria, followed by membranes (microsomes), in agreement with previous observations in HeLa cells [70]. PON2 was not detected in the cytosolic, nuclear, or cytoskeletal fractions. In contrast, the upper band PON2 alloform was expressed at highest levels in the nucleus and the cytoskeleton, neither of which contained significant levels of the 43 kDa band [66].

As in other peripheral tissues, PON2 exerts a protective effect toward oxidative stress and neuroinflammation in brain cells. The cytotoxicity of two known oxidants, H_2O_2 and 2,3-dimethoxy-1,4-naphtoquinone (DMNQ), was investigated in cerebellar and striatal astrocytes and neurons isolated from wild-type ($PON2^{+/+}$) and $PON2^{-/-}$ mice. In all instances, cells from mice lacking PON2 were

more susceptible to the toxicity of both compounds, by a factor of 5–11-fold [66]. The protection afforded by PON2 to neurons and astrocytes was related to its ability to scavenge reactive oxygen species (ROS) upon exposure to oxidants. For example DMNQ (10 μM) increased ROS to ~400% of basal in neurons from *PON2*$^{-/-}$ mice, and only 170% in the same cells from *PON2*$^{+/+}$ mice [66]. Levels of glutathione (GSH), which represents the main cellular defense factor against oxidative stress, did not differ between cells isolated from *PON2*$^{-/-}$ and *PON2*$^{+/+}$ mice, suggesting that the differential susceptibility to oxidants was primarily due to the presence or absence of PON2 [66].

3.4. Gender differences in PON2 expression

The higher levels of PON2 in tissues, including the brain, from female mice may be related to a positive modulatory effect by estrogens. In striatal astrocytes from male mice, 17β-estradiol caused a time- and concentration-dependent increase in the levels of PON2 protein; a 12–24 h exposure with 200 nM estradiol increased PON2 expression to the levels found in female striatal astrocytes [94]. Interestingly, in female astrocytes, estradiol could further increase PON2 expression, by a factor of about 2.5-fold. The estradiol effect was due to transcriptional activation of the *PON2* gene, and was mediated by activation of estrogen receptor-alpha [94]. In ovariectomized mice, PON2 levels (protein and mRNA) were significantly reduced in striatum, cerebral cortex and liver, approaching the levels found in male mice. Striatal astrocytes and neurons from male mice were more sensitive to H_2O_2 and DMNQ-induced oxidative stress and ensuing cytotoxicity [94]. Though gender-dependent differences in other cell defense mechanisms cannot be excluded, it is noteworthy that levels of GSH did not differ between genders. Another important aspect is the lack of gender difference in susceptibility in cells from *PON2*$^{-/-}$ mice. Striatal astrocytes from *PON2*$^{-/-}$ mice of either gender were highly susceptible to oxidant-induced toxicity, as expected, but there were no significant female/male differences. In CNS cells from *PON2*$^{+/+}$ male mice, exposure to estradiol (200 nM, 24 h) provided protection toward toxicity induced by the two oxidants. This is not surprising, as neuroprotective actions of estrogens are well known [100–103]. However, the protective effect of estradiol was absent in cells from *PON2*$^{-/-}$ mice, suggesting that a major mechanism of estrogen neuroprotection may be represented by induction of PON2 [94]. The functional consequences of a higher expression of PON2 in females may have several ramifications. First, similar gender differences were also found in rats, humans [94], and non-human primates [93]. With regard to neurodegenerative diseases, the role of oxidative stress in the etiopathology of Parkinson's disease (PD) is well established [104]; of note is that the incidence of PD is 90% higher in males [105,106]. Even though dopaminergic areas (striatum, substantia nigra, and nucleus accumbens) have the highest levels of PON2 in both genders, levels in females are still 2- to 3-fold higher than in males [66,94,95]. Lower PON2 levels in dopaminergic neurons in males may thus provide fewer defenses against oxidative stress. In this regard, of much interest are the recent findings that activation of dopamine D2 receptors in the kidney positively modulates PON2 expression, leading to a decrease in ROS production [107]. In the CNS, the highest levels of dopamine D2 receptors are found in the striatum, nucleus accumbens, substantia nigra and olfactory tubercle [108], areas that also have the highest level of PON2 expression [66] (Giordano et al., unpublished results). If a similar mechanism as observed in kidneys also occurs in the CNS, the loss of dopamine associated with PD would lead to decreased PON2 levels, thus fostering a spiral of events further aggravating neurodegeneration. Furthermore, as PON2 is expressed in most tissues, and levels appear to be higher in females in each tissue examined [66], the reported higher sensitivity of males to oxidative stress in heart, the higher susceptibility of males to atherosclerosis and to infections, may all be related to a differential expression of PON2 [109–112].

3.5. Modulation of PON2

While there is a substantial amount of work on the modulation of PON1, which has been summarized in several reviews [113–115], more limited research has been carried out on PON2. In macrophages, PON2 expression is increased by oxidative stress [67], and in vascular cells by endoplasmic reticulum stress modulated via an endoplasmic reticulum stress element-like sequence found to be present in the promoter region of *PON2* [71]. Arachidonic acid [116], unesterified cholesterol [117], the licorice phytoestrogen glabidrin [118], and the hypocholesterolemic drug atorvastatin [119] also upregulate PON2 expression in various cell types. Urokinase plasminogen activator upregulates PON2 in macrophages via NADPH oxidase and the transcription factor SREBP-2 [120,121], while in mouse fibroblasts dexamethasone increases PON2 mRNA levels [122]. In one study, pomegranate juice was found to increase PON2 in macrophages [123], while quercetin was reported to increase PON2 mRNA and protein in macrophages *in vitro* [124]. The latter was confirmed in mouse astrocytes [95]. Extracts of Yerba mate (*Ilex paraguariensis*) have been reported to increase PON2 mRNA and lactonase activity in macrophages *in vitro* and after *in vivo* administration to healthy women [125].

4. PON3

PON3 is a 40-kDa glycoprotein mainly synthesized by the liver and at lower levels by the kidney. Like PON1, PON3 is found in circulation tightly bound to HDLs [126,127], with PON3 protein also identified in multiple mouse tissues [5]. In addition, PON3 expression has been described in endoplasmic reticulum of intestinal cells [128] and more recently in mitochondria of selected tissues [129,130].

4.1. PON3 activity and polymorphisms

PON3 was the last member of the PON family of proteins to be described [2] and is the least characterized. Like PON2, PON3 cannot hydrolyze OPs [72], but it retains lipo-lactonase and N-acyl homoserine lactone activities. The activity of PON3 has been reported to be calcium-dependent, like PON1 [131]. Interestingly, PON3 has a higher catalytic activity for statin lactones than PON1 [73,127]. It is for this reason that statin lactones (such as lovastatin, spironolactone and canrenone) are commonly used to monitor PON3 activity. There are very few studies on polymorphisms in the *PON3* gene. In a study with healthy subjects from southern Italy, the authors identified 3 silent (G51G, G73G, G99G) and 2 missense (S311T, G324D) variants in the exons III, IV and IX of *PON3* [132]. These SNVs exhibited very low frequency in comparison with the frequency of *PON1* and *PON2* coding region SNVs. The effects of these missense variants on PON3 activity have yet to be evaluated. The same polymorphisms were studied in children with diagnosed inflammatory bowel disease, but no relationship between the *PON3* genetic variants and disease was observed [133]. Haplotype associations between several SNVs in the *PON* gene cluster and AD were reported in a cohort of Caucasian and African Americans [134], suggesting a possible important role of PONs in AD that has not been ascertained to date. More recently, six *PON3* promoter SNVs in linkage disequilibrium were significantly associated with changes in serum PON3 concentration in a healthy Mediterranean cohort [135]. The same authors studied these six SNVs in human

immunodeficiency virus (HIV)-infected patients [136] and in coronary artery disease and peripheral artery disease patients [137]. However, no differences were found in these *PON3* gene promoter SNVs and their haplotypes between patients and controls, suggesting that *PON3* genotype neither influences serum PON3 concentration nor the course of these human diseases.

4.2. Physiological function of PON3

The physiological function of PON3 is less clear as this PON member has been poorly investigated. PON3 seems to be more potent than PON1 in protecting LDL from oxidative modification *in vitro*, although PON3 concentration in serum is about 2 orders of magnitude lower than PON1 [127]. In addition, unlike PON1, liver PON3 expression is not affected by oxidized phospholipids (HepG2 cells) or a high-fat diet (mouse liver) [126].

Circulating PON3 has been studied in a variety of human oxidative stress-related diseases with the objective to explore if disease states are associated with changes in the levels of PON3 concentration, as seen with PON1. Indeed, a significant increase of PON3 concentration has been reported in chronic liver disease [138], HIV-infection [136], and coronary and peripheral artery disease [137]. However, a more recent study in patients with autoimmune disease (systemic lupus erythematosus and type 1 diabetes) has shown significant depletion of PON3 protein in HDLs of patients with autoimmune disease and subclinical atherosclerosis [139]. Of note is that the technique used to measure PON3 in these studies is different (in-house serum ELISA [135] in the first studies vs. HDL LC-MS/MS in the latter study). Interestingly, in the HIV study, the authors also studied possible changes in the distribution of PON3 in lipoproteins with disease. Lipoproteins were fractionated by FPLC. They found that in non-infected participants, PON3 was exclusively detected in HDLs, while in HIV-infected subjects a substantial amount of PON3 was measured in the smallest HDL and LDL particles [136]. The HDLs measured in patients with autoimmune disease with and without subclinical atherosclerosis were separated by ultracentrifugation, and presence of PON3 in LDLs was not studied [139]. All in all, PON3 may be a useful analytical biomarker of human oxidative-stress related diseases.

PON3 has been shown to protect murine macrophages against oxidative damage, although cellular PON3 activity is decreased under oxidative stress [67]. These early *in vitro* results suggested an atheroprotective role for PON3 *in vivo* that was demonstrated in mice overexpressing human PON3 [140]. In the first study, Shih and colleagues found that elevated PON3 expression in human *PON3* transgenic mice [either wild-type or *LDL receptor* knockout ($LDLR^{-/-}$) mice on the C57BL/6J background] significantly reduced diet-induced atherosclerotic lesions [140]. Surprisingly, this protective effect was male-specific and not driven by HDL, as the authors could not detect PON3 activity in mouse serum. Another striking finding from Shih and colleagues was a role for PON3 in attenuating the development of obesity in male mice. In the study led by Ng et al., adenoviral expression of human PON3 protected *apolipoprotein E* knockout ($apoE^{-/-}$) mice against progression of atherosclerosis [141]. In particular, elevated levels of PON3 enhanced cholesterol efflux, decreased LDL oxidation, and increased antioxidant properties of HDL, factors that promote slowing down the atherosclerotic process. Furthermore, the authors demonstrated that although human and rabbit PON3 associate with HDL [126,127], endogenous mouse PON3 is undetectable in serum, in accordance with similar previous observations by the same group [140,142]. Neither mice with adenoviral human PON3 expression nor mice that did not receive adenoviral PON3 had detectable PON3 protein in serum or HDL [141], suggesting that PON3 remains associated with cells in mice. In fact, adenovirus-mediated transgene expression was detected in a number of tissues, with liver showing a 2-fold increase in PON3 lactonase activity. This observation was supported by immunohistochemical analysis of PON3 expression in normal mouse tissues [5] and by *Pon3* mRNA expression via *in situ* hybridization [143]. Both studies described *Pon3* expression in a wide range of mouse tissues, with much higher *Pon3* mRNA expression in newborn mice compared to adult mice [143]. It should be noted that *Pon3* mRNA and protein expression in murine macrophages [67] and in specific segments of human and mouse gastrointestinal tract [96] had already been reported. Altogether, these results suggest that mouse PON3 may exert its anti-oxidative effect locally in a variety of epithelia and cells.

Following up on these observations, a protective role of PON3 in obesity has recently been confirmed *in vivo* in *PON3* knockout ($PON3^{-/-}$) mice [130]. In this study, the authors found that lack of PON3 led to alterations in bile acid metabolism, increased body weight and increased atherosclerotic lesions compared to wild-type mice on a high fat diet. In addition, PON3 deficiency seemed to result in impaired mitochondrial respiration and mitochondrial superoxide levels, and increased hepatic expression of inflammatory genes. A recent study in patients with systemic lupus erythematosus reported an inverse association between PON3 concentration in HDLs and body mass index, supporting a role of PON3 in adiposity [139].

PON3 has also been related to immune-mediated enteropathies such as inflammatory bowel disease and celiac disease. In the study by Rothem et al., the authors found that PON3 (and PON1) mRNA expression was repressed in certain parts of the large intestine of Crohn's patients and non-treated celiac disease patients, and in patients with ulcerative colitis [128]. They also localized PON3 in the endoplasmic reticulum of cultured *PON3-GFP* transfected HT29 and CaCo-2 cells, suggesting local synthesis and secretion of PON3 by intestinal cells.

Despite PON3's beneficial role in protecting against a variety of oxidative-stress related diseases, an unexpected finding by Schweikert and colleagues demonstrated that PON3, like PON2, has an oncogenic role in human cancers [129]. PON3 was found to be upregulated in various human cancer tissues, protecting those cells against mitochondrial superoxide-mediated apoptosis. In this regard, PON2 and PON3 seem to play similar roles in cancer, with PON3 being much more overexpressed in cancer cells than PON2. Although knocking down PON3 from certain cancer cells did not enhance susceptibility to chemotherapeutic drugs *in vitro*, PON3 may have potential as an anti-cancer target.

The finding that PON2 and PON3 have similar roles in cancer prompted the same research group to study if, similar to PON2, PON3 is involved in protecting against *Pseudomonas aeruginosa* infections [91]. It is known that pyocyanin, an essential virulence factor secreted by the bacterium, causes oxidative stress (ROS) *in vivo*, which results in cell damage [144], and leads to a pro-inflammatory response resulting in interleukin-8 release [145]. Schweikert and colleagues described that *in vitro* exposure of pyocyanin to different cell lines led to ROS production, and activation of NF-*k*B, the major pathway involved in inflammatory response (via interleukin-8 release) [91]. More importantly, PON3 (and PON2) overexpression significantly prevented pyocyanin-induced ROS production and had a dramatic effect diminishing NF-*k*B-mediated inflammatory response. Furthermore, the quorum sensing signal N-acyl homoserine lactone promoted calcium influx in cells, which resulted in a calcium-dependent inactivation of PON2 (as previously demonstrated [76]) and PON3 activity. Altogether, PON3 and PON2 have important roles in the defense against *P. aeruginosa* virulence, although the bacterium can inactivate PON2

and PON3 activity during the infection.

5. PONs and drug metabolism

The paraoxonases are involved in both bioactivation and inactivation of specific drugs. A few examples are provided here with comments on the importance of catalytic efficiency of hydrolysis.

The first report on the contribution of A-esterases (PON1) to the activation of prodrugs was by Tougou and colleagues [146]. In this report, the authors demonstrated that PON1 could activate prulifloxacin (NM441), the prodrug of a carboxylic acid antibacterial agent (NM394). Later, lactone hydrolysis by PON1 was reported for the first time with glucocorticoid γ-lactones and cyclic carbonates [147]. These topically administered agents were rapidly inactivated by plasma PON1, limiting systemic side effects and improving therapeutic indices. The lack of PON1 in the lungs, the target tissue of these drugs, made them an ideal "antedrug" to treat diseases such as asthma. In the same year, the laboratory of Dr. La Du described 30 lactones and cyclic carbonate esters as being hydrolyzed by PON1, with four lactone-containing drugs (spironolactone, mevastatin, simvastatin, and lovastatin) described as PON1 substrates [59]. However, once rabbit PON3 was purified, it was observed that PON3 hydrolyzes statin lactones at a much higher rate than PON1, while PON1 hydrolyzes a much broader spectrum of lactones at higher rates than PON3 [127]. Based on the homology of the three PONs, Billecke et al. speculated that PON2 and PON3 may also have lactonase activity, and that PON1s' original activity was that of a lactonase, with POase and AREase activities being acquired during evolution [59]. The same laboratory further described PON1's and PON3's lactonizing activities of hydroxyl acids, indicating the potential of PON1 and PON3 for metabolizing drugs and endogenous compounds [148]. Furthermore, Khersonsky et al. [149] reported a thorough study of the mechanism of hydrolysis of more than 50 substrates by PON1. It was concluded that the three PON1 activities that had been reported to date (phosphotriesterase, esterase and lactonase) all resided in the same active site, and that PON1 was in fact a lactonase, in agreement with previous results by La Du and colleagues. PONs 1, 2 and 3 were confirmed as being lactonases/lactonizing enzymes, when they were expressed recombinantly with a baculoviral expression system [73]. The authors concluded that PONs are lactonases with overlapping and distinct substrate specificities, with OPs being exclusively hydrolyzed by PON1, bulky drug substrates (such as lovastatin and spironolactone) hydrolyzed only by PON3, and PON2 mainly inactivating long-chain homoserine lactones.

Another example of the hydrolysis of a prodrug bioactivated by PON1 includes the anti-hypertensive drug olmesartan medoxomil which is also metabolized by carboxymethylenebutenolidase (CMBL) [150]. Examples of other drugs inactivated by PON1 to confine their site of action to target tissues include roflumilast analogs, inhibitors of phosphodiesterases such as PDE4 which are useful in treating inflammatory or autoimmune diseases [151].

5.1. Clopidogrel

The case of clopidogrel (an anti-platelet drug used in treating coronary artery disease, peripheral vascular disease and cerebrovascular disease) requires special comment. In 2011, Bouman et al. reported that PON1 was a major determinant of clopidogrel efficacy [152]. In a letter to the editor, many members of the PON1 research community expressed their concerns about the methodology used in their study [153]. PON1 was subsequently shown to generate an endothiol inactive metabolite [154,155] while the two steps required for the bioactivation of clopidogrel to the active metabolite involve cytochromes P450.

The lesson learned from the studies of PONs in considering their roles in metabolism of various endogenous metabolites as well as xenobiotics is that the catalytic efficiency of hydrolysis is key in determining the physiological relevance of a given hydrolytic activity. For example, it was thought for a half-century that PON1 would provide protection against parathion/paraoxon. The studies by Li et al. [19] showed that while PON1 hydrolyzed PO *in vivo*, the catalytic efficiency was too low to provide protection against exposure whereas, the catalytic efficiency of DZO and CPO hydrolysis was sufficient to protect against exposures to these two OPs. The clopidogrel and homocysteine thiolactone studies described above provide additional examples of the importance of assuming a physiological relevance of a given PON in metabolism of a specific compound without solid *in vivo* data to support the conclusion.

6. Concluding remarks and future directions

PON1 was the first member of the PON family of enzymes discovered and characterized. Since it is a hydrophobic protein that retains its leader sequence, it was difficult to purify and the purified PON1 would change positions to higher molecular weight bands following purification [15]. Purification and sequencing of rabbit PON1 facilitated the isolation of the rabbit and human PON1 cDNAs which in turn allowed for the characterization of the PON1 gene structure [156,157]. Development of the genetically modified mouse model for three PONs by Shih, Lusis and co-workers at UCLA [33,84,130] has provided an invaluable resource for understanding the physiological roles of PON1 from modulating OP exposures to risk of disease.

Perhaps the most important conclusion from all of the studies reported to date is that any epidemiological studies related to PON1 genetic variability and risk of exposure or disease need to include measurements of PON1 activities. This point should be immediately evident from examining Figs. 1 and 2. The more activity one has, the more rapidly they are going to metabolize endogenous or xenobiotic toxins whether insecticide metabolites or products of oxidative stress. Fig. 2 also illustrates the importance of analyzing PON1 status for each of the $PON1_{192}$ functional genotypes (Q/Q; Q/R and R/R) separately. In addition to these quite evident points, there is a second point that is not yet so clear, which relates to the observation by several authors of a decrease in PON1 activity that is accompanied by an increase in PON1 concentration in a variety of oxidative stress-related diseases. Further research on this PON1 inactivation by oxidative stress is needed.

The earlier proposals that the thiolactonase activity of PON1 is important for detoxifying homocysteine thiolactone [57] and bioactivating clopidogrel [152] do not make sense in light of the much higher activity of biphenyl hydrolase-like protein in the case of homocysteine thiolactone [61] and the role of the cytochromes P450 in the case of clopidogrel bioactivation [154,155].

PON2 is emerging as a potentially important intracellular defense mechanism against oxidative stress, particularly given its widespread tissue distribution and mitochondrial localization. Its identification and initial characterization in brain tissue suggest that this enzyme may play a relevant role in determining susceptibility to oxidative stress and neuroinflammation, and that its positive modulation may represent a novel strategy for neuroprotection. Gender differences in PON2 expression also represent a finding of much interest, as gender is a variable that is often ignored in toxicological and neurotoxicological studies, though most scientists would readily acknowledge that major differences may exist between males and females in their response to toxicants, which may be ascribed to differences in exposure, toxicokinetics and metabolism, and to pharmacodynamic factors [110,158,159]. As many adverse health outcomes in the CNS and other organs involve

oxidative stress, this finding may explain the gender-dependent differential incidence of several diseases. The lactonase activity of PON2 and its potential anti-inflammatory actions may also pertain to other pathological processes, providing the stimulus for numerous investigations addressing gender effects. The protective action of PON2 toward oxidative stress and neuroinflammation suggest that attempts aimed at increasing its levels of expression may be useful. So far, limited research has been carried out in this area. However, dietary or pharmacological modulation of PON2 may be of interest and may provide new avenues for neuroprotection or explanations for known neuroprotective effects. A caveat to this strategy is represented by the findings that in tumor cells PON2 is up-regulated, by still unknown mechanisms [97]. Though CNS tumors have not been specifically investigated, the finding appeared to be valid for tumors of different tissues. Given its characteristics, it is not surprising that PON2 would thus provide resistance of these cells to apoptosis and that a useful therapeutic strategy would be one causing a decrease of PON2 [97].

Recent studies on PON3 have demonstrated the potential antioxidant and anti-inflammatory role that this less characterized member plays in a variety of human diseases, from atherosclerosis, to metabolic syndrome, HIV infection, chronic liver disease and innate immunity. Of note is that the antioxidant capacity of PON3 is higher than that of PON1. The intriguing oncogenic role of PON3 demands further research. The functions and effects of PON2 and PON3 seem to be very similar. Thus, it could be speculated that PON2 and PON3 act by a common antioxidant and anti-inflammatory mechanism. It should be noted that PON2 and PON3 have very distinct substrate specificities, with PON2 showing a dominant lactonase activity and PON3 preferably hydrolyzing large lactones (i.e. statins) or arylesters. A link between PON3 polymorphisms and disease has yet to be identified, which may suggest a more important role by environmental factors in modulating PON3 activity and expression.

In summary, it is clear that PONs 1, 2 and 3 are potent antioxidant and anti-inflammatory enzymes. Their distinct substrate specificity and localization point out that they may have different functions in the human body. The role of PON1 in protecting against exposure to specific OP compounds has been solidly confirmed with the PON1 genetically modified mouse model systems.

Based on the value demonstrated for determining PON1 status, it will be important to develop protocols for determining status assays for PONs 2 and 3. Such protocols would greatly facilitate the understanding of the contribution of genetic variability of PONs 2 and 3 to disease. The ability to produce recombinant PONs may have utility in restoring function of PONs in individuals with defective *PON* genes and for treating OP exposures with recombinant engineered PON1. The initial studies on the roles of PONs 2 and 3 in preventing apoptosis in cancer cells suggest an important future direction in understanding and modulating these activities. The recent studies showing the gender differences in PON2 levels and the importance of PONs in protecting against oxidative stress and infectious disease point directions for additional research. Understanding the role of gender differences in PON2 levels and disease susceptibility to and dietary modification that may increase PON2 levels and resistance to oxidative stress are important areas of future research.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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