

Paraoxonase 1 and homocysteine metabolism

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Abstract Paraoxonase 1 (PON1), a component of high-density lipoprotein (HDL), is a calcium-dependent multifunctional enzyme that connects metabolisms of lipoproteins and homocysteine (Hcy). Both PON1 and Hcy have been implicated in human diseases, including atherosclerosis and neurodegeneration. The involvement of Hcy in disease could be mediated through its interactions with PON1. Due to its ability to reduce oxidative stress, PON1 contributes to atheroprotective functions of HDL in mice and humans. Although PON1 has the ability to hydrolyze a variety of substrates, only one of them—Hcy-thiolactone—is known to occur naturally. In humans and mice, Hcy-thiolactonase activity of PON1 protects against *N*-homocysteinylation, which is detrimental to protein structure and function. PON1 also protects against neurotoxicity associated with hyperhomocysteinemia in mouse models. The links between PON1 and Hcy in relation to pathological states such as coronary artery disease, stroke, diabetic mellitus, kidney failure and Alzheimer's disease that emerge from recent studies are the topics of this review.

Keywords Paraoxonase 1 · Homocysteine · *N*-homocysteinylation · Thiolactonase · Atherosclerosis · Alzheimer's disease

Introduction

Paraoxonase 1 (PON1) exhibits a wide range of physiologically important activities, including homocysteine (Hcy)-thiolactone detoxification, drug metabolism, and detoxification of nerve agents. PON1 hydrolyzes the toxic oxon metabolites of organophosphorous insecticides, including parathion, diazinon, and chlorpyrifos, nerve agents, such as sarin and soman, aromatic esters, a variety of aromatic and aliphatic lactones (Draganov et al. 2005), as well as Hcy-thiolactone (Jakubowski 2000a). It has been extensively investigated in the context of human pathology, including cardiovascular disease, kidney disease, diabetes, and Alzheimer's disease. PON1 is believed to have antiatherogenic properties; however, the mechanisms by which it protects against hardening of arteries are not well understood. A growing body of evidence links PON1 with Hcy, a risk factor for coronary artery disease (CAD) (Anderson et al. 2000). Hydrolysis of Hcy-thiolactone by PON1 prevents the accumulation of *N*-Hcy-protein in humans (Perla-Kaján and Jakubowski 2010; Jakubowski 2011, 2012) and protects against neurotoxicity associated with hyperhomocysteinemia in mouse models (Borowczyk et al. 2012a). In this study, we review the recent advances in the PON1 research field with special emphasis on the links between PON1 and Hcy metabolism.

Structure and catalytic mechanism

Paraoxonase 1 is expressed in the liver (Kelso et al. 1994) and circulates in the blood in association with

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apolipoprotein (Apo) A1 in HDL particles (Blatter et al. 1993). Recent studies, however, indicate that PON1 is more widely distributed and present in other organs, including the brain (Marsillach et al. 2008). It belongs to the paraoxonase family consisting of three proteins: PON1, PON2 and PON3 that are all coded by genes located on the long arm of chromosome 7 (Humbert et al. 1993).

Human PON1 is a 45-kDa enzyme whose polypeptide chain consists of 354 amino acids (Hassett et al. 1991) and has two bound calcium ions, one playing a structural and the other a catalytic role (Harel et al. 2004). Early studies, which found that phenyl acetate is a *mixed type* inhibitor of the paraoxonase (POase) activity (Gan et al. 1991) while phenyl acetate and paraoxon are *non-competitive* inhibitors of the Hcy-thiolactonase (HTase) activity of PON1 (Jakubowski 2000a), suggested that different substrates bind at different active sites of the human PON1 enzyme. Recent structural studies (Ben-David et al. 2012) provide a mechanistic explanation for these observations.

Due to instability and tendency to form aggregates in the absence of detergents, native PON1 has been difficult to crystallize for structural studies. These limitations however were overcome by generating well-soluble rabbit PON1 variants in bacteria by directed evolution. According to the crystal structure determined at pH 4.5, the engineered PON1 variant is a six-bladed β -propeller, where each blade consists of four β -sheets. The top of the propeller is covered by three α helices, involved in the anchoring to the HDL particle (Harel et al. 2004). Recently, two other crystal structures of PON1 at pH 6.5 have been described, apo-protein and a complex with a lactone analog 2-hydroxyquinoline (2HQ). 2HQ inhibits all three activities of PON1 (lactonase, esterase, and phosphotriesterase) by binding to the catalytic calcium ion and displacing the phosphate ion that is present in both the apo structures. Comparison of different PON1 structures, namely apo at pH 4.5, apo at pH 6.5, and complexed with 2HQ at pH 6.5, disclose the existence of various conformations that result in different active site shapes and sizes, which account for the binding of different substrates to PON1's active site. The authors identified three conformations of PON1: (1) closed, characterized by structured active-site loop (Lys70–Lys81) and Tyr71 pointing into the active site and represented by PON1 complexed with 2HQ; (2) open, observed

at pH 4.5, where the active-site loop is flexible but Tyr71 is fixed and points outside the active site; (3) open, at pH 6.5 with the entire active-site loop being flexible (Ben-David et al. 2012).

Site-directed mutagenesis was used to identify PON1 active site residues and elucidate its catalytic mechanism. The postulated catalytic site includes the upper calcium atom, the phosphate ion, and the His115–His134 dyad. Activity assays indicate that His115 and His134 are responsible for the arylesterase (AREase) activity, but not for the POase activity of PON1. It was proposed that the two catalytic activities may be catalyzed by different active site residues (Harel et al. 2004). Indeed, certain Ala mutations of residues 70–81 showed significantly different effects of different activities. The most marked variation was observed for Ile74Ala which resulted in a large decrease of AREase and POase activities, while having no effect on the lactonase activity (Ben-David et al. 2012).

Computational docking was employed to reveal the binding modes of PON1 and putative reaction intermediates for different substrates: aliphatic lactones, aryl esters, and paraoxon. The differences of catalytic details proposed for PON1's activities are summarized in Table 1 (Ben-David et al. 2012).

The data indicate that the lactonase activity takes place in closed conformation. The Tyr71 contacts the lactone ring, and the Ile74 contacts the alkyl side chain, but the structure of PON1 does not indicate a specific crevice for the alkyl side chain (Ben-David et al. 2012). Probably, this is why PON1 does not show a strong preference for the size of the alkyl side chain (Khersonsky and Tawfik 2005). The closed form is also likely to mediate the AREase activity. Phenyl acetate docks similarly to 2HQ, and Ile74 is involved in the catalysis. Moreover, it binds in a similar fashion to lactones and utilizes the same catalytic mechanism. Paraoxon on the other hand docks only to the open conformation.

The studies of (Ben-David et al. 2012) showing that different active-site residues or conformations are responsible for the hydrolysis of different substrates provide a possible explanation for the *non-competitive* inhibition by paraoxon or phenyl acetate of the HTase activity of PON1 (Jakubowski 2000a). Other inhibitors such as penicillamine and isoleucine (with D-stereoisomers being more potent

Table 1 Different PON1 structural conformers accept different substrates (Ben-David et al. 2012)

	Aliphatic lactones	Phenyl acetate	Paraoxon	Dihydrocumarin (lactone)
PON1's conformation	Closed	Closed	Open	Closed
Bond cleaved	C–O	C–O	P–O	C–O
Putative reaction intermediate	Tetrahedral	Tetrahedral	Pentavalent	Tetrahedral
Intermediate reacts with	H115 E53	H115 E53	D269 E53	D269 E53

than L-stereoisomers) are also non-competitive with respect to Hcy-thiolactone (Jakubowski 2000a), suggesting the presence of a separate specific amino acid-binding effector site(s) on the PON1 protein.

Polymorphism

There are several polymorphisms in the coding and non-coding regions of the *PON1* gene, of which Q192R and L55M (Hassett et al. 1991; Adkins et al. 1993) are the most often studied.

Functional effects of the polymorphism at position 192 on PON1 activities are substrate dependent. For example, subjects with 192RR genotype have the highest mean POase activity, while diazoxonase, sarinase, and somanase have the lowest activities compared with other genotypes (192QR and 192QQ). All three genotypes give approximately the same activity toward chlorpyrifos oxon and phenyl acetate (Davies et al. 1996). High HTase activity is associated with R192 and L55 alleles, whereas low HTase activity is associated with Q192 and M55 alleles. HTase activity is strongly correlated with POase activity, which suggests that the POase is a good surrogate for the biological HTase activity (Jakubowski et al. 2001; Lacinski et al. 2004a, b).

Human allozymes Q192 and R192 PON1 have been recently purified and their activities tested with phenyl acetate, paraoxon, and Hcy-thiolactone. When Hcy-thiolactone was used as a substrate, the R192 PON1 isoform had 2.5- and 2.6-fold higher V_{max} and k_{cat}/K_m , respectively, than the Q192 PON1 isoform. K_m values of Q192 and R192 PON1 variants for Hcy-thiolactone were 23.5 and 22.6 mM, respectively (Bayrak et al. 2011). These values are in agreement with previously reported K_m value for Hcy-thiolactone of 23 mM (Jakubowski 2000a). Rabbit liver PON1 have also been purified and characterized recently. The molecular weight of this protein is close to 40 kDa. The K_m of the rabbit enzyme for phenyl acetate and Hcy-thiolactone are 0.55 ± 0.024 and 17.31 ± 1.2 mM, respectively (Bayrak et al. 2010).

The influence of polymorphism at position 192 on PON1 activity is consistent with the fact that the residue at this position is part of the active site wall (in the crystallized PON1 variant there is Lys at position 192) (Harel et al. 2004).

The polymorphism at position 55 does not affect the catalytic efficiency of the enzyme, but it has been shown that the *PON1* M55 allele is correlated with decreased mRNA and protein levels (Garin et al. 1997; Leviev et al. 1997; Mackness et al. 1998; Brophy et al. 2000). Isoform M55 is more susceptible for proteolysis than isoform L55 (Leviev et al. 2001). This is consistent with the role of

Leu55 in packing the propeller's central tunnel and of its neighboring residues Glu53 and Asp54, which interact with calcium ions (Harel et al. 2004).

PON1 function from animal studies

Paraoxonase 1 has been studied by toxicologists since the 1960s, but it has been molecular biologists working on animal models, who provided important insights into the physiological roles of PON1. *Pon1*^{-/-} mice are more sensitive to atherosclerosis induced by high-fat diet and to organophosphate toxicity than the wild-type (wt) animals. *Pon1*-deficient mice develop significantly larger atherosclerotic lesions in their aortas compared to their wt counterparts (Shih et al. 1998, 2000). HDL from *Pon1*^{-/-} mice does not protect low-density lipoprotein (LDL) from oxidation (Shih et al. 1998). *Pon1*-deficient mice are also more susceptible to neurotoxicity induced by intraperitoneal injections of Hcy-thiolactone than wt animals (Jakubowski 2011, 2012; Borowczyk et al. 2012a).

Additional data on PON1 anti-atherosclerotic function came from studies with transgenic mice overexpressing human PON1. Human PON1 expressed in mice protects against a high-fat diet-induced atherosclerosis (Tward et al. 2002). An increased protection against oxidative stress induced by copper ions, as indicated by lecithin:cholesterol acyltransferase (LCAT) activity, has also been observed. Excess PON1 inhibits lipid hydroperoxide formation on HDL. Thus, overexpression of PON1 protects HDL integrity and function (Oda et al. 2002). Furthermore, human PON gene cluster transgenic overexpression in mice represses atherogenesis and promotes atherosclerotic plaque stability (She et al. 2009). PON3 is also an HDL-associated enzyme with biological activity similar to PON1 (Reddy et al. 2001). PON3 transgenic mice exhibit decreased atherosclerosis compared to their wt littermates (Shih et al. 2007).

In *PON1*-transfected macrophages and in *Pon1*-deficient mice, PON1 directly reduces macrophage and aortic oxidative status: a phenomenon associated with decreased superoxide anion production and increased glutathione content (Rozenberg et al. 2005). A study of the effects of PON1 on macrophage oxidative stress and on streptozotocin-induced diabetes development in mice has shown that PON1 overexpression is associated with decreased diabetes-induced macrophage oxidative stress, decreased diabetes development, and decreased mortality, in comparison to wt and *Pon1*-deficient mice (Rozenberg et al. 2008).

PON1 and protection of LDL against oxidative damage

Even though experiments in mouse models show that manipulation of PON1 expression affects atherosclerosis,

the mechanisms involved are unclear. It is often *incorrectly* stated in the literature that hydrolytic enzymatic activities of PON1 toward oxidized lipids can account for its anti-atherosclerotic function, e.g., (Bhattacharyya et al. 2008). However, the preponderance of available evidence indicates that PON1 does not have an intrinsic anti-oxidative function (Vos 2008; Perla-Kaján and Jakubowski 2010) and that the protective effect of PON1 is *indirect*. Although Mackness et al. have shown that PON1 as well as HDL inhibits the Cu²⁺-induced generation of lipid peroxides in LDL (Mackness et al. 1993), their findings are controversial, as other investigators have shown that PON1 does not have intrinsic redox activity (see below). There is also controversy regarding whether the protective activity of PON1 depends on the polymorphism at position 192 (Aviram et al. 1998) or not (Cao et al. 1999). The degree of protection tends to relate to POase activity (Mackness et al. 1993), but is independent of POase activity (Cao et al. 1999). The protection occurs in a concentration-dependent manner (Mackness et al. 1993; Aviram et al. 1998) and is not sensitive to EDTA (Aviram et al. 1998; Cao et al. 1999), meaning that the putative protective activity does not require calcium ions, while other PON1 activities are calcium dependent (POase, AREase, HTase, diazoxonase). The protection of LDL is not eliminated by heating PON1 protein (56 °C, 10 min) (Cao et al. 1999); however, more intensive heat treatment (60 °C, 15 min) inhibits LDL protection (Aviram et al. 1998). Blocking the PON1's free sulfhydryl group at position 283 with *p*-hydroxymercuribenzoate inhibits both AREase activity and protection of LDL from oxidation (Aviram et al. 1998). However, HTase activity is not affected by iodoacetate or iodoacetamide, which indicates that thiol groups are not required for activity (Jakubowski 2000a). Mutation of Cys 283 to Ala or Ser abolishes antioxidative function of PON1 (against LDL oxidation), even though the enzyme retains POase and AREase activities. It has been suggested that PON1's AREase/POase activities and the protection against LDL oxidation do not involve the active site on the enzyme in exactly the same way (Aviram et al. 1998).

Some investigators reported that PON1 directly protects LDL against oxidation, but subsequent studies have shown that PON1 does not have an intrinsic antioxidant function. For example, Marathe et al. showed that trace amounts of platelet-activating factor (PAF) acetylhydrolase, a serine esterase associated with HDL particles, contaminate PON1 preparations and that PON1 lacking PAF acetylhydrolase does not have phospholipase activity toward PAF or pro-atherogenic oxidized phospholipids (Marathe et al. 2003).

Teiber et al. have studied the antioxidant properties of serum PON1 using *in vitro* assays with copper or the free radical generator 2,2'-azobis-2-amidinopropane hydrochloride. The antioxidant activity of different purified

PON1 preparations did not correlate with their AREase, lactonase, or phospholipase A2 activities and could largely be accounted for the "antioxidant" activity of the detergent present in PON1 preparations (Teiber et al. 2004).

Recombinant PONs do not protect human LDL against Cu²⁺-induced oxidation *in vitro*, and no antioxidant activity copurifies with any of the PON1 preparations. The authors stated that antioxidant activity of human serum PON1 was attributable to a low molecular mass contaminant and to the detergent in the preparation (Draganov et al. 2005).

Hcy-thiolactonase activity of PON1 protects against protein *N*-homocysteinylation

More light on the mechanism of cardioprotective functions of PON1 was shed by the discovery of its natural substrate, Hcy-thiolactone (Jakubowski 2000a). Hcy-thiolactone is a product of Hcy editing reaction catalyzed by methionyl-tRNA synthetase (Jakubowski 2011, 2012). The presence of an HTase activity in human sera was first reported by Dudman et al. (Dudman et al. 1991), but it was not yet known which enzyme was responsible for the HTase activity. Serum HTase has been purified to homogeneity about a decade later and shown to be identical with PON1 (Jakubowski 2000a). Substrate specificity and steady-state enzyme kinetic studies of human serum HTase/PON1 have shown that phenyl acetate and paraoxon are non-competitive inhibitors of HTase, and that Hcy-thiolactone, phenyl acetate, and paraoxon are hydrolyzed at different sites of the HTase/PON1 enzyme (Jakubowski 2000a). HTase activity of PON1 strongly correlates with POase activity in various populations (Jakubowski et al. 2001; Lacinski et al. 2004; Perla-Kaján and Jakubowski 2010).

Hcy-thiolactone is a reactive metabolite that modifies protein lysine residues in a process known as *N*-homocysteinylation (Jakubowski 1997, 1999). Plasma Hcy-thiolactone and *N*-linked protein Hcy (*N*-Hcy-protein) are present in the human body (Jakubowski 2000b; Jakubowski et al. 2000; Chwatko and Jakubowski 2005a, b) and are greatly elevated in genetic or nutritional hyperhomocysteinemia (Glowacki and Jakubowski 2004; Chwatko et al. 2007; Jakubowski et al. 2008; Perla-Kaján et al. 2008). Hcy-thiolactone and *N*-Hcy-protein are also elevated in hyperhomocysteinemic mouse models (Chwatko et al. 2007; Jakubowski et al. 2009).

N-Homocysteinylation affects protein structure and function, e.g., enzymes lose their activity (Liu et al. 1997; Jakubowski 1999; Ferretti et al. 2003), *N*-Hcy-fibrinogen acquires prothrombotic properties (Sauls et al. 2006; Undas et al. 2006), and *N*-Hcy-proteins become autoimmunogenic (Perla et al. 2004; Undas et al. 2004). *N*-Homocysteinylation of HDL

changes the conformation and properties of apolipoprotein. The modification causes significant decrease of POase and lactonase activities of the PON1 protein, which could affect the ability of HDL to protect against oxidative damage and protein *N*-homocysteinylation and contribute to atherosclerosis in patients with hyperhomocysteinemia (Ferretti et al. 2010). *N*-Hcy-ApoA1 is present in normal human sera and exhibits great inter-individual variability (Ishimine et al. 2010).

Protein *N*-homocysteinylation could account for detrimental effects of hyperhomocysteinemia. A hypothesis of Hcy-thiolactone-mediated vascular disease (Jakubowski 1997) explains the toxicity of Hcy by its conversion to Hcy-thiolactone, which then modifies proteins to generate *N*-Hcy-protein, leading in turn to cell death, inflammation, autoimmune response, and in consequence to atherosclerosis, thrombosis, and neurotoxicity (Jakubowski 2008, 2010, 2011, 2012) (Fig. 1).

The discovery of HTase activity of PON1 led to a hypothesis that PON1 could be atheroprotective also by its ability to detoxify Hcy-thiolactone and minimize protein *N*-homocysteinylation (Jakubowski 2000a) (Fig. 1). Support for that hypothesis came from discoveries that PON1 protects against the accumulation of *N*-Hcy-protein in vitro (Jakubowski et al. 2000, 2001) and in vivo (Perla-Kaján and Jakubowski 2010). Plasma *N*-Hcy-protein is negatively correlated with serum HTase activity ($r = -0.43$, $P = 0.01$) in cystathionine β -synthase-deficient patients. Enzymatic activities of the PON1 protein measured with artificial substrates correlate less strongly ($r = -0.36$, $P = 0.025$ for POase activity) or do not correlate at all (phenyl acetate hydrolase and TBLase activities) with plasma *N*-Hcy protein. Furthermore, the inverse in vivo relationship between *N*-Hcy-protein and HTase activity was recapitulated in separate in vitro *N*-homocysteinylation experiments. These findings provide evidence that the HTase activity of PON1 is a major determinant of plasma *N*-Hcy-protein levels in vivo (Perla-Kaján and Jakubowski 2010), which is also in agreement with earlier in vitro findings that high activity forms of PON1 afford better protection against protein *N*-homocysteinylation than low activity forms (Jakubowski et al. 2001).

Lowering serum HTase activity of PON1 by leptin administration in rats increases the level of *N*-linked Hcy in plasma proteins, but has no effect on plasma total Hcy. It has been suggested that the decreased capacity to metabolize Hcy-thiolactone and concomitant increase in protein *N*-homocysteinylation contribute to the pro-atherogenic effect of chronic hyperleptinemia, which is independent of oxidative stress (Beltowski et al. 2010).

Recent studies on *Pon1*^{-/-} mice have shown that PON1 plays a protective role against neurotoxicity of Hcy-thiolactone (Borowczyk 2012a). *Pon1*^{-/-} mice, compared to

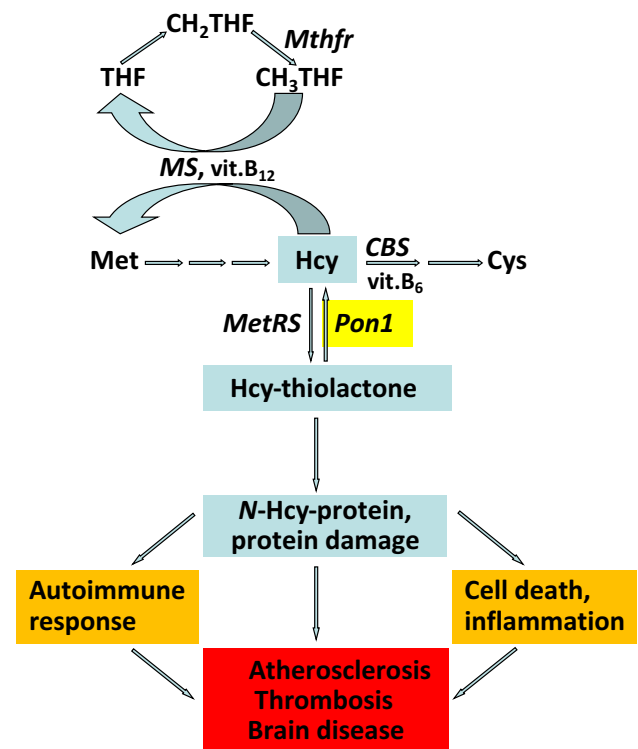


Fig. 1 The Hcy-thiolactone hypothesis. In humans and animals, Hcy is formed from dietary protein Met as a result of cellular methylation reactions. In this pathway, Met is first activated by ATP to yield *S*-adenosylmethionine (AdoMet). As a result of the transfer of its methyl group to an acceptor, AdoMet is converted to *S*-adenosylhomocysteine (AdoHcy). Enzymatic hydrolysis of AdoHcy is the only known source of Hcy in the human body. Levels of Hcy are regulated by remethylation to Met, catalyzed by Met synthase (MS), and transsulfuration to cysteine, the first step of which is catalyzed by cystathionine β -synthase (CBS). The remethylation requires vitamin B12 and 5,10-methyl-tetrahydrofolate (CH₃-THF), generated by 5,10-methylene-THF reductase (MTHFR). The transsulfuration requires vitamin B6. Hcy is also metabolized to a thioester, Hcy-thiolactone, by methionyl-tRNA synthetase. Hcy-thiolactone modifies proteins generating *N*-Hcy-protein. Hydrolysis of Hcy-thiolactone by PON1 protects against its neurotoxicity and protein *N*-homocysteinylation [adapted from (Chwatko et al. 2007)]

wt animals, have elevated levels of Hcy-thiolactone in the brain, while in other organs the levels of Hcy-thiolactone are not influenced by *Pon1* gene deletion. When hyperhomocysteinemia was induced in *Pon1*^{-/-} and *Pon1*^{+/+} mice, the levels of plasma tHcy increased 5.6- and 10.4-fold, respectively. *Pon1*^{-/-} mice excreted 2.4-fold more Hcy-thiolactone in urine than wt animals, while urinary tHcy levels were similar. A surprising finding of this study was that after Hcy-thiolactone was injected intraperitoneally (i.p.) into the mice, the turnover of plasma Hcy-thiolactone was similar in *Pon1*^{-/-} and wt littermates. The half-life of plasma Hcy-thiolactone in vivo was about 5 min, while in vitro in serum from *Pon1*^{+/+} and *Pon1*^{-/-} mice it was 73 and >1,000 min, respectively. These values suggest that PON1

contributes at most 10 % to Hcy-thiolactone clearance from the mouse blood in vivo and indicate that there are other more efficient mechanisms of Hcy-thiolactone clearance from the circulation. One of such mechanisms involves kidneys as *Pon1*^{-/-} mice excrete more Hcy-thiolactone than wt counterparts. After i.p. injection of Hcy-thiolactone, plasma tHcy increased to higher levels in *Pon1*^{+/+} mice than in knockout animals.

Pon1^{-/-} mice are more sensitive than wt littermates to i.p. injection of Hy-thiolactone, as manifested by higher incidence of seizures, shorter seizure latency, and higher levels of Hcy-thiolactone and N-Hcy-protein in the brain (Borowczyk 2012a). These findings suggest that PON1 protects against neurotoxicity caused by Hcy-thiolactone.

Association of *PON1* genotypes and activity

As mentioned in previous sections, the two most commonly studied PON1 polymorphisms, M55L and Q192R, influence PON1 function. The HTase activity of PON1 is modified by polymorphisms at position 55 and 192 in American and European populations: products of *PON1* L55 and R192 alleles have higher HTase activity than *PON1* M55 and Q192 alleles (Jakubowski et al. 2001; Lacinski et al. 2004). In a study of a Tunisian population, *PON1* 192RR, and *PON1* 55MM genotypes were associated with higher HTase activity (Koubaa et al. 2009).

Other PON1 activities are also modified by gene polymorphism. For example, a large, prospective study of a US population of patients carried out at the Cleveland Clinic ($n = 1,399$) demonstrated a significant association between *PON1* genotype and serum PON1 POase and AREase activities. Genotypes *PON1* 192QQ and *PON1* 192RR had lowest and highest activity, respectively. Moreover, participants with the *PON1* 192QQ genotype showed higher risk of mortality and of major adverse cardiac events than subjects with the *PON1* 192RR or 192QR genotype. Elevated systemic levels of fatty acids oxidation products that are increased in atherosclerotic plaque and plasma of participants with cardiovascular disease were associated with low PON1 activity and the *PON1* 192QQ genotype (Bhattacharyya et al. 2008).

The association of PON1 polymorphisms and activity was also investigated in Turkish patients with CAD and healthy controls. Genotypes *PON1* 55 and *PON1* 192 were associated with PON1 activity for all subjects. Genotypes RR, QR, and QQ have the highest, intermediate, and lowest activity, respectively. When subjects were divided into the patient and control groups, *PON1* 55 and *PON1* 192 genotypes were significantly associated with PON1 activity in patients, but not in controls. PON1 activity in patients with *PON1* 55LL genotype was significantly elevated compared with individuals with MM genotype. Similarly,

in patients carrying RR and QR alleles, PON1 activity levels were significantly increased compared to patients with QQ genotype. In contrast, *PON1* 55/192 was not associated with the analyzed parameters in controls (Aydin et al. 2009).

Also in another Turkish population of CAD and healthy subjects, POase and HTase activity of PON1 was affected by Q192R polymorphism. Genotype QQ had the lowest activity while QR and RR had intermediate and highest activity, respectively (Bayrak et al. 2011).

The association of PON1 polymorphisms with risk factors for cardiovascular disease has been studied. Fan et al. examined gene polymorphisms in genes associated with Hcy metabolism (among which was *PON1*) with cardiovascular risk markers such as serum Hcy, CRP, and plasma fibrinogen in a study of adult women in USA ($n = 3,409$). They did not find any association of polymorphism in *PON1* with Hcy levels (Fan et al. 2010). On the other hand, the results of another study that examined polymorphisms Q192R and L55M in the population on the Island of Crete showed significant association of the polymorphisms with blood pressure, fasting blood glucose, triglycerides, apolipoprotein B, serum iron, and Hcy (Zafiroopoulos et al. 2010).

PON1 genotype and disease

Observation of the atheroprotective function of PON1 that emerged from animal studies has directed the interest of epidemiologists to the role of PON1 in human disease. As the PON1 allozymes differ in their enzymatic activities, one of the questions to answer is whether there are differences in *PON1* genotype frequencies between patients and controls. After almost two decades of studies, the answer to this question is ambiguous. *PON1* genotype frequencies differ in human populations; high activity alleles are more prevalent in blacks than in whites, while low activity alleles are more frequent in whites than in blacks (Jakubowski et al. 2001). Some studied populations show no differences in allele frequency between patients and controls, while others indicate an association of a particular genotype with a disease state. For example, the frequency of *PON1* 192RR genotype did not differ between CAD cases and healthy subjects in a UK (Domagala et al. 2006), Turkish (Bayrak et al. 2011), and Southeastern USA population (Coombes et al. 2011). However, in North-West Indian Punjabis (Gupta et al. 2011) and another Turkish population (Aydin et al. 2009), R allele was significantly elevated in CAD patients compared to controls. QR and RR genotypes showed significant association with type 2 diabetes mellitus in a North-West Indian population (Gupta et al. 2011).

A recent study claims that PON1 participates in the bioactivation of the antithrombotic pro-drug, clopidogrel, to an active thiol, and that *PON1* Q192R polymorphism is a major determinant of clopidogrel efficacy in individuals with CAD who underwent stent implantation (Bouman et al. 2011). The bioactivation involves a thiolactone intermediate, which is formed in a cytochrome P450 (P450)-dependent monooxygenation of its thiophene ring leading to 2-oxo-clopidogrel. The opening of the thiolactone ring of 2-oxo-clopidogrel is catalyzed by PON1. Compared with PON1 192RR homozygous individuals, 192QQ subjects showed risk of stent thrombosis, lower PON1 plasma activity, lower plasma concentrations of active metabolite of clopidogrel, and lower platelet inhibition (Bouman et al. 2011). However, other investigators immediately expressed reservations regarding the validity of the Bouman et al. findings (Camps et al. 2011; Dansette et al. 2011) and subsequent studies do not support those findings. For example, Dansette et al. (2012), have shown that there are two metabolic pathways for the opening of the thiolactone ring of 2-oxo-clopidogrel. The major one that was previously described results from a P450-dependent redox bioactivation of 2-oxo-clopidogrel and leads to two thiol diastereomers bearing an exocyclic double bond. The second, minor one results from a hydrolysis of 2-oxo-clopidogrel, which is dependent on PON1, and leads to an isomer, in which the double bond has migrated from an exocyclic to an endocyclic position in the piperidine ring. Furthermore, the major active thiol isomer present in the plasma of clopidogrel-treated subjects is the one that is formed via a cytochrome P450-dependent pathway. In addition, overwhelming evidence from clinical studies involving thousands of patients now indicates that the response to clopidogrel therapy does not depend on *PON1* Q192R polymorphism (Kreutz et al. 2012; Lewis and Shuldiner 2012).

The distribution of *PON1* Q192R genotypes differed significantly between Han Chinese patients with stroke and controls. Genotype QQ was more frequent in controls, while QR and RR were more frequent in patients with concurrent stenosis (Man et al. 2010) and in another group of ischemic stroke patients (Can Demirdöğen et al. 2008). However, a large study on young survivors of ischemic stroke ($n = 501$) and matched controls ($n = 1,211$) did not identify significant association between PON1 polymorphism and premature ischemic stroke (Giusti et al. 2010). Genotype distribution for PON1 SNPs was not significantly different between patients suffering from abdominal aortic aneurysm (AAA) and controls (Giusti et al. 2008). The gene frequency for the *PON1* 192 polymorphisms (QQ, RR, and QR) was not significantly different between pre-eclamptic women and healthy pregnant women in a Turkish population. The same observation was drawn for

the *PON1* 55 polymorphisms (LL, MM, and LM) (Isbilen et al. 2009).

The distribution of *PON1* 55 MM, ML, and LL genotypes between patients and controls also differs depending on the population. LL and LM genotypes and L allele of *PON1* 55 were more frequent in Turkish patients with CAD, while MM genotype and M allele were more frequent in healthy controls (Aydin et al. 2009). In L55M polymorphism, there was no difference in allele frequency between CAD subjects and controls (Gupta et al. 2011).

A study with a Korean population of ischemic stroke patients and controls did not identify significant differences in PON polymorphisms between the ischemic stroke and control subjects. This study confirmed that there was an association between allele L of *PON1* 55 and tHcy. Homozygotes for *PON1* 55L had a higher plasma concentration of tHcy (Shin et al. 2008).

On the other hand in a study with a Turkish population with acute hemispheric ischemic stroke and controls, *PON1* genotypes were associated with the risk of stroke. *PON1* 55LL genotype was associated with a 1.78-fold increase in the risk of ischemic stroke (Demirdöğen et al. 2009).

PON1 activity and disease

Since the gene frequencies for *PON1* 192R and *PON1* 192Q vary significantly among different ethnic groups (Richter et al. 2010), it was suggested that all epidemiological studies into the role of PON1 and disease, in addition to the genetic polymorphisms, should include a measurement of the enzyme itself (Mackness et al. 2001). Enzyme activity (POase and HTase) was found to be a better predictor for cardiovascular disease than the *PON1* genotype (Brophy et al. 2000; Jakubowski et al. 2001), and PON1 activity but not Q192R polymorphism was related to the extent of atherosclerosis (Bayrak et al. 2011).

In clinical studies, enzymatic activity of PON1 is measured with several substrates: paraoxon (POase), phenyl acetate (AREase), diazoxon (Furlong 2006), 5-thiobutyl butyrolactone (TBLase) (Kosaka et al. 2005), and Hcy-thiolactone (HTase) (Jakubowski 2000a). Out of these, only Hcy-thiolactone is a natural substrate of PON1. Activity measured with artificial substrates may not reflect physiological activity of the enzyme. It has been shown that HTase activity correlates strongly with POase (Jakubowski et al. 2001; Lacinski et al. 2004; Perla-Kaján and Jakubowski 2010; Sztanek et al. 2012) and less strongly with TBLase. However, HTase does not correlate significantly with AREase activity (Perla-Kaján and Jakubowski 2010). Measuring activity with phenyl acetate measures the AREase activity of PON1, which is considered a reliable

measurement of enzyme level because rates of phenyl acetate hydrolysis do not differ between PON1 192Q and R alloforms as they do for paraoxon and Hcy-thiolactone hydrolysis. Recently, a new chemiluminescent method employing a derivative of methylacridinium triflate, measuring AREase activity of PON1, was evaluated (Mu et al. 2012).

The majority of studies on healthy subjects and patients have shown that in pathological states such as CAD, renal failure, Alzheimer's disease, diabetes mellitus, age-related macular degeneration (AMD), and ischemic stroke, the activity of PON1 is diminished, and usually negatively correlated with Hcy level. Serum from hyperhomocysteinemic subjects has an impaired ability to induce cholesterol efflux from lipid-loaded macrophages compared with healthy controls. The activity of POase in serum is significantly reduced in subjects with elevated Hcy concentration (Holven et al. 2008), although acute mild hyperhomocysteinemia caused by L-Met loading does not influence POase activity (Türkeli et al. 2010). As mentioned in previous sections, PON1 circulates in the blood associated with ApoA1 in HDL (Blatter et al. 1993). Hcy reduces liver expression of ApoA1 and decreases its blood level in mouse models (Liao et al. 2006; Mikael et al. 2006). In humans, ApoA1 and HDL-cholesterol are also negatively correlated with plasma Hcy concentrations (Qujeq et al. 2001; Lacinski et al. 2004; Domagala et al. 2006; Liao et al. 2006; Mikael et al. 2006; Guéant-Rodriguez et al. 2011).

A meta-analysis of 43 studies showed significant decrease of POase and AREase activity in CAD patients compared with controls (Zhao et al. 2012). Since HTase activity of PON1 was suggested to contribute to an atheroprotective role of PON1 (Jakubowski 2000a, 2010; Jakubowski et al. 2001; Perla-Kaján and Jakubowski 2010), studies on the relationship between CAD and HTase activity of PON1 should provide useful information and have recently drawn more attention. In a study of a Japanese population, HTase activity was diminished in CAD patients compared with healthy controls and decreased inversely with the number of affected vessels and according to PON1 polymorphism. CAD patients had lower levels of HDL cholesterol and increased levels of tHcy and ox-LDL. HTase activity in patients was negatively associated with tHcy and Hs CRP levels, but positively associated with apoB and triglyceride levels (Koubaa et al. 2009).

Also a study on a Turkish population showed that POase and HTase activities were significantly decreased in CAD patients compared to controls. In the patients group, a negative correlation was observed between POase and HTase activities and the extent of atherosclerosis (expressed as Gensini score). It is worth mentioning that the negative correlation between the Gensini score and HTase

activity was higher and more significant than that with POase activity, which could suggest that HTase activity was a better indicator of physiological function of PON1 than activity measured with an artificial substrate. Both PON1 activities positively correlated with HDL levels. HDL concentration was lower and the cholesterol/HDL ratio was higher in the patient group, but neither of them correlated with the severity and extent of CAD (Bayrak et al. 2011). PON1 activity was found to be diminished also in CAD patients from Morocco compared with controls (Amine et al. 2011). Similar results were obtained in a study of North-West Indian Punjabis (350 angiographically proven CAD patients and 300 healthy controls), a distinct ethnic group with high incidence of CAD. The serum POase and AREase activities were significantly lower in CAD patients as compared to the controls. POase activity was modulated by all studied polymorphisms except L55M, while AREase activity was not affected by them (Gupta et al. 2011).

Not in all studies though CAD patients are characterized by lower PON1 activity. In a study of a Manchester and Blackpool UK population, a group of CAD patients had higher mean HTase activity than a control subject group (Domagala et al. 2006). However, mean HTase activity did not differ between CAD subjects, myocardial infarction (MI) subjects, and controls in a study of population from Poznan, Poland. This discrepancy could be due to the small number of subjects analyzed (51 CAD patients, 73 MI patients, and 60 controls) (Lacinski et al. 2004).

Serum POase and HTase activities were also investigated in patients after acute coronary syndrome receiving treatment with aspirin and ticlopidine. The two activities of PON1 were significantly associated with each other and did not correlate with any parameters for platelet aggregation, hypertension, sleep apnea, and diabetes mellitus. In contrast, serum PON1 activities seemed to be involved in cardiac function; brain natriuretic peptide and ejection fraction were significantly correlated with serum HTase ($r = -0.2767$, $P = 0.0214$) and POase activity ($r = 0.2558$, $P = 0.0339$), respectively. POase activity also demonstrated a significant association with increased levels of ankle-brachial index (Ohmori et al. 2012).

Decreased PON1 activity (AREase and POase) (Itahara et al. 2000; Rajković et al. 2010) and concentration (Suehiro et al. 2002) are found in patients undergoing hemodialysis (HD). Also, subjects with chronic kidney disease (CKD) have lower PON1 activity compared to controls (Atamer et al. 2008). HD and renal transplanted (RT) patients have significantly lower TBLase activities compared to the control subjects (Sztanek et al. 2012). Significantly lower POase activities are found in HD patients compared to the RT group (Kimak et al. 2011; Sztanek et al. 2012). Patients with end-stage renal disease

(ESRD) have decreased lactonase activity compared to control subjects, which increased after HD to levels similar to those of control subjects. One mechanism for lower lactonase activity in ESRD patients may be inhibition by uremic toxins and oxidative stress (Gugliucci et al. 2011). Another possible mechanism of diminished PON1 activity in renal disease patients involves Hcy and/or its metabolite. High Hcy levels are commonly present in plasma of CKD patients (Perna et al. 2004). As a consequence, hemodialyzed patients with ESRD have increased levels of S- and N-homocysteinylated proteins (Perna et al. 2006).

Paraoxonase 1 activity is negatively correlated with Hcy in HD, RT (Varga et al. 2009), and CKD patients (Atamer et al. 2008; Varga et al. 2009). PON1 activity is also negatively correlated with cystatin C (Varga et al. 2009), malondialdehyde (MDA), lipoprotein (a), apoA1 (Atamer et al. 2008), and asymmetric dimethylarginine (AMDA) levels (Sztanek et al. 2012). Decreased PON1 activity in CKD patients could be one of the reasons for the higher chance of developing atherosclerosis in those subjects. In fact, serum PON1 activity predicts the cardiovascular-related and all-cause mortality of HD patients (Ikeda et al. 2007) and is a new biomarker of HDL dysfunction.

Demirdöğen et al. found POase and AREase activities and PON1 activity ratio (POase/AREase) to be lower in ischemic stroke patients than in controls (Can Demirdöğen et al. 2008). The same authors did not find differences in POase, AREase, and diazoxonase activities between stroke and controls subjects from a Turkish population (Demirdöğen et al. 2009).

Paraoxonase activity is decreased (Wehr et al. 2009; Zengi et al. 2011) and negatively correlated with Hcy levels (Wehr et al. 2009) in Alzheimer's disease (AD) patients. Furthermore, HTase activity is diminished in the brains of subjects with AD, suggesting that this dysfunctionality could contribute to the pathology of AD (Suszynska et al. 2010; Borowczyk 2012b).

Decrease of serum PON1 activities is associated with type 2 diabetes mellitus in several populations (Amine et al. 2011; Gupta et al. 2011). In these patients, POase activity is significantly correlated with the plasma HDL level, age, and myocardial flow reserve (Dunet et al. 2011). There is a tendency for a negative association between TBLase activity and the thickness of the carotid intima media. Coronary heart disease is the number one cause of mortality in type 2 diabetes mellitus. Reduced PON1 activity could lead to accelerated hardening of arteries, and TBLase activity may be an indicator of atherosclerosis severity in patients suffering from type 2 diabetes mellitus (Kosaka et al. 2005).

A growing body of evidence suggests that Hcy is implicated in many diseases of the ocular system, including retinal atherosclerosis (Ghorbanihaghjo et al. 2008), central

retinal vein occlusion (CRVO) (Sodi et al. 2008), and exudative AMD (Javadzadeh et al. 2010). Intravitreal Hcy-thiolactone injection in mice causes degeneration of multiple retinal cells and homocysteinylated of retinal proteins (Chang et al. 2011). A negative correlation between serum PON1 and plasma Hcy was detected in CRVO patients that show a significantly lower serum PON1 AREase activity and higher plasma Hcy level compared with controls (Angayarkanni et al. 2008).

Subjects with AMD also have significantly lower serum PON1 activity and higher serum levels of Hcy than controls. In those patients, there is a negative correlation between PON1 AREase activity and Hcy level as well as between the activity and MDA, a lipid peroxidation product, used as a marker of oxidative stress (Ates et al. 2009). Disequilibrium between oxidative stress and antioxidant levels has been proposed to be one of the mechanisms of exudative AMD. Javadzadeh et al. investigated Hcy level, PON1 activity (using paraoxon and phenyl acetate as substrates), and ox-LDL levels in patients with exudative AMD and healthy controls. The distribution of PON1 phenotypes was significantly different between patients with exudative AMD and control subjects, i.e., the phenotype with the lowest activity (AA) was significantly more frequent in exudative AMD patients compared with healthy subjects, while phenotypes with intermediate (AB) and high (BB) activity were more frequent in controls. Except in BB phenotype, patients with AA and AB phenotypes had higher plasma Hcy levels in comparison to those of controls. The mean ox-LDL levels were significantly higher in the patients than controls. However, no significant differences in the comparison of Hcy and ox-LDL levels between the three PON1 phenotypes in both control and patients were found (Javadzadeh et al. 2012).

Barathi et al. reported that Hcy-thiolactone is positively correlated with HTase activity of PON1 in the eye's vitreous in patients with proliferative diabetic retinopathy ($n = 13$) and macular hole ($n = 8$) as well as in ex vivo cultured bovine retinal capillary endothelial cells. Furthermore, vitreous Hcy-thiolactone levels and HTase activity were significantly elevated in patients with proliferative diabetic retinopathy, compared with macular hole patients, and thus can be used as markers of diabetic retinopathy (Barathi et al. 2010).

Conclusions

Paraoxonase 1, carried on HDL in the circulation, is an enzyme with several activities enabled by different conformations of the active site. It can contribute to atheroprotective functions of HDL due to its ability both to reduce oxidative stress and to minimize protein damage by

N-homocysteinylation. Links between PON1 polymorphisms 192 and 55 and disease were investigated. Some studied populations show differences in allele frequency between patients and controls, while others do not.

The discovery of an atheroprotective function of PON1 has led to intensive studies of PON1 association with other risk factors for atherosclerosis and CAD, including homocysteine. Because of its essential role in protecting against toxic pesticides and cardiovascular disease, much attention has been devoted to pharmacological and dietary modulators of PON1 activity (Costa et al. 2005, 2011). Enzymatic activities of PON1 are diminished in pathological states, thus it would seem worthy to evaluate whether elevation of PON1 levels improves outcomes in patients. As shown in many studies, plasma Hcy is negatively correlated with HTase and POase activities of PON1. Thus, lowering Hcy levels should be beneficial by elevating PON1 activities. Indeed, short-term oral folic acid (5 mg/day) supplementation with or without methylcobalamin is an effective approach to decrease Hcy levels and increase HTase/PON activity in patients with type 2 diabetes. This could be a promising novel approach to protect against vascular complications in patients with diabetes (Weijun et al. 2008). Antioxidants also have been shown to up-regulate PON1. One of them is quercetin, a plant flavonoid present in red wine, with a number of bioactivities (Gong et al. 2009). Moderate alcohol up-regulates liver PON1 gene expression and serum activity, whereas heavy alcohol consumption has the opposite effects in animal models and humans (Lakshman 2009). To paraphrase a popular proverb: a glass of wine a day keeps your PON1 awake.

In spite of intensive studies and impressive advances especially concerning the structure, in the PON1 research, there are still questions awaiting examination. Results from computational docking and experiments collected for lipophilic lactones, phenyl acetate, and paraoxon provide an explanation of PON1's promiscuity. As the data show that HTase activity of PON1 could contribute to its athero- and neuroprotective function, it would be worth performing computational docking for Hcy-thiolactone as well as structure–function studies of the HTase activity of PON1. Other questions to answer are: Why does the deletion of *Pon1* gene not influence Hcy-thiolactone levels in mouse organs other than the brain? What are the mechanisms of Hcy-thiolactone detoxification in those organs?

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