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COMPREHENSIVE INVITED REVIEW

The Human Paraoxonase Gene Cluster As a Target in the Treatment of Atherosclerosis

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Abstract

The paraoxonase (*PON*) gene cluster contains three adjacent gene members, *PON1*, *PON2*, and *PON3*. Originating from the same fungus lactonase precursor, all of the three *PON* genes share high sequence identity and a similar β propeller protein structure. PON1 and PON3 are primarily expressed in the liver and secreted into the serum upon expression, whereas PON2 is ubiquitously expressed and remains inside the cell. Each PON member has high catalytic activity toward corresponding artificial organophosphate, and all exhibit activities to lactones. Therefore, all three members of the family are regarded as lactonases. Under physiological conditions, they act to degrade metabolites of polyunsaturated fatty acids and homocysteine (Hcy) thiolactone, among other compounds. By detoxifying both oxidized low-density lipoprotein and Hcy thiolactone, PONs protect against atherosclerosis and coronary artery diseases, as has been illustrated by many types of *in vitro* and *in vivo* experimental evidence. Clinical observations focusing on gene polymorphisms also indicate that PON1, PON2, and PON3 are protective against coronary artery disease. Many other conditions, such as diabetes, metabolic syndrome, and aging, have been shown to relate to PONs. The abundance and/or activity of PONs can be regulated by lipoproteins and their metabolites, biological macromolecules, pharmacological treatments, dietary factors, and lifestyle. In conclusion, both previous results and ongoing studies provide evidence, making the PON cluster a prospective target for the treatment of atherosclerosis. *Antioxid. Redox Signal.* 16, 597–632.

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I. Introduction

The human paraoxonase (*PON*) enzyme was initially characterized as an organophosphate hydrolase, because it catalyzes the hydrolysis of paraoxon organophosphate insecticides and sarin nerve gases as well as other similar compounds (74). PON has also been demonstrated to detoxify oxidized low-density lipoprotein (oxLDL) and protect against protein modification (N-homocysteinylation) by detoxifying homocysteine (Hcy) thiolactone. PON has a protective effect on the cardiovascular system and can reduce the incidence cardiovascular diseases (CVD), especially atherosclerosis. Atherosclerosis is still considered to be the most prevalent cardiovascular cause of disabling illness and death in developed societies (106, 201), although the lives of countless individuals suffering from atherosclerosis-related disease have been improved and/or saved by the currently available interventions (244). Thus, new strategies for preventing and treating atherosclerosis based on an enhanced understanding of the contributing factors are very much needed. The notion that the human *PON* gene cluster could be a target for the treatment of atherosclerosis has shed some light on this challenge (276).

Studies exploring the relationship between PON and CVD (especially atherosclerosis) constitute a flourishing field that has accumulated abundant data that could be useful for developing PON-related therapeutic strategies for atherosclerosis. In this review, we examine the reasons to use the *PON* gene cluster as a target in atherosclerosis by reviewing the progress made toward understanding the roles of PON in atherosclerosis and the regulation of the PON cluster. A better understanding of the possibilities for use of the human *PON* gene cluster as a target for the treatment of atherosclerosis may enable us to design novel and more effective strategies to combat atherosclerosis, the leading cause of death in humans.

II. The PON Family

In 1946, Mazur described the enzymatic hydrolysis of organophosphorus compounds by animal tissues (227). The esterases identified were named PONs by Aldridge based on their capability to hydrolyze their canonical substrate paraoxon (10, 11). In 1996, it was established that the gene responsible for the PON/arylesterase activities is a member of a multigene family; three such esterases (*PON1, PON2*, and *PON3*) have been identified (268). They are named according to their order of discovery.

A. Evolution of the PON genes

As a gene family, all three genes are located adjacent to one another in a cluster on the long arm of human chromosome 7

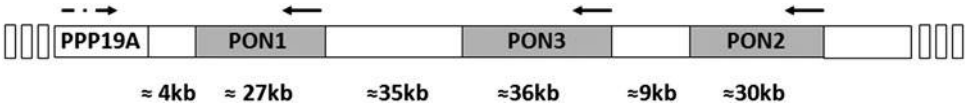

FIG. 1. Genetic map of the human *PON* gene cluster. *PON*, paraoxonase.

and on mouse chromosome 6, between q22.3 and q23.1 (268) (Fig. 1). The three gene members contain nine exons of approximately the same length in both species and share considerable structural homology. Within a given mammalian species, *PON1*, *PON2*, and *PON3* share ~70% identity at the nucleotide level and 60% identity at the amino acid level. However, each of the three genes share 81%–90% identity at the nucleotide level and 79%–90% identity at the amino acid level between mammalian species (206). Also, polymorphic variants are known to be common in at least the human and rabbit *PONs* (352). All *PON1s* have extra three additional nucleotide residues in exon 4, which code for amino acid 106 (lysine in human PON1), compared with *PON2* and *PON3* (268).

The whole gene cluster may have arisen from the tandem duplication of a common evolutionary precursor. The lactonase of the fungus *Fusarium oxysporium*, based on its appreciable structural homology and similar substrate spectrum (dihydrocoumarin and homogentisic acid lactone) with human serum PON1, is an attractive candidate for the common ancestor of this family (42, 169) (Fig. 2). *PON*-like genes can also be found in bacteria, plants, the worm *Caenorhabditis elegans*, the fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), frogs (*Xenopus laevis*), chickens (*Gallus gallus*), turkeys (*Meleagris gallopavo*), and a number of mammals (86). Based on these findings, Draganov *et al.* came up with a phylogenetic tree of the vertebrate *PONs* (Fig. 2) (86). Of these three members in the gene cluster, *PON2* appears to be the oldest based on the structural homology and predicted evolutionary distance among the family members. *PON3* arose from *PON2*, followed by the appearance of *PON1*. (295) PON2 and PON3 also exhibit lactonase activity but not PON activity (88). The gene duplication suggests that the PONs have important, as-yet unknown physiological roles, hence the redundancy and the number of polymorphic variants (86). Lactonase activity may be the common role of the PON enzyme family, because lactones are commonly found in plants, natural flavoring agents, and many food products. This activity may protect the *PON*-carrying species against dietary and environmental lactones, which could be the selective forces responsible for maintaining the balanced polymorphisms in the PON enzymes in mammals (295).

B. Structure of the PONs

1. Primary structure. The human *PON1* gene encodes a protein of 355 amino acids with a molecular mass of 43 kDa (210). The mature PON1 protein starts with an *N*-terminal hydrophobic sequence (125), which mediates PON1's association with high-density lipoprotein (HDL) (316, 317). There are three cysteine (Cys) residues at positions 42, 284, and 353 in the serum form of PON1. Cys42 and Cys353 form a disulfide linkage; Cys284, however, is free (178). The activity of PON1 can be abolished by mutating Cys42 or Cys353 to alanine; either of these mutations also significantly decreases the secretion of the protein (161). Mutation of Cys284 (to alanine or serine) decreases but does not abolish the PON and arylesterase activities (318). However, Cys284 has been demonstrated to be required for PON1's ability to protect LDL against copper-induced oxidation (22). Aviram *et al.* thus speculate that that PON1 possesses two catalytic sites, one that is required for the hydrolytic activity and another that is necessary for the antioxidant activity (21, 22). Two calcium-binding sites have been identified. The higher-affinity site is essential for enzyme stability, whereas the other is essential for the enzymatic hydrolytic activity. The activity and stability of PON1 are irreversibly destroyed when Ca^{2+} is removed by chelating agents. Some divalent ions, such as Zn, Mn, and Mg, however, can keep PON1 in a stable but inactive state

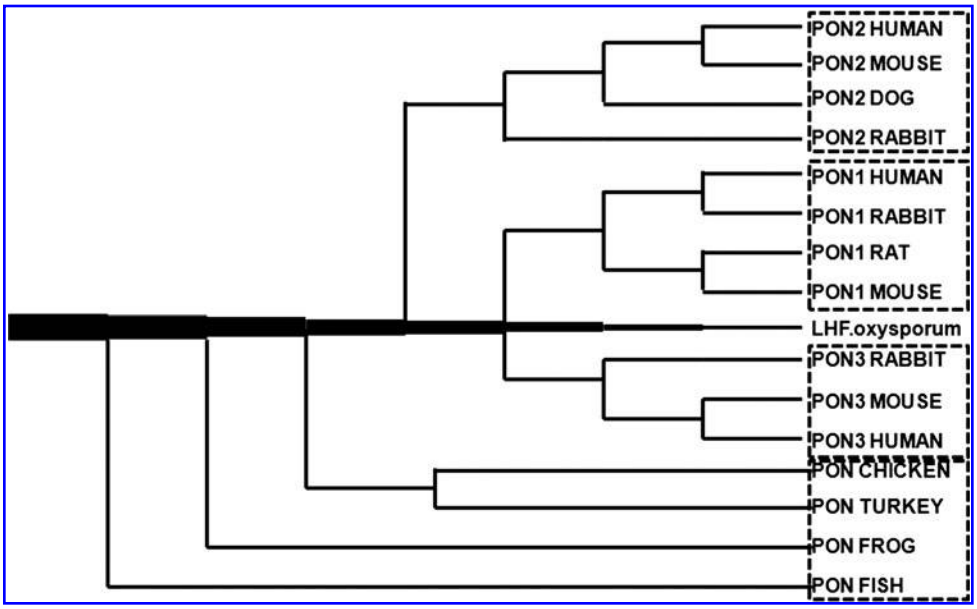

FIG. 2. Phylogenetic tree of vertebrate *PONs*. The tree begins at the fungal lactone hydrolase (LH) [for details, see ref. (86)].

(179). Several other amino acid residues (His115, His134, His155, His243, and Trp281) that are essential for PON1's esterase activity have also been identified using group-selective labeling and site-directed mutagenesis (160).

2. Three-dimensional structure. The three-dimensional structure of PON1 has been by resolved by Harel *et al* by crystallizing and analyzing a recombinant variant of rabbit PON1 at a resolution of 2.2 Å. This PON1 variant is highly similar to human PON1 (Fig. 3A, B) (121). PON was the first HDL-associated protein to be structurally elucidated (121). All the residues except the N-terminal residues (residues 1–15) and a surface loop (residues 72–79), two calcium ions, a phosphate ion, and 115 water molecules are shown in the structure (121). The overall architecture of PON1 is a β-propeller with six blades and a central tunnel; each blade consists of four β-sheets. A disulfide bridge between Cys42 and Cys353 forms a covalent closure between the N and C termini, which are conserved throughout the PON family (121). Two calcium ions, one at the top of the structure and one in the central section, are present in the central tunnel at a distance of 7.4 Å (121). The top one is considered to be the catalytic calcium and interacts with the side chain oxygens of Asn224, Asn270, Asn168, Asp269, and Glu53 (121, 179). The central calcium ion may contribute to the protein's structural stability (121, 179). PON1 is a glycosylated protein, and Asn253 and Asn324 have been proposed to be the two glycosylated sites (121).Throughout the whole gene cluster, the aforementioned residues that are important for maintaining the basic structure and catalytic activity are highly conserved, suggesting that all of the members maintained a common active site structure and catalytic machinery while diverging into three independent genes during evolution and expansion (121). PON1 and PON3 have additional N-terminal regions that are rich in hydrophobic residues and compatible with a transmembrane helix structure (at residues 7–18 and 19–28, respectively); these regions are hypothesized to contribute to HDL binding by acting as major hydrophobic interfaces, along with other hydrophobic residues in the second helix (121) (Fig. 3C, D).

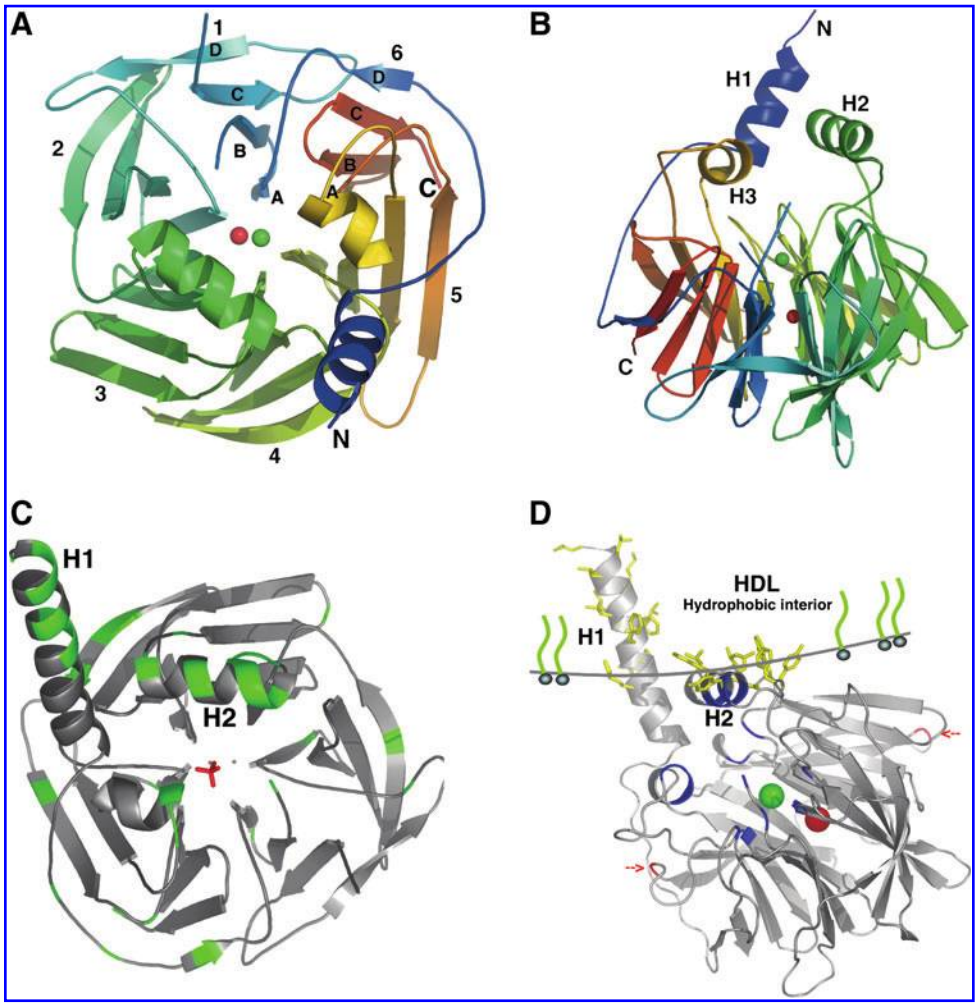

FIG. 3. PON1 protein structure and its docking to HDL. **(A)** *Top* view of the six-bladed propeller-like structure of PON1. The top of the propeller shows the face that carries the loops linking the outer strand of each blade (strand D) with the inner strand (A) of the next blade. The N and C termini and the two calcium atoms (Ca1, *green*; Ca2, *red*) are located in the central tunnel of the propeller. 1–6, six blades of PON1 molecule. **(B)** One of the sides of the propeller. H1, H2, and H3 helices appear at the top of the propeller. Residues 1–15 (at the N-terminus) and residues 72–79 (in a surface loop between strands 1B and 1C) are invisible in this structural model [adapted from ref. (121) with permission]. **(C)** Tertiary structure of rePON1. The hydrophobic surfaces are shown to be exposed. N-terminal residues 7–18 are predicted to be helical and are thus modeled as part of H1, which is actually not displayed in the crystal structure. All the hydrophobic residues appear with an accessible surface area of 20 $Å^2$. **(D)** PON1 is anchored to HDL *via* its hydrophobic side chains (shown in *yellow*). The line models the interface between the hydrophobic interior and the exterior aqueous portions of HDL, which is defined by the side chains of Tyr 185, Phe 186, Tyr190, Trp194, and Trp202 on helix H2 and the adjacent loops and Lys21 on helix H1, respectively. The hydrophobic side chains of the leucine and phenylalanine residues of H1 are located in the apolar region. The active site and the selectivity-determining residues are shown in *blue*. Glycosylation sites on Asn253 and Asn324 are shown in *red*. The high-resolution images were kindly supplied by Prof. Joel L. Sussman from the Weizmann Institute of Science, Israel. HDL, high-density lipoprotein.

C. Tissue and cellular distribution of PONs

In humans, PON1 and PON3 are expressed primarily in the liver, though a low level of PON3 expression can also be found in the kidney (277). Human PON2, however, is expressed in many tissues including heart, kidney, liver, lung, placenta, small intestine, spleen, stomach, and testis (245, 268). Cells of the artery wall, including endothelial cells, smooth muscle cells (SMCs), and macrophages, can also express PON2 (245, 268) (Fig. 4A). In mice, we found that the expression of mouse PON1 is limited highly to the liver (high expression) and the lung (low expression). Mouse PON2 and PON3 are expressed more universally in a variety of tissues including the heart, kidney, liver, lung, muscle, intestine, spleen, stomach, ovary, aorta, and brain at various levels (301) (Fig. 4B). PON3 mRNA and protein are also present in murine but not in human macrophages (284). In rabbits (254) and rats (282), PON3 can be purified from liver microsomes. Upon expression, PON1 and PON3 associate with HDL in the circulation (Fig. 5) (245); PON2, however, localizes intracellularly, notably to the perinuclear region, where it associates with the endoplasmic reticulum and nuclear envelope instead of the plasma membrane (Fig. 5) (134). In atherosclerotic plaques, PON1 and PON3 are present due to their association with HDL; PON2 is synthesized and retained by the major cellular components of plaques, that is, endothelial cells, SMCs, and macrophages, to protect them against risk factors (Fig. 6).

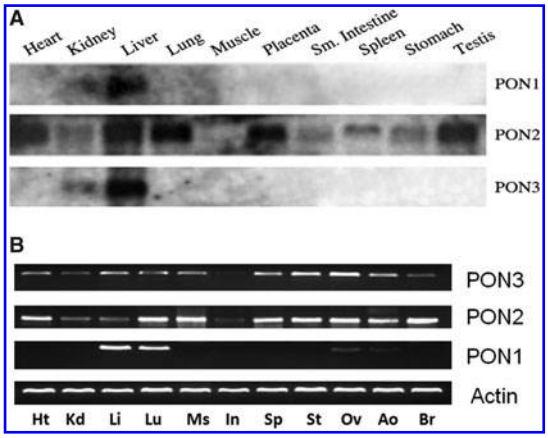

FIG. 4. Tissue distribution of the PON enzymes. (A) Northern blotting was used to detect the mRNA levels of the PONs in different human tissues using PON1, PON2, and PON3 probes [adapted from ref. (244) with permission]. Sm, small. **(B)** Reverse transcription–polymerase chain reaction was used to detect the expression of the PONs in mouse heart (Ht), kidney (Kd), liver (Li), lung (Lu), muscle (Ms), intestine (In), spleen (Sp), stomach (St), aorta (Ao), ovary (Ov), and brain (Br) with primers specific for mouse PON1, PON2, and PON3 messenger RNA [adapted from ref. (301) with permission]. β-Actin was used as a control.

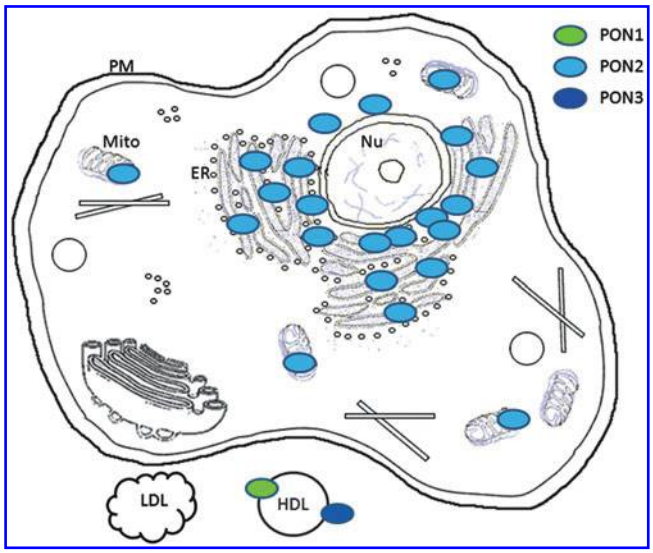

FIG. 5. Cellular distribution of the PONs. Upon expression, PON1 and PON3 are secreted from the cells and associate with HDL in the circulation (245); PON2 protein remains in the perinuclear region, where it associates with the ER and nuclear envelope rather than the PM (134). ER, endoplasmic reticulum; LDL, low-density lipoprotein; Mito, mitochondria; Nu, nuclear; PM, plasma membrane.

D. Substrates of PONs

1. Chemical substrates. Human PON1, which is classified as an "A-esterase" (10), is able to hydrolyze various toxic oxon metabolites of insecticides with different efficiencies. Its activity toward paraoxon, parathion, diazinon, chlorpyriphos, and similar compounds is low (79, 86), but it exhibits much higher catalytic activity to other organophosphates, such as diazoxon (DZO) and chlorpyriphos oxon (198). Some nerve agents, such as sarin and soman, can also be hydrolyzed by PON1 (79, 86). In addition, phenylacetate, thiophenylacetate, 2-naphthylacetate, and other aromatic esters are substrates for PON1 (42, 86). PON1 has also been found to be able to hydrolyze a variety of aromatic and aliphatic lactones and cyclic carbonates, for example, homogentisic acid lactone, dihydrocoumarin, γ-butyrolactone, and Hcy thiolactone (41, 42, 145). Nevertheless, under nonphysiological conditions, PON1 is even reported to be able to catalyze the lactonization of γ- and δ-hydroxycarboxylic acids, which is thought to be a reverse reaction (328). Human PON2 and PON3 exhibit very limited or no PON and arylesterase activities. Like PON1, however, they can hydrolyze aromatic and long-chain aliphatic lactones, for example, dihydrocoumarin and 5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid lactone (88). Using a baculoviral expression system, Draganov *et al.* characterized the catalytic spectrum of all three PON members. The results support the idea that the PONs are lactonases/lactonizing enzymes. The three members share some overlapping substrates (*e.g.*, aromatic lactones) but also have distinctive substrate specificities (88) (Fig. 7). Nevertheless, some drugs containing lactone or cyclic carbonate moieties can also be metabolized by the PONs. For example, the unsaturated cyclic carbonate prodrug prulifloxacin can be hydrolyzed by PON1 to the active quinolone antibiotic NM394 (333). PON3 can hydrolyze some of the statin lactone drugs (lovastatin and simvastatin) and the diuretic spironolactone (86). However, none of these chemicals is present in the normal human body; therefore, none of them can be PON3's physiological substrate.

2. Physiological substrates. Based on the substrate spectrum elucidated by many lines of research, the PONs are now proposed to be lactonase enzymes. Oxidized metabolites of polyunsaturated fatty acids (PUFAs) could be physiological

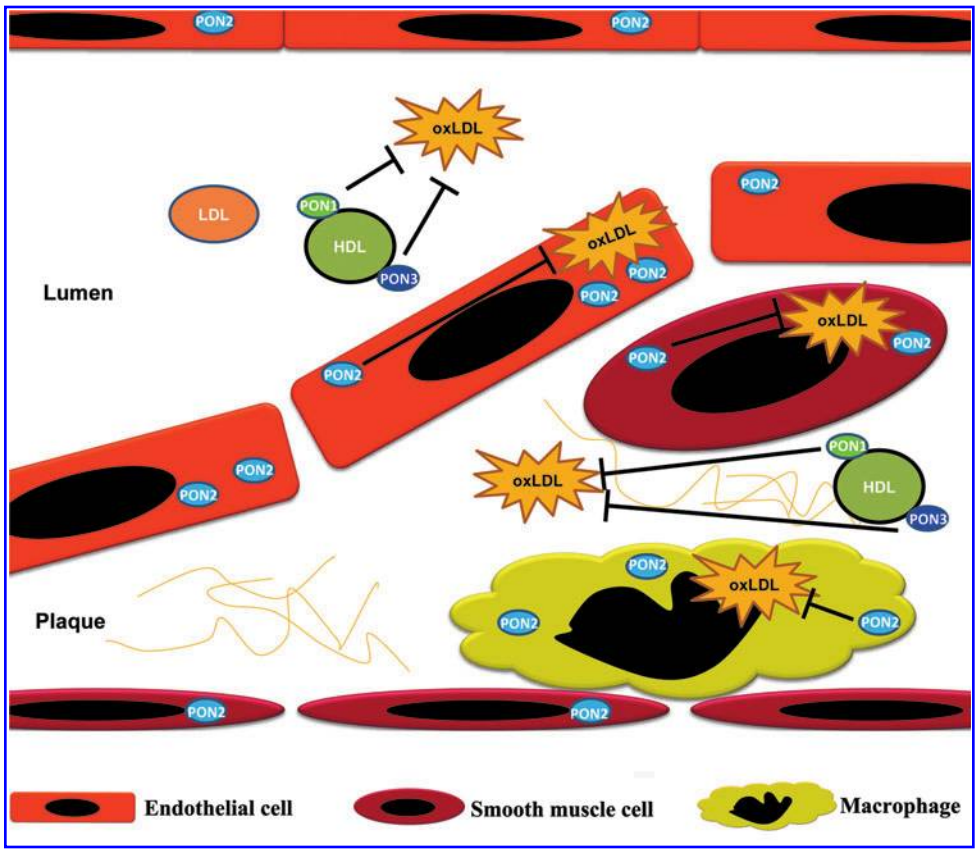

FIG. 6. Distribution of PONs and their functions in blocking the effects of oxLDL in atherosclerotic plaques. PON1 and PON3 appear in atherosclerotic plaque by associating with HDL. PON2 is expressed in plaque endothelial cells, smooth muscle cells, and macrophages. PON1 and PON3 exert their effect at the extracellular level by associating with HDL in either the serum or the atherosclerotic plaque. PON2, however, protects plaque competent cells by acting intracellularly. oxLDL, oxidized LDL.

substrates of PONs, because the structure of many of these molecules is similar to that of lactones (88, 128, 167). Purified PON1 can destroy the biologically active lipids in mildly oxLDL (351). Further experiments indicated that PON1 is capable of mediating the hydrolysis of 19% of the lipid peroxides and 90% of the cholesteryl linoleate hydroperoxides in oxidized HDL. Both HDL-associated PON1 and purified PONs were able to substantially hydrolyze hydrogen peroxide (H_2O_2) (27). PON1 was also confirmed to be able to cleave the ester bound between the cholesterol and the linoleic acid hydroperoxide or hydroxide (23).

Accumulated data have shown that Hcy-thiolactone is also very likely to be a natural substrate of PON1 (349). Jakubowski has demonstrated that a single specific enzyme, present in mammalian but not in avian sera, hydrolyzes thiolactone to Hcy. Thiolactonase, in the presence of the 45-kDa protein component of HDL in human serum, requires calcium for its activity and stability and is inhibited by isoleucine and penicillamine. Substrate specificity studies suggest that Hcy-thiolactone is a likely natural substrate of this enzyme. The unanticipated outcome of this study was that Hcy-thiolactonase is identical with serum PON (145). Hcy-thiolactone is a reactive metabolite that causes protein N-homocysteinylation through the formation of amide bonds with protein lysine residues, which impairs or alters protein function (147). It has also been demonstrated that the Hcy-thiolactonase activity of PON1 is a determinant of plasma N-Hcy-protein levels, leading to the conclusion that PON1 protects proteins against *N*-homocysteinylation *in vivo* (263). PONs can thus prevent protein homocysteinylation and related protein inactivation and cell damage.

In addition, PON1 has also been shown to have the ability to hydrolyze *N*-(3-oxododecanoyl)-L-homoserine lactone (3-OC12-HSL), which is an important quorum-sensing component of *Pseudomonas aeruginosa* (330). PON2 has also been demonstrated to be able to lower H_2O_2-induced intracellular oxidative stress (245), reduce 2,3-dimethoxy-1,4-naphthoquinone-induced reactive oxygen species (ROS) production in cells, and even decrease ROS levels during endoplasmic reticulum stress (134). Among the PON family, PON2 exhibits the greatest capability to degrade acylhomoserine lactones (88) and 3-OC12-HSL (330), which are related to pulmonary infections and cystic fibrosis (355).

The ability to hydrolyze oxidized metabolites and Hcy-thiolactone allows the PONs to protect against oxidative stress and inflammatory diseases, including atherosclerosis.

III. PONs Protect Against Atherosclerosis and Coronary Heart Disease by Decreasing the Toxicity of LDL

A. Atherosclerosis-related CVDs do great harm to human health

CVD has accounted for more than half of all deaths in the United States and other industrialized nations since the 1950s. Worldwide, CVD is projected to become the most common cause of death, with more than 36% of all deaths, for the first time in human history in 2020. This is more than twice the number of deaths from cancer (53). Thus, CVD has become one of the most serious health problems for the present and foreseeable future. Atherosclerosis, which is related to coronary artery disease (CAD), stroke, abdominal aortic aneurysm,

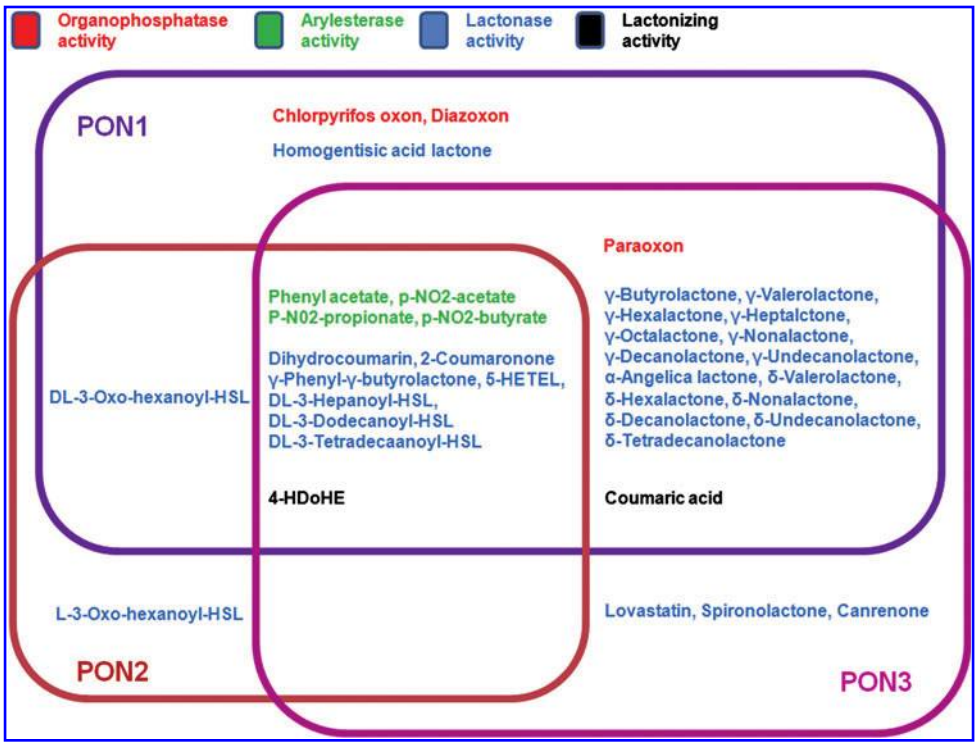

FIG. 7. Specific enzymatic activities of the purified recombinant human PONs and relationships among the enzymatic activities of various PONs. Some chemicals are substrates for more than one PON. However, the catalytic efficiency sometimes dramatically differs among PON members. None of the substrates shown is the physiological substrate of PONs [see ref. (88) for details]. 5-HETEL, (±)5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid 1,5-lactone; HSL, homoserine lactone.

and peripheral artery disease, constitutes the single most important contributor to this growing burden of CVD (201). Thus, developing effective strategies to treat atherosclerosis is among the most important ways scientists can work to overcome CVD.

B. OxLDL is one of the most important risk factors for atherogenesis

Numerous genetic and environmental risk factors for atherosclerosis have been revealed by epidemiological studies over the past 50 years (Fig. 8) (203). Increased levels of atherogenic lipoproteins, mainly LDL and very LDL (VLDL), are a prerequisite for most forms of the disease (202). As the major cholesterol carrier in human plasma, the surface of LDL is composed of a monolayer of 700 phospholipid molecules, consisting primarily of lecithin, small amounts of sphingomyelin and lysolecithin, and 600 molecules of cholesterol. The apolipoprotein (Apo)B-100 molecule embeds in the outer layer. Approximately 1600 molecules of cholesterol ester and 170 molecules of triglyceride reside in the central cores of LDL particles (111). Half of the fatty acids inside LDL are PUFAs

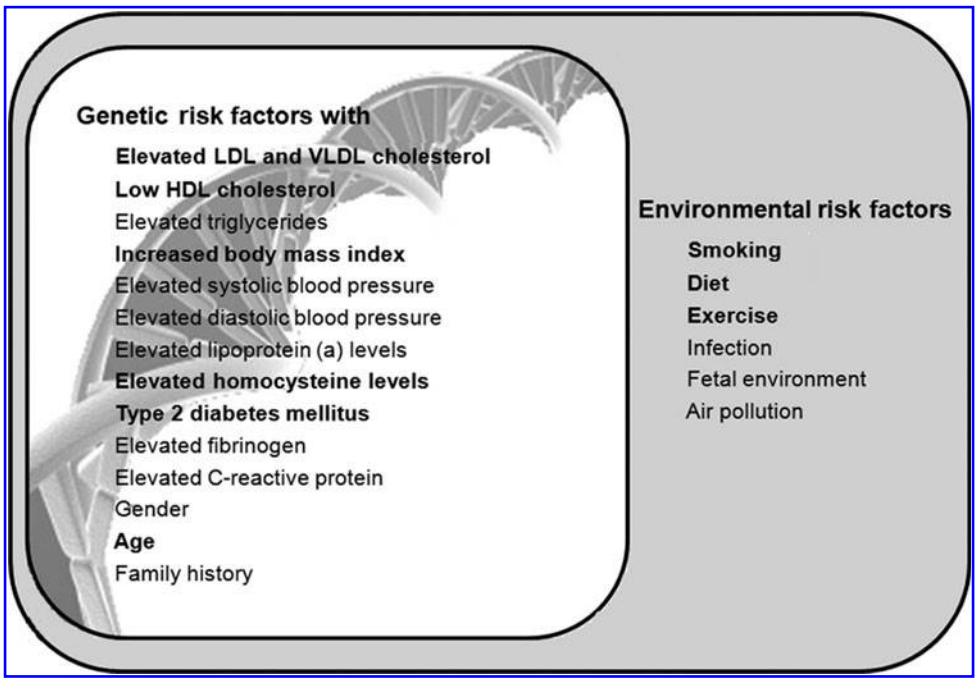

FIG. 8. Risk factors for atherosclerosis. Susceptibility to atherosclerosis is determined by many genetic factors; some of these (typed in *bold*) are also related to *PON* gene cluster. Environmental factors can also affect the development of atherosclerosis; amongst, factors shown in *bold* have been indicated to affect PON status [modified from ref. (203)]. VLDL, very LDL.

that are mostly composed of linoleic acid but also include arachidonic acid and docosahexaenoic acid. All of these PUFAs are usually protected against free radical attack and oxidation by alpha-tocopherol and other antioxidants (272). Whenever there is an imbalance in the levels of antioxidants and the amount of PUFAs, LDL is oxidized. LDL can be oxidized by metal ions, lipoxygenases, myeloperoxidase, and reactive nitrogen species, mainly under the aorta intima; this process is mediated by the cells residing in the aorta wall (230). Based on the extent of modification, oxLDL can be classified as minimally modified LDL, mildly modified oxLDL, moderately modified oxLDL, heavily modified oxLDL, or extensively modified oxLDL. Transitional products of the oxidation process including aldehydes, for example, malondialdehyde (MDA), and 4-hydroxynonenal, interact with the positively charged ε-amino groups of lysine residues on apoB. This interaction not only changes the structure of LDL and decreases its affinity for liver LDL receptor but also renders the LDL more negatively charged and increases its affinity for scavenger receptors (SRs). OxLDL plays a pivotal role in triggering proinflammatory events that initiate and exacerbate atherogenesis (201, 320).

OxLDL plays important roles in endothelial injury. First, oxLDL is toxic to cells; increased oxLDL levels can cause endothelial cell degeneration, necrosis, and detachment from the aorta wall. OxLDL was reported to dose-dependently promote the death of endothelial cells when added to an endothelial culture system (131, 173 199, 273). OxLDL can also induce increased production of intracellular ROS and apoptosis by binding to lectin-like oxLDL receptor-1 (65, 72). OxLDL also affects the expression of various key genes in endothelial cells, thus altering endothelial function (126). In addition, oxLDL can also induce cytoskeletal rearrangements such as F-actin distribution, cell contraction, and the formation of intercellular gaps (92, 364). Lastly, oxLDL can induce endothelial cells to express and secrete various inflammatory and adhesive molecules such as intercellular adhesion molecule 1 (ICAM-1) and monocyte chemotactic protein 1 (MCP-1), which facilitate the adherence and migration of monocytes and T lymphocytes to injured sites.

Recent studies have shown that oxLDL promotes morphological changes in cultured human vascular smooth muscle cells (VSMCs), suggesting that oxLDL prompts VSMCs to switch from the contractile to the synthetic type (279). In addition, oxLDL can dose-dependently stimulate SMC migration *via* a chemotactic mechanism; native LDL has no such activity (20). It has also been reported that oxLDL but not native LDL stimulates DNA synthesis in cultured SMCs and that alpha-tocopherol (vitamin E) inhibits this proliferative response (183). These effects are partly mediated by oxidative stress, which causes the release of fibroblast growth factor-2 and a subsequent autocrine or paracrine response (63).

In addition, Okura *et al.* found that oxLDL increases the susceptibility of VSMCs to apoptosis (251). At lower concentrations, oxLDL may stimulate VSMCs to proliferate and develop into foam cells; at higher concentrations, it may induce apoptosis in VSMCs (365). OxLDL has been shown to induce tissue factor gene expression mediated by both early growth response protein 1 (Egr-1) and specificity protein 1 (Sp1) in SMCs (77). Collagen production by SMCs is stimulated by oxLDL but not native LDL, suggesting that elevations in oxLDL could lead to collagenosis in atherosclerosis (159). OxLDL may also promote osteogenic differentiation of VSMCs and thus vascular calcification (35).

Minimally modified oxLDL (MM-LDL) stimulates the endothelium to express adhesion molecules such as ICAM-1 and vascular adhesion molecule 1, which mediate adhesion of monocytes to the injured endothelium (71). MM-LDL also induces endothelial cells to express monocyte colony stimulating factor (M-CSF), thus promoting monocyte proliferation and differentiation into macrophages (223, 271). Nevertheless, MCP-1 expression and secretion from endothelial cells and SMCs can also be stimulated by oxLDL, which induces the infiltration of monocytes into the subendothelial space (78, 195). Mertens and Holvoet hypothesized that, because it is a potent inhibitor of macrophage motility, oxLDL may also promote macrophage retention in the arterial wall (230).

Upon being taken up as a ligand of the macrophage surface receptor (319), oxLDL promotes foam cell formation. OxLDL, especially MM-LDL, can upregulate the expression of the macrophage SR by activating activator protein 1 (AP-1), nuclear factor kappa B (NF-κB), and other transcription factors (143, 226), cluster of differentiation 36 *via* peroxisome proliferator-activated receptor γ (PPAR-γ) activation (94, 119), and macrosialin (361). Upregulation of the oxLDL receptor leads to increased uptake of oxLDL by macrophages, so there is no negative feedback between oxLDL and its receptors. OxLDL is also very resistant to degradation by the lysosome. Because of these properties, oxLDL stimulation facilitates lipid accumulation in macrophages, leading to foam cell formation (16, 174, 230, 362).

It has been demonstrated that the growth of murine macrophages is induced by oxLDL (289). Many signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) (136), extracellular signal-regulated kinase 1/2 (ERK1/2) or p38 mitogen-activated protein kinase (MAPK) (297), and adenosine monophosphate-activated protein kinase pathways (141), have been reported to be involved in the mitogenic effect of oxLDL. Oxidatively modified apoB is thought to be the main growth-stimulating component of oxLDL, although oxidized phospholipids may play a secondary role (222).

OxLDL can injure macrophages, especially when the levels of phagocytosed oxLDL exceed their degradation capability. Cell death can occur through necrosis or apoptosis depending on the oxidation level of oxLDL, the concentration of oxLDL, and the length of stimulation. Most of the toxicity is caused by the lipid fraction of oxLDL, which contains a wide variety of oxidized lipids (130, 239). OxLDL not only activates caspase-8 through the Fas-FasL pathway but also induces caspase-9 activation by promoting mitochondria to release cytochrome *c* (70, 197, 225, 246, 256).

When stimulated with oxLDL, macrophages present in atherosclerotic plaques become activated and release many cytokines, tissue factors, and inflammatory mediators. These molecules can induce and regulate repair, migration, mitosis, and synthesis of lipid and proteins in neighboring cells, thus affecting atherogenesis, vasomotion and blood coagulation. For example, interleukin (IL)-1 and tumor necrosis factor α (TNF-α) released by oxLDL-activated macrophages in atherosclerotic plaques induce VSMCs to express platelet-derived growth factor (PDGF), which promotes VSMC proliferation. TNF-α also induces VSMCs to express SRs (43, 166).

C. PON1 contributes to the anti-atherosclerotic functions of HDL

In contrast to LDL, HDL levels have been shown to be inversely related to the development of cardiovascular atherosclerosis (108). Gotto and Brinton reported that every 1 mg/dl increase in HDL is associated with up to 3% decrease in the risk of adverse cardiovascular events (110). HDL has been thought to be at least as important in the pathogenesis of atherosclerosis as LDL. Nevertheless, it has been shown that low serum levels of HDL more strongly predict future adverse cardiovascular events than elevated LDL levels (288). Even in patients with low or close to normal LDL levels, the serum levels of HDL are strongly predictive for coronary heart disease (CHD) (33, 108). In addition to being a useful predictor for atherosclerosis-related diseases, HDL has also been suggested to have a direct protective effect on atherosclerosis (59, 107, 187, 200).

HDL exerts its protective effect through multiple mechanisms. The first proposed mechanism is that HDL facilitates reverse cholesterol transport (RCT), through which free cholesterol produced by peripheral tissues and cells is transferred to the liver (54, 110), where cholesterol is converted to sterol metabolites and then excreted into the bile. HDL can also accept cholesterol and facilitate its efflux from macrophages and foam cells that reside within atherosclerotic plaques through this RCT mechanism (200).

In addition, HDL exerts protective effects by reducing systemic and local inflammation (34). This process is mostly accounted for by HDL's protection of LDL from oxidation, which prevents the recruitment and migration of inflammatory cells to the arterial wall (54, 212, 238). The fact that HDL is able to reduce LDL oxidation has been shown *in vitro* in various experimental models of LDL oxidation in many independent studies (236, 241), including our own (301). The induction of LDL oxidation was always shown to be significantly prevented or reduced when HDL particles were present in the incubated system compared with conditions without HDL present. In such cases, the HDL complex enzymatically hydrolyzes the oxidized phospholipid molecules of LDL particles and transfers the modified phospholipid particles to HDL itself. Reduction of the levels of oxLDL therefore attenuates the vicious atherogenic cycle originating from LDL oxidation. Many other antiatherogenic effects, such as reductions in endothelial death, SMC apoptosis (131), and macrophage foam cell formation (236), also result from reductions in LDL oxidation. The antiatherogenic and antioxidant effects of HDL should be attributed to HDL-associated proteins such as apoA I, lecithin cholesterol acyltransferase, and PON1, among others (211, 343, 348). Of these proteins, PON1 contributes significantly more antioxidant activity to HDL (90) than the others, because many of the lipid oxidative metabolites can be degraded by members of the PON family, as discussed earlier. However, several studies show that PON1 does not have intrinsic antioxidant activity and previous reports of such activity *in vitro* may have been due to contaminations present in PON1 preparations (73, 216, 329).

Moreover, HDL has the ability to hydrolyze Hcy-thiolactone and thus is hypothesized to protect against Hcy-thiolactone toxicity by preventing thiolactone formation and limiting the extent of protein modification (149). Earlier work by Jakubowski suggests that HDL is capable of hydrolyzing Hcy-thiolactone and blocking the accumulation of N-Hcy-modified proteins *in vitro*, and PON1 has the appropriate enzyme activity. The Hcy-thiolactonase activity of plasma is associated with PON1 polymorphic variants (Leu/Met 55 and Arg/Gln 192) (148). In the latest study by Perla-Kajan and Jakubowski, the use of new assays for the quantification of N-linked Hcy content in proteins and for the determination of Hcy-thiolactonase activity showed that the Hcy-thiolactonase activity of PON1 influences the serum N-Hcy-protein level and protects against the atherogenic effects of N-Hcy-protein accumulation *in vivo*. These findings provide evidence for atheroprotective roles for HDL in humans (263).

D. PON activity decreases atherosclerosis

1. *PON1* and its protection against atherosclerosis. *PON1*, the first member of the *PON* gene cluster to be discovered, has been studied for many years and is by far the best understood member of the family. It is highly conserved in mammals but is absent in fish, birds, and invertebrates such as arthropods (181). Changes in the size and shape of HDL particles strongly influences the binding affinity and stability of PON1 and results in a reduced antioxidative capacity (90). PON1 is the main factor the nervous system uses to protect itself against neurotoxic organophosphates that enter the circulatory system (82).

In addition to its established important roles in organophosphate metabolism, PON has also been shown to play roles in lipid metabolism. A relatively new area of investigation, the study of the links between PON1 and LDL oxidation, led to an extensive investigation of the potential roles of PON1 in atherosclerosis. The capacity of PON1 to prevent LDL and HDL against oxidation by a variety of pro-oxidant factors, including cell-induced LDL oxidation, has been demonstrated in both *in vitro* and *in vivo* studies (82, 90, 181, 237, 351). It has been shown that the antioxidant activity of PON1, by blocking LDL oxidation, prevents a number of pathological characteristics associated with atherogenesis, monocyte recruitment, and gene activation (305). The strongest evidence for PON1's roles in hydrolyzing oxLDL and providing atheroprotection comes from studies of PON1-knockout mice (303, 305). Both HDL and LDL isolated from PON1 null mice are more susceptible to oxidation in the presence of cocultured cells than those from wild-type littermates. Also, HDL isolated from PON1 null mice was no longer able to prevent LDL oxidation in a cell coculture model of the artery wall. Thus, PON1 null mice are more susceptible to both lipoprotein oxidation and atherosclerosis than wild-type mice. The association between low plasma PON1 activity and features of atherosclerosis has been verified by additional studies in mice, humans, and other species. In apo E-knockout mice, LDL receptor-knockout mice, and apoAII-overexpressing transgenic mice, the plasma PON1 levels are greatly reduced, and the mice exhibit hypercholesterolemia and atherosclerosis (196).

As reported in 2002, Tward *et al.* constructed human PON1 transgenic mice to test whether high levels of PON1 protect against LDL oxidation and decrease atherosclerosis *in vivo* (338). The results indicated that transgenic overexpression of human PON1 dose-dependently protects mice against both early and late stages of atherogenesis. This study demonstrated, for the first time, that the human PON1 protein exhibits antioxidative and antiatherogenic functions *in vivo*. In the PON1 transgenic mice, HDL exhibited three times as

much PON1 activity as wild-type HDL and was better able to protect LDL against oxidation. It has also been reported that PON1 preserves HDL function during oxidative stress (250). Using PON transgenic mice, Oda *et al.* found that overexpression of PON1 inhibits lipid hydroperoxide formation on HDL and protects HDL integrity and function. PON1 also reduces monocyte chemotaxis and adhesion to endothelial cells (7). The antioxidative functions of PON1 are attributable either to its phospholipase A2-like activity, which hydrolyzes biologically active oxidized phospholipids, and its peroxidase-like activity, which destroys lipid hydroperoxides and H_2O_2 (8, 23, 27, 155), or to the contamination of other enzymes during PON1 purification (73, 216, 329). In addition to preventing LDL oxidation, PON1 can also promote cellular cholesterol efflux, which is the first step in RCT. Thus, PON1 is hypothesized to affect the efficiency of lipid transfer between HDL cholesterol and LDL cholesterol (206). It has also been demonstrated that PON1 plays a role in protection against bacterial endotoxins, thus stabilizing cellular membranes during either acute or chronic exposure to oxidative agents and free radicals that challenge the selective permeability of the membrane (283). All of these biochemical functions contribute to PON1's protective effect in the development of atherosclerosis and CVDs.

At the same time, clinical investigations also support the idea that PON1 protects against atherosclerosis-related disease. The PON1 activity in patients with CHD is approximately half that in disease-free control participants (28). Low PON1 activity increases the development of atherosclerosis (353). The group of men with the highest levels of serum PON1 activity were nearly 60% less likely to have CHD than those with the lowest (205). These results show that serum PON1 concentration is inversely correlated with susceptibility to atherosclerosis. Other lines of evidence for PON1's atheroprotective effect include studies showing that purified PON1 reduces the uptake of oxLDL by macrophages by ~68%. PON1 also inhibits cholesterol biosynthesis in macrophages (by ~84%) and increases the efflux of cholesterol from macrophages (by ~70%) (205).

2. PON2 and its protection against atherosclerosis. As the second member of the *PON* gene family, *PON2* gene shares 79%–90% identity with *PON1* (268). PON2 is thought to be able to lower intracellular oxidative stress and prevent cellular oxidation of LDL, although its aromatic ester and PON hydrolyzing activities are lower than those of PON1 (245). Cells overexpressing PON2 are less able to oxidize LDL and show significantly less intracellular oxidative stress when exposed to either H_2O_2 or oxidized phospholipids (245), suggesting that PON2 plays a protective role in atherosclerosis. Indeed, when PON2-knockout, apoE null mice were challenged with a high-fat diet, these mice developed significantly larger atherosclerotic lesions than their wild-type counterparts, although the serum levels of VLDL and LDL cholesterol were significantly lower in PON2-deficient mice compared with wild-type mice. Enhanced inflammatory signaling by LDL, an attenuated antiatherogenic capacity of HDL, and a heightened state of oxidative stress, along with an exacerbated inflammatory response in PON2-deficient macrophages, were also detected in the PON2-deficient mice (241). Conversely, adenoviral overexpression of PON2 in apoE null mice significantly enhances the efflux potential and antioxidant capacity of serum and increases the anti-inflammatory properties of HDL, thus protecting mice against atherogenesis *in vivo* (243). Further investigation showed that the antiatherogenic effects of PON2 are partly contributed by its protection of mitochondrial against oxidative stress (84). PON2 has been found to be able to prevent mitochondrial superoxide formation and apoptosis of cells, which is independent from its lactonase activity (12). These studies are sufficient to show that PON2 strongly inhibits the development of atherosclerosis.

3. PON3 and atherosclerosis. *PON3*, the third member of the *PON* gene family to be identified, encodes the PON3 protein, which is composed of 353 amino acid residues. Rabbit PON3 is more efficient at protecting LDL from copper-induced oxidation than rabbit PON1 (87). PON3 overexpression can prevent the formation of mildly modified oxLDL and reduce the concentration of previously formed mildly modified oxLDL in cultured human aortic endothelial cells (277). Interestingly, PON3 expression is not regulated by oxidized phospholipids in HepG2 cells or by high-fat atherogenic diet feeding in mouse liver (277). Further, infecting apoE-deficient mice with adenovirus expressing human PON3 resulted in a significant decrease in lesion formation compared with mice infected with a control adenovirus. Serum from mice overexpressing human PON3 contained significantly lower levels of lipid hydroperoxides and was better able to release cholesterol from cholesterol-loaded macrophages than serum from control mice. In addition, the LDL from these mice was less susceptible to oxidation, and their HDL was better able to protect against LDL oxidation (242). Human PON3 transgenic mice developed significantly smaller atherogenic diet-induced atherosclerotic lesions compared with their nontransgenic littermates either on a B6 background or an LDL receptor knockout background, although no significant differences in total plasma, HDL, VLDL/LDL cholesterol, triglyceride, or glucose levels were observed between the PON3 Tg and the control mice. Nevertheless, the aortic expression of monocyte chemoattractant protein-1 was also decreased compared with the control littermates. More impressively, decreased adiposity and lower circulating leptin levels were also observed in both lines of human PON3 transgenic mice compared with the nontransgenic mice. These results demonstrate that PON3 may play an important role in protection against not only atherosclerosis but also obesity (306).

4. Roles of the *PON* gene cluster in atherosclerosis. Based on the findings discussed earlier, Reddy *et al.* concluded that all of the three members of the *PON* gene family protect against atherosclerosis and could be used as therapeutic targets for the treatment of atherosclerosis (276). However, the role of the entire *PON* gene cluster in atherogenesis remains to be elucidated. Therefore, we generated human *PON* gene cluster transgenic mice to analyze the functions of the *PON* gene cluster in atherogenesis. We found that *PON* cluster transgenic apoE null mice formed significantly fewer atherosclerotic lesions than nontransgenic apoE null mice under a high-fat diet challenge, although there was no change in total plasma cholesterol, HDL cholesterol, VLDL/LDL cholesterol, triglycerides, or glucose levels. Contrary to our expectations, no observable additive effect on atherogenesis of overexpressing the entire gene cluster *versus*

Table 1. Comparison of Atherogenesis Phenotypes Among Mouse Strains with Various Genetic Modifications of Paraoxonase

	Diet				
Mice model	*Fat*	*CHO*	*Sodium cholate*	*Period*	*Plaque quantification*
PON1 ko	15.75%	1.25%	0.50%	15 weeks	Cross-section
PON1 ko/ApoE ko	42.00%	0.15%	0	16 weeks	*En face*
PON1 tg	15.75%	1.25%	0.50%	15 weeks	Cross-section
PON1 tg/ApoE ko	Regular chow			28 weeks	Cross-section
PON2 ko	15.80%	1.25%	0	15 weeks	Cross-section
PON3 tg	15.75%	1.25%	0.50%	15 weeks	Cross-section
PON3 tg/LDLR ko	42.00%	0.15%	0	8 weeks	Cross-section
PON3 ad	Regular chow			3 weeks	Cross-section
PC tg/ApoE ko	10%	1.25%	0	10 or 16 weeks	*En face* and section

CHO, cholesterol.

expressing only PON1 or PON3 was found. We hypothesize that overexpression of any single *PON* gene might saturate oxLDL inhibition, rendering overexpression of all three *PONs* redundant. This idea is partly supported by our results showing that sufficient amounts of wild-type HDL attenuate copper-induced lipid hydroperoxide production by human LDL to the same extent as an equal amount of HDL isolated from PON cluster transgenic mice (301). In addition, oxLDL, although important, is not the only risk factor for atherosclerosis. Thus, although the PON cluster potentiates most of the functions of oxLDL, it cannot completely inhibit atherogenesis. It is also possible that an additive effect of the PONs does exist but that we failed to find it because of differences between the experimental systems; the background of the mice, the diet, induction period, and the evaluation strategies all differ among our studies and the studies investigating the effects of single gene overexpression (Table 1). It would be better to compare the effect of overexpressing the PON cluster *versus* single *PON* genes on atherogenesis in the same experimental system. It is also possible that other genomic elements in our transgenic construct affected atherogenesis *via* unknown mechanisms, because the construct we used was much longer than those used in the PON1 and PON3 single transgenic mice (Fig. 9).

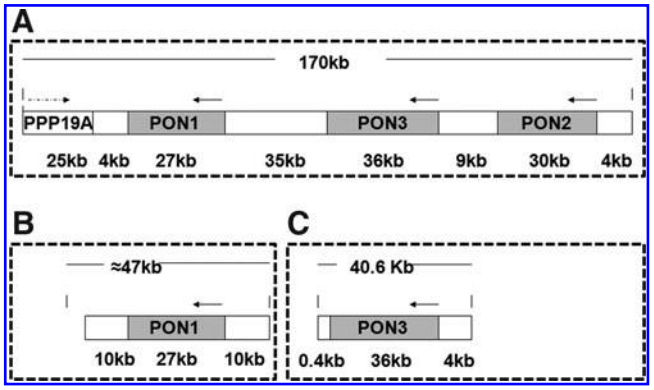

FIG. 9. Comparison among PON1, PON3, and PON cluster transgenic mice. (A) Human BAC DNA fragment used to make human PON cluster transgenic mice. **(B)** Human BAC DNA fragment used to make human PON1 transgenic mice. **(C)** Human BAC DNA fragment used to make human PON3 transgenic mice. BAC, bacterial artificial chromosome.

Like transgenic overexpression of PON1 or PON3 alone, PON cluster overexpression enhanced the ability of HDL to protect LDL from oxidation *in vitro*. In addition, serum expression of ICAM-1 and monocyte chemoattractant protein-1 were also repressed by PON cluster overexpression, as were proatherogenic reactions (ROS generation, inflammation, matrix metalloproteinase-9 expression, and foam cell formation) by peritoneal macrophages induced by oxLDL. More importantly, we found that plaques from PON cluster transgenic apoE null mice exhibited increased levels of collagen and SMCs and reduced levels of macrophages and lipids compared with those from apoE null mice, indicating that the lesions of PON cluster transgenic apoE null mice more closely resemble stable plaques than those of apoE null mice. Thus, the PON cluster transgene not only represses atherogenesis but also promotes atherosclerotic plaque stability *in vivo* (301). It seems that, as a gene cluster, PONs exert their antiatherosclerotic functions at the following two levels: PON1 and PON3 exert their effect on HDL in the serum, and PON2 protects macrophages and possibly other cells by acting intracellularly (Fig. 6).

IV. PONs Protect Against Hcy Toxicity and Associated Atherogenesis

A. Hcy is an important risk factor in atherosclerosis

Hcy is a product of the methionine cycle that is generated by transfer of a methyl group to methionine. Methionine can also be converted to produce *S*-adenosyl methionine (SAM), which is the most important biological donor of methyl groups, by adenosyl transferase. SAM can then be demethylated to produce *S*-adenosyl Hcy, which is converted to Hcy after deadenylation. Endogenous Hcy can be metabolized *via* two pathways: the methionine cycle and transsulfurylation (218). This transsulfurylation reaction requires vitamin B6 as an essential cofactor. In cases of methionine deficiency, Hcy can be remethylated into methionine by methionine synthase (MS) using vitamin B12 as a cofactor and 5-methyltetrahydrofolate as a substrate (218). Deficiency of any of the enzymes or cofactors aforementioned will lead to an accumulation of Hcy. High levels of Hcy [$>15\,\mu M$ is considered "abnormal" (278)] are now considered to be an independent risk factor for CVD (50). The first report that homocysteinemia was related to the pathogenesis of arteriosclerosis was made by McCully in 1969 (228). Such a notion is supported by almost all of the large

meta-analyses that have been conducted. The first large meta-analysis (reported in 1995) stated that Hcy is strongly associated with vascular disease and that Hcy accounts for up to 10% of the population's CAD risk (50). A 5 μM increment in total Hcy concentration is correlated with a 20 mg/dl plasma cholesterol increase (50). A recent meta-analysis indicated that a 3 μM reduction of the plasma Hcy concentration could lead to a reduction of the relative risk of 11% for CAD and 19% for stroke (131a). An increased plasma Hcy concentration in patients with acute coronary syndromes is an independent predictor for recurrent cardiovascular events (240, 322, 347). Roles for Hcy in atherosclerosis have been elucidated by these clinical studies and also by experimental investigations (68).

Impairment of endothelium-dependent dilation has been well documented in subjects with homocystinuria (85). In addition, high levels of Hcy can lead to vascular endothelial injury, especially when blood pressure is high. These effects could be accounted for by one of several possible mechanisms. First, homocysteinemia increases the vascular oxidative burden by inducing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inducible nitric oxide synthase activity (340), altering superoxide dismutase and glutathione peroxidase (354), elevating asymmetrical dimethylarginine levels (323), and uncoupling endothelial nitric oxide synthase (eNOS) (13, 14). Second, Hcy not only inactivates eNOS by inducing its phosphorylation at threonine 495 (158) but also downregulates eNOS by interfering with intracellular redox signaling in endothelial cells (231, 363). Third, high levels of Hcy induce apoptosis in endothelial cells by affecting the PI-3 kinase (36), Fas-FasL (325), p53 (188), and p38 MAPK (31) pathways, among others. Fourth, the level of Hcy is important for thiolactone formation and protein homocysteinylation, which is known to induce cell death (149, 264) and a variety of immunological consequences (264).

Hcy also dose-dependently promotes the proliferation of rat, human, and pig aortic VSMCs (64, 334). Hcy can also upregulate collagen synthesis and accumulation by arterial SMCs (213), which could lead to the increased collagen content of the atherosclerotic plaques observed in patients with untreated homocystinuria. In addition, Hcy indirectly promotes VSMC migration through a paracrine or endocrine effect involving adipocyte-derived resistin (157).

Severe homocysteinemia resulting from genetic homocystinuria has been reported to be closely related to recurrent vascular thrombosis (52, 229). Endothelial injury is thought to be the major mechanism for Hcy-mediated promotion of this prothrombotic state, because platelet aggregation and thrombi formation always occur at sites of injury endothelium in both human and animal homocysteinemia (122). Some studies suggest that, in humans, the plasma total Hcy level has effects on fibrin clot structure, representing a novel prothrombotic effect of Hcy (40, 146, 339). In addition, high levels of Hcy can shift the balance between procoagulation and antithrombus factors. For example, Hcy may be able to inhibit the expression of thrombomodulin and promote the expression of tissue factor in endothelial cells (99). Hcy can also induce the expression of clotting factors II, V, X, and XII (122) and attenuate the activation of protein C and antithrombin III (124). Hcy levels have been used as an independent predictor of thrombotic events in these individuals (40).

B. PONs protect against Hcy toxicity

Hcy metabolites are thought to be the major cause of Hcy toxicity. Elevated Hcy levels lead to protein N-homocysteinylation. Accumulating evidence has suggested that protein N-homocysteinylation can cause enzyme inactivation and protein aggregation and precipitation. Studies by Paoli *et al.* revealed how the low levels of protein N-homocysteinylation induce mild conformational changes leading to the formation of native-like aggregates that evolve over time to produce amyloid-like structures. (257) Hui and coworkers have hypothesized that plasma levels of Hcy-thiolactone adducts could be a more direct predictive index of CHD than plasma Hcy levels. They concluded that high plasma levels of Hcy-thiolactone adducts are independent of traditional risk factors and that Hcy-thiolactone (HTL) adducts might play a role in atherosclerotic vascular diseases (359). Given that Hcy is formed in all cell types (145) and that Hcy-thiolactone harms the body in many ways, the ability to detoxify Hcy is essential for biological integrity. Jakubowski and coworker demonstrated that human PON1 has Hcy-thiolactonase activity and provided evidence that Hcy-thiolactone is a physiological substrate of PON1. They also suggested that the plasma *N*-Hcy-protein levels are mainly determined by the Hcy thiolactonase activity of PON1 and that PON1 protects against accumulation of proatherogenic *N*-Hcy-proteins *in vivo* (263). This may be a novel mechanism for the atheroprotective role of PON1 (Fig. 10).

In clinical investigations, serum PON activity has been reported to be negatively associated with the serum Hcy concentration (118, 163, 165, 182, 185). One study reported that the hydrolytic activity of human PON1 toward Hcy-thiolactone is associated with the PON1-192-Arg/Arg and PON1-55-Leu/Leu genotypes and strongly correlated with PON1's hydrolytic activity toward organophosphate paraoxon substrates (182). The T allele of *PON1*/T(−107)C and the Ser allele of the *PON2*/Cys (311)Ser polymorphism were reported to be associated with lower plasma Hcy and Hcy thiolactone complex levels (148, 269). The Hcy thiolactonase activity of PON1 is negatively associated with the thickness of the carotid intima media in patients with type 2 diabetes mellitus. The Hcy thiolactonase activity of serum PON1 may thus reflect the status of atherosclerosis in patients with type 2 diabetes mellitus (172).

V. *PONs* Polymorphisms and Coronary Artery Disease

A. PON1 *polymorphisms*

1. *PON1* polymorphisms and PON1 activity. Two major gene polymorphisms in the coding region of the human *PON1* gene have been reported. One is at position 192 and leads to a glutamine to arginine substitution (Arg/Gln192). Another mutation encodes a leucine to methionine substitution at position 55 (Met/Leu55). Each has been reported being independently associated with PON1 activity and defined as the molecular basis for interindividual variability (4, 105, 135). These two polymorphisms are also hypothesized to be the major determinants of the well-known biochemical polymorphism in serum PON activity toward various organophosphates (Table 2).

PON1 activity levels vary widely among individuals, which may partly account for differences in susceptibility to

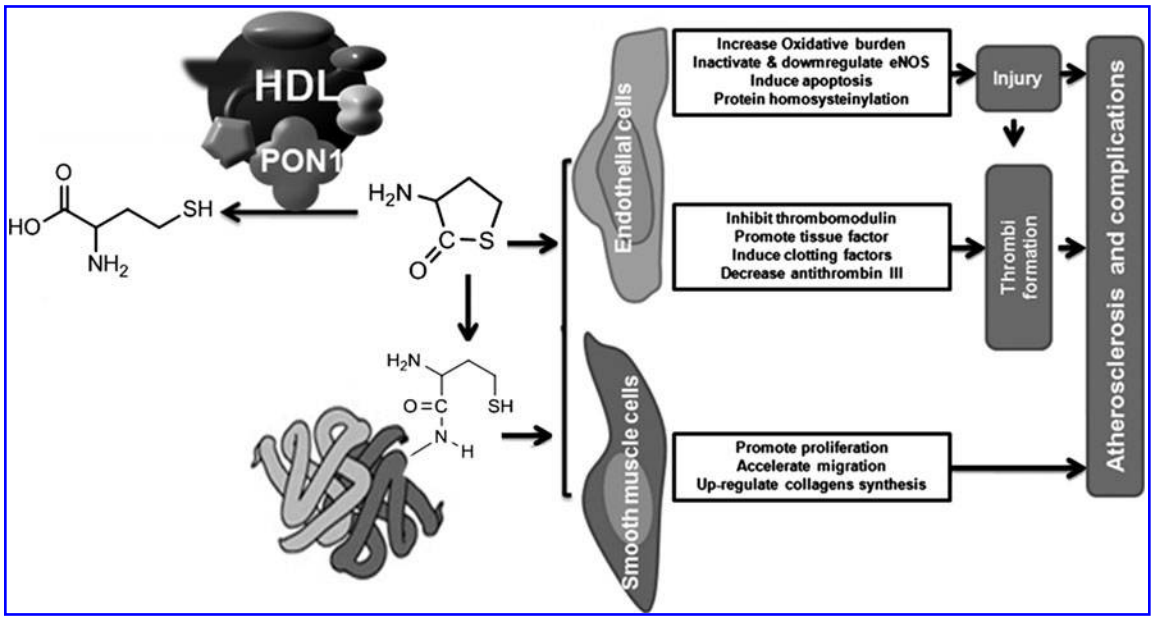

FIG. 10. PON1 can protect against atherogenesis by detoxifying Hcy. Hcy can promote atherogenesis by inducing atherogenic alterations of vascular endothelial cells and smooth muscle cells, either by direct toxicity or by homocysteinylating intracellular protein. PON1 has been reported to be able to detoxify Hcy by hydrolyzing it to cysteine. eNOS, endothelial nitric oxide synthase; Hcy, homocysteine.

organophosphate poisoning. The molecular basis for these differences was reported to be associated with *PON* gene polymorphisms. Purified PON1 from PON1 192 Arg/Arg and *PON1* 55 Leu/Leu individuals has the greatest hydrolytic activity toward paraoxon, whereas that from 192 Gln/Gln and *PON1* 55 Met/Met individuals has the least; heterozygotes have intermediate levels of activity (140, 352). A similar pattern of substrate specificity was observed when other oxons, such as methyl paraoxon, chlorthion-oxon, and amine, were used as substrates (352).

However, the capacity of PON1 to protect LDL against oxidation follows the opposite trend as that of paraoxon

Table 2. Major *PON1* Gene Polymorphisms

Site	*Amino acid/nucleotide*	*Effects on expression or activity*	*Association with atherosclerosis-related condition*	*References*
55	Leu *vs.* Met	Leu/Leu: highest expression; highest PON, arylesterase, and lactonase activity	Met/Met: lowest total and LDL cholesterol and ApoB/ApoAI ratio. Leu/Leu: higher HDL and ApoAI. Leu/Leu: risk factor for CHD or no association.	(15, 44, 56, 74, 128, 189, 192, 214, 215, 270, 291, 358)
192	Gln *vs.* Arg	Gln/Gln: highest activity to phenylacetate, lactonase and sarinase activity; Arg/Arg: highest PON and arylesterase activity	Gln/Gln: lowest total and LDL cholesterol and ApoB/ApoAI ratio. Gln/Gln: lower oxLDL	(56, 79, 135, 137, 189, 270)
−108/−107	C *vs.* T	−108C: two times higher activity; CC: highest serum concentration and activity to phenylacetate		(55, 56, 190, 324)
−126		No differences		(55)
−160/−162	A *vs.* G	AA: highest activity to phenylacetate		(55, 56)
−824/−832	A *vs.* G	−824A: 1.7 times higher activity or no difference; AA: highest activity to phenylacetate		(55, 81, 190)
−907/−909	C *vs.* G	GG: highest activity to phenylacetate		(55, 56, 81, 190)

apo, apolipoprotein; CHD, coronary heart disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PON, paraoxonase.

hydrolytic activity. PON1 from 55 Met/Met/-192 Gln/Gln individuals exhibits the greatest protective capacity for LDL oxidization. This type of PON1 is also able to best hydrolyze DZO and the nerve gases sarin and soman (352). For other substrates, such as phenyl acetate, chlorpyrifos oxon, and 2-naphthyl acetate, all polymorphic variants of PON1 have similar hydrolytic activities (352).

In addition to the coding region polymorphisms, five gene polymorphism sites have been reported in the *PON1* upstream transcription regulatory region, at −107/−108, −126, −160/−162, −824/−832, and −907/−909 (55, 56, 102, 190, 191, 193, 194). Brophy *et al.* reported that the −108 polymorphism has a significant effect on PON1 transcription, whereas the −162 polymorphism has a lesser effect (56).

The −909 polymorphism is in linkage disequilibrium with the other sites and has no independent effect on PON1 activity. In addition, the −107T, −824G, and −907G genotypes are correlated with high PON1 concentration and activity, and −107T seems to have a dominant effect on *PON1* gene expression (81).

2. *PON1* polymorphisms and plasma lipoprotein levels. Because PON1 polymorphisms affect serum PON1 activity and serum PON1 activity is associated with variations in the concentrations of plasma lipoproteins including serum apoAI, LDL cholesterol, and HDL cholesterol, *PON1* gene polymorphisms could affect plasma lipoprotein levels. This hypothesis has been tested in several studies. One study shows that PON1 polymorphisms were associated with variations in most plasma lipoproteins, including LDL and HDL cholesterol, in samples taken from the genetically isolated Alberta Hutterites and aboriginal Oji-Cree from northern Ontario (128).

PON1 Gln192 homozygotes exhibit a better plasma lipoprotein profile, with lower levels of total cholesterol and plasma apoB-related biochemical variables and lower apoB/apoA-I ratios than heterozygotes and *PON1* Arg192 homozygotes. A significant difference was detected in the mean total cholesterol and LDL cholesterol levels between subjects with the *PON1* Leu/Leu55 and Met/Met55 genotypes; *PON1* Met/Met55 subjects have a better plasma lipoprotein profile (189). *PON1* Gln192 patients have been found to have lower plasma oxLDL levels than control subjects (137). Mean plasma concentrations of total and LDL cholesterol and apoB are higher in *PON1* Met55 carriers than those of noncarriers in samples from two Canadian aboriginal Oji-Cree and Inuit populations (93). Malin *et al.* reported that *PON1* Leu/Leu55 homozygotes have increased basal HDL concentrations and also tended to have higher apoAI concentrations (215). They also found that the Arg/Gln192 polymorphism is a more powerful predictor of changes in HDL cholesterol and apoAI concentration during pravastatin therapy than the Met/Leu55 polymorphism.

However, some studies claim that no association between *PON1* polymorphisms and plasma lipoproteins exists (48, 204, 353). Contradictions among the results from different studies could result from partially intrinsic differences in serum PON levels among populations. Thus, it is important to compare the results obtained from association studies in diverse genetic isolates with different ethnicities. Also, the mechanisms by which *PON* genotypes affect the total cholesterol, HDL cholesterol, and LDL cholesterol levels and the apoB/apoAI ratio remain to be elucidated.

3. *PON1* polymorphisms and CHD. In recent years, a relationship between the *PON1* 192 polymorphism and CHD has been proposed (142, 253), although some studies have reported no association between *PON* genotype and CHD risk (128, 353). Such dichotomies often occur with association studies, which are thought to have several biases (196). Some recent studies that combine association studies with functional data have suggested that the PON activity/concentration, in addition to the *PON* genotype, contribute to the risk of CHD *in vivo* (215). One recent study that involved 1399 patients undergoing diagnostic coronary angiography systematically investigated the relationship between *PON1* genotypes and functional activity with systemic oxidative stress and CVD risk in humans. The results indicated that (i) the *PON1* genotype is dose-dependently associated with decreased serum PON1 activity (Gln/Gln192 > Gln/Arg192 > Arg/Arg192) and with increased levels of systemic oxidative stress (Gln/Gln192 < Gln/Arg192 < Arg/Arg192); (ii) participants with the Gln/Gln192 genotype, compared with participants with either the *PON1* Arg/Arg192 or Gln/Arg192 genotype, demonstrated an increased risk of overall mortality and of major adverse cardiac events; and (iii) the incidence of major adverse cardiac events was significantly lower in participants with the highest PON1 activity compared with those with the lowest PON1 activity. (39) In addition, Blatter-Garin *et al.* reported that homozygosity for the 55Leu allele of PON1 is an independent risk factor for CHD in non–insulin-dependent diabetics (44), which is supported by other studies (74, 128). In contrast, some studies conducted in patient groups of different ethnicities found no significant association between the 55 polymorphism and the presence of CHD (15, 214, 291, 358).

These inconsistent results suggest that the association between the *PON1* genotype and CHD might exist but be weak or variable and that it might be influenced by other polymorphisms or by environmental factors. Thus, the *PON1* genotype cannot convincingly be used as a predictor of CHD. Instead, PON1 activity level appears to be a better predictor of CHD than genotype. Jarvik *et al.* showed low levels of PON1 activity in CHD subjects compared with age- and race-matched controls, although the *PON1* 192 ($p = 0.75$) and PON1 55 ($p = 0.83$) genotypes could not predict case–control status (154). PON1 activity, however, predicted CHD independently of traditional risk factors. These results influence the earlier report of decreased PON1 activity in 50 myocardial infarction survivors, making it clear that PON1 activity is a risk factor for, rather than a result of, infarction. It was also reported that PON1 activity was lower in type 2 diabetic patients with CHD than in those without CHD (152). Significantly lowered PON1 activity was detected in 417 CHD patients *versus* 282 controls (204).

B. PON2 *polymorphisms*

1. *PON2* polymorphisms. The human *PON2* gene has two common polymorphisms at residues 148 and 311, both of which lead to amino acid substitutions (245). The alleles encode either glycine or alanine at codon 148 and either Cys or serine at codon 311. Accordingly, these two polymorphisms are designated Gly/Ala148 and Cys/Ser311 (234). Almost total linkage disequilibrium exists between these two polymorphic sites in four different human populations, which

Table 3. Major *PON2* Gene Polymorphisms

Site	*Amino acid/nucleotide*	*Association with atherosclerosis*	*References*
148	Gly *vs.* Ala	Ala/Ala: highest LDL cholesterol and ApoB; Lowest HDL cholesterol and ApoAI. Gly/Gly: highest HDL cholesterol and ApoAI.	(49, 128, 307)
311	Cys *vs.* Ser	Ser/Ser: highest LDL cholesterol and ApoB; Ser allele: risk for CHD, Cys allele: protect against CHD.	(66, 128, 189, 255, 307)

indicate that the genotype at one site can be used as a surrogate for the genotype at the other site. In other words, the Ala148 and Ser311 variants form one common allelic haplotype, and the Gly148 and Cys311 variants form the second common allelic haplotype in white, South Asian, and African samples (128) (Table 3).

2. *PON2* polymorphisms and plasma lipoprotein levels. *PON2* polymorphisms are associated with variations in plasma lipoprotein levels. By analyzing a sample of 334 nondiabetic Oji-Cree individuals, Hegele found that *PON2* Ala148/Ser311 homozygotes exhibited significantly higher plasma total and LDL cholesterol and apoB than subjects with the other two genotypes (128), which was confirmed by an investigation by Shin *et al.* (307). Another study showed that individuals homozygous for *PON2* Gly148 had the highest plasma concentrations of total and HDL cholesterol and apoAI, Ala148 homozygotes the lowest, and heterozygotes had an intermediate phenotype (49). Leus *et al.* reported that the common *PON2* polymorphisms are associated with clinical manifestations of CVD in familial hypercholesterolemia patients. The results also indicated that PON2 Ser311 carriers seem to be at risk for CHD due to significantly higher total and LDL cholesterol; conversely, subjects with the Cys/Cys311 genotype seem to be protected against the development of premature CVD (189). The authors also claimed that the associations were not related to linkage disequilibrium between the two sites, because the associations for *PON2* were independent of the associations for *PON1*. The finding of associations between *PON2* polymorphic variants and plasma lipoprotein levels suggests a relationship between these polymorphisms and lipoprotein, although the corresponding mechanisms are still unknown.

3. *PON2* polymorphisms and CHD. The *PON2* Ser311 polymorphism is also consistently reported to be associated with CHD in various populations (66, 189, 255), leading to a speculation that PON2 is even more important for protection against CHD than PON1. Other interpretations are that an as-yet undiscovered functional mutation is in stronger linkage disequilibrium with the *PON2* 311 polymorphism than the *PON1* polymorphism or that there are other polymorphisms of *PON1* that are more strongly related to CHD risk than the 192 polymorphism. More extensive data from independent studies are needed to make a final conclusion about the associations between *PON2* polymorphisms and CHD.

VI. Other Diseases Related to PONs

In addition to being directly related to atherosclerosis and CHD, PONs have also been shown to play roles in many other diseases, probably because of their multiple enzymatic capacities. Many of these diseases can also contribute to atherogenesis and CHD.

A. PONs' relations to diabetes mellitus and corresponding complications

Diabetic patients may overproduce ROS as a result of chronic hyperglycemia, hyperinsulinemia, elevated free fatty acids, and dyslipidemia (219). Their PON1 activities, however, have been found to be lower than normal (139). Glycation of the PON1 protein, rather than reduced expression, is considered to be the main reason for the low enzyme activity in these patients (127). Another possibility is that PON activity is inactivated by abnormally high levels of ROS, because it has been shown that components of ox-LDL are able to strongly downregulate PON1 activity (26).

The Met allele of the *PON1* Leu/Met 55 polymorphism is more frequently in type 1 and 2 diabetes patients than in controls, whereas the Arg allele of the Gln/Arg192 polymorphism is less frequent in type 1 and 2 diabetics than healthy controls. Serum PON1 activity is significantly decreased in both type 1 and 2 diabetes patients compared with the controls. The Met/Met and Gln/Gln genotypes are more tightly associated with lower PON1 activity than the Leu/Leu and Arg/Arg genotypes. Also, PON levels are lower in diabetic patients with complications such as neuropathy, nephropathy, and retinopathy (98). Van den Berg *et al.* also found that the Arg/Arg genotype was found at a significantly higher frequency in newly diagnosed type 2 diabetes patients compared with subjects with normal glucose tolerance (341). An association between coding region polymorphisms in *PON1* or *PON2* and the presence of diabetic complications such as diabetic nephropathy and retinopathy in diabetes has also been reported. Pinizzotto *et al.* showed that *PON2* polymorphisms are strongly and significantly associated with diabetic nephropathy in Swiss type 2 diabetes patients and were independent of traditional risk factors (266). A strong association between *PON1* Met/Leu55, but not Gln/Arg192, and diabetic retinopathy was reported in Australian adolescents with type 1 diabetes (162). Young type 1 diabetes patients with the L/L polymorphism at position 55 of *PON1* gene were found to be more susceptible to retinal complications (171). The 192Arg allele of *PON1* predisposes to microangiopathy in type 2 diabetes mellitus patients, as shown in a case–control study with a total of 280 patients with type 2 diabetes with or without microangiopathies (235). The *PON2*-311 polymorphism is associated with the presence of microvascular complications in diabetes mellitus, although no association was found between the PON2-311 polymorphism and lipid or lipoprotein concentrations. In this study, over-representation of the Cys/Cys 310 genotype in patients with diabetes and

microvascular complications was found (207). The presence of specific *PON* gene polymorphisms in diabetic patients provides evidence for a relationship between the *PON* gene family and diabetes.

Oxidative stress can accelerate the development of diabetes. PON1-overexpressing mice had decreased diabetes-induced macrophage oxidative stress, decreased risk of development of diabetes, and decreased mortality compared with wild-type mice; this phenotype was even more dramatic when compared with that of PON1 knockout mice. PON1 was therefore hypothesized to be able to attenuate the development of diabetes because of its antioxidative properties (287). Low PON1 activity is also related to higher CRP, which is independent of adipokines, obesity, and lipids. Decreased PON1 activity may enhance systemic low-grade inflammation and thus contribute to increased cardiovascular risk in type 2 diabetes mellitus (89).

A significantly increased incidence of CVD was found in diabetes patients who had a lower level of PON1 activity than the median value. Low concentration and enzymatic activity of PON1 may be used as independent predictors of cardiovascular events in diabetic patients (138). Decreased PON activity also accounts for the increased risk of cardiovascular events in diabetic patients.

B. PONs' associations with metabolic syndrome and obesity

Metabolic syndrome is a group of metabolic abnormalities including dyslipidemia, abdominal obesity, high blood pressure, and thrombotic and inflammatory states (235a) and has been recognized as a major risk factor for CVD mortality. Metabolic syndrome is considered to result primarily from insulin resistance (115), which may be partially caused by oxidative stress (258). Thus, because of their antioxidant activities, the PONs are hypothesized to protect against metabolic syndrome. In a study of 1364 randomly recruited subjects, of whom 285 were found to be suffering from metabolic syndrome, Senti *et al.* found that serum PON1 activity levels were significantly lower and lipid peroxide concentrations were significantly higher in subjects with metabolic syndrome compared with the unaffected subjects. They also reported that, in this population, as the number of metabolic disturbances increased, PON1 activity decreased significantly and lipid peroxide concentrations increased significantly. Although no differences in the prevalence of *PON1* codon 192 genotypes were found among the subjects with various metabolic abnormalities, metabolic syndrome severity was hypothesized to be associated with increased oxidative stress and lower antioxidant PON1 enzymatic capacity (298). Rizos *et al.* also found that serum PON1 activities were lower in the metabolic syndrome group compared with the non-MS group (280). In studies by Garin *et al.*, the metabolic syndrome was found to be characterized by the presence of smaller, denser lipoprotein particles, which are more susceptible to oxidative modifications and decreases in serum PON1 levels (103). The Leu/Met 55 polymorphism has effects on insulin resistance in healthy subjects. The presence of the Leu/Leu *PON1* genotype is associated with a more severe degree of insulin resistance (32). A significant interaction was found between the metabolic syndrome and both the *PON1* Leu/Met55 and Gln/Arg192 polymorphisms in determining the risk for coronary artery disease. Subjects who had metabolic syndrome and both the 55Leu and 192Arg alleles had significantly increased risk compared with subjects without metabolic syndrome and with the Met/Met55-Gln/Gln192 genotype (224). An investigation of the relationship between adiponectin and PON1 showed that PON1 activity correlated positively with HDL-C and adiponectin levels and negatively with body–mass index, waist circumference, systolic blood pressure, levels of HbA(1C), insulin, homeostatic model assessment–insulin resistance, and other markers of metabolic syndrome (29). Some studies, however, have failed to find a positive relationship between metabolic syndrome and serum PON activity (184, 327). In leptin- and LDL receptor-deficient mice, which serve as a model for metabolic syndrome, adenoviral overexpression of human PON1 significantly reduced the total plaque volume, the plaque macrophage volume, and plaque-associated oxLDL. It also increased the percentage of SMCs in the plaques. Expressing human PON1 lowered the titer of autoantibodies against MDA-modified LDL, although no effect on plasma total cholesterol and triglycerides was found (208). These results suggested that increasing PON1 activity could decrease the cardiovascular risk of metabolic syndrome patients.

As an independent risk factor for CVD, obesity has been shown to be related to low PON activity, because the activity of HDL-PON in obese subjects is significantly lower than that in controls (96, 170). The HDL composition is altered in obese individuals, exhibiting a decrease in protein content and an increase in the cholesterol levels and triglyceride/protein and cholesterol/protein ratios. These changes may decrease the binding of PON1 to the surface of HDL, resulting in inhibition of PON1 enzyme activity (97). PON1 arylesterase activity showed an inverse univariate correlation with leptin levels and a positive correlation with adiponectin levels (170). The high concentrations of leptin present in obese individuals may decrease plasma PON1 activity and induce oxidative stress, which may account for some of the proatherogenic effects of obesity (38). Also, transgenic overexpression of human PON3 in mice decreases adiposity and circulating leptin levels in addition to inhibiting atherogenesis, suggesting that PON3 may play an important role in protecting against obesity as well as atherosclerosis (306).

C. PONs and aging

Aging is thought to result partially from an imbalance between pro-oxidants and antioxidants, in which excess accumulation of pro-oxidants promotes aging (123).

It has been reported that plasma and HDL PON activities decrease significantly with aging (27, 67, 232). Serum PON1 activity was found to be significantly decreased with age, although its arylesterase activity and concentration in the serum exhibited no significant change. Decreased PON1 activity may contribute to the increased susceptibility of HDL to oxidation modification observed with aging (299). Jaouad *et al.* have suggested that age-related decreases in the antioxidant capacities of HDL and PON1 are due to alterations in PON1's free sulfhydryl groups (153).

Increased age is also negatively correlated with basal and stimulated PON activities of the PONs and the 192 (Gln/Arg) polymorphic variant of *PON1*. Thus, *PON1* gene and

activities may contribute to the aging process (357). An investigation of the *PON1* polymorphisms at residues 192 and 55 showed that the frequency of the 192Arg allele was significantly increased in centenarians over young people, suggesting a small survival advantage for Arg allele carriers (48). A more extended follow-up study confirmed this result (275). These polymorphic variants have differences in PON activity, with Arg+ and Met–carriers having significant higher PON activities than their Gln and Leu counterparts. Thus, not only the levels of PON activity but also the *PON1* genotype may significantly increase longevity (217). However, in a recent large association study, no significant association of the *PON1* 192 Gln/Arg genotype with longevity was observed. Moreover, a potential interaction of *PON1* 192 Gln/Arg with ApoE epsilon 4 was found to exist in one German population but not in the other three populations examined. These results do not exclude the possibility of population-specific effects of PON1 on longevity, however, which could result from differences in gene–environment interactions (57).

D. Other diseases related to PONs

Because oxidative stress is an important etiological factor for carcinogenesis (290) and PONs are important free-radical scavenging molecules, the relationships between PONs and many types of cancer have been extensively explored. PONs have been shown to be able to protect against many kinds of cancer, including prostate cancer, lung cancer, gastrointestinal tumors, glioma, ovarian malignancy, and breast cancer, among others. In addition, PONs are involved in some types of neurological disorders, such as autism, Alzheimer's disease, Parkinson's disease, schizophrenia, amyotrophic lateral sclerosis, depression, and acute ischemic stroke. Recently, mixed connective tissue disorder, Behchet's disease, systemic lupus erythematosus, rheumatoid arthritis, fibromyalgia, osteoarthritis, and other connective tissue diseases have also been reported to be influenced by *PON1* polymorphisms. PONs are also reported to be associated with some gynecological disorders, such as endometriosis. Many liver disorders, such as viral hepatitis, nonalcoholic steatohepatitis, cirrhosis, hepatosteatosis, and even alcoholic hepatitis are also related to PONs. PONs have also been reported to protect against certain renal diseases and disorders. As the main means by which the nervous system protects itself against neurotoxicity arising from organophosphate exposure, PONs are a key for the response to organophosphorus and lead poisoning (109).

VII. Regulation of PONs

The results discussed earlier show that the PONs not only protect against atherosclerosis, atherosclerosis-related diseases, and many other diseases but also detoxify organophosphorus pesticides and nerve agents; therefore, PON regulation and the use of PONs to treat disease are worthy of further investigation.

Human serum PON1 activity increases from 15 to 25 months of age onwards and plateaus in adults (69). PON1 activity is theoretically determined by the genetic background of the individual, including the *PON1* regulatory region polymorphisms. However, variations in environmental factors such as lipoproteins, cytokines, environmental chemicals, drugs, physiological and pathological states, diet, and lifestyle have been demonstrated to affect PON expression and activity.

A. Transcriptional regulation of PONs

Shih *et al.* have claimed that the expression of *PON1* is under genetic control, based on the finding that a high-fat diet decreases the expression of PON1 in C57BL/6J mice but has no such effect in C3H/HeJ mice (302). Bioinformatic analysis of the sequence immediately upstream of the *PON1* gene revealed that the *PON1* promoter region contains neither a canonical TATA nor a CAAT box and is GC rich, which is typical of TATA-less promoters (55). Serial deletion analysis of 11.5 kb of the *PON1* promoter region showed that cell-type-specific promoter elements for liver and kidney are present in the first 200 bp upstream of the coding sequence (190). The polymorphisms at positions −909 and −162 of upstream of coding region, which are potential NF-I transcription factor binding sites, have approximately a twofold effect on expression level, as does the −108 position. The effects of these three polymorphisms may depend on the effects of the surrounding sequences because they are not strictly additive (190). Other single-nucleotide polymorphisms (SNPs) in the *PON1* promoter region that are able to affect the serum concentration of PON1 have been reported, namely, −107(T or C), −824(G or A), and −907(C or G). The latter residues are correlated with higher concentrations and activities than the former, as shown in a reporter system and by direct inspection (252). Of these three SNPs, −107 is a putative Sp1 binding site. Research has indicated that overexpression of full-length Sp1 dramatically enhances *PON1* promoter activity. It had also been found that the GC box sequence located at residues −111 to −106 may be the most important factor for transcriptional regulation by Sp1. PKC can upregulate the expression of PON1, partially through its direct or indirect interaction with Sp1 (83). Because very few explorations of the transcriptional elements of *PON2* and *PON3* have been reported, we are now working in this area to attempt to elucidate the transcriptional regulation of *PON2* and *PON3*.

B. Lipoproteins and their metabolic products regulate PONs expression

HDL is the main serum carrier of PON1 and plays important role in determining the enzyme concentration; this effect is clearly observed in HDL deficiency syndromes, in which PON1 levels are reduced (150). PON1 is synthesized in the liver and secreted into the serum. PON1's secretion is dramatically decreased if lipoproteins are absent, whereas the addition of phospholipid micelles or HDL promotes its secretion. However, LDL and lipid-free ApoA-I have no such effect. This suggests that an appropriate acceptor is required by PON1 for its release into serum. HDL appears to be the predominant physiological acceptor (82). Binding to HDL is necessary for PON1 to maintain optimum activity and stability (317). Human Apo A-I overexpression significantly increases the PON activity in both wild-type C57BL/6J and ApoE-deficient C57BL/6J mice, although adenoviral Apo A-I gene transfer does not have this effect, probably

because of cytokine production in the liver (80). Thus, factors that increase the level of HDL may increase the enzyme activity of PON1. OxLDL and oxidized lipids can down-regulate PON1 expression when administered to the HepG2 liver cell line (238, 344). When C57BL6 mice were fed a cholate-containing, high-fat, high-cholesterol diet for 15 weeks, the PON1 message levels in their livers were reduced compared with their chow-fed counterparts. Strangely, however, this effect was absent in ApoE null mice given the same challenge (244). More interestingly, the high-fat diet failed to decrease the expression of human PON1 in human PON1 transgenic mice (in which the transgene included the *PON1* gene with 10 kb of the upstream and downstream regions), although endogenous mouse PON1 expression dramatically decreased by a mechanism that remains unclear (338). This result could be accounted for by differences in PON1 regulation between human and mouse, although it is also possible that the necessary regulatory elements were included in the transgenic construct. PON2 and PON3 have different responses to oxidative stress compared with PON1. Unlike PON1 expression, PON2 message and activity were upregulated upon exposure to oxidative stress (244), whereas PON3 expression is not significantly altered by oxidative stress (277). Treating mouse peritoneal macrophages with oxidative stress-inducing agents increases PON2 expression and lactonase activity but has no effect on PON3 mRNA levels, although the PON3 lactonase activity decreases (286). *In vivo*, C57BL6 or apoE null mice fed a high-fat, high-cholesterol, cholate-containing diet for 8 or 15 weeks, respectively, exhibit significantly higher levels of PON2 mRNA but no change in PON3 expression in their livers compared with their chow-fed counterparts, contrary to the changes observed in PON1 under the same conditions (244). However, in humans, PON2 message levels in monocyte-derived macrophages from hypercholesterolemic individuals are lower than in those derived from their normal counterparts (286). This effect can be reversed by the administration of atorvastatin (286). Oxidative stress was shown to inactivate serum PON1 and PON3, whereas macrophage PON2 expression increases under oxidative stress (24), which may be a compensatory mechanism.

C. Biological macromolecules modulate PONs expression

With increased research into PON regulation, various biological macromolecules and medications have been found to increase the expression of PON1. Incubating the liver cell line HepG2 with IL-6 has been shown to rapidly decrease PON1 expression (344). Treating HepG2 cells with IL-1beta and TNF-alpha decreases the relative promoter activities of the sequences at –589 to –6 upstream of PON1, resulting in a decrease in PON1 mRNA expression; however, the opposite effect was observed upon treatment with IL-6 (175). The discrepancy seen in the regulation of PON1 mRNA by IL-6 may be due to differences in the treatment protocols (100 ng/ml for 16 h *vs.* 10 ng/ml for 24 h) and methods of analysis (northern blot *vs.* reverse transcription–polymerase chain reaction) used (244) or to the complexity of PON1 regulation. These results suggest that PON1 mRNA expression in hepatocytes is regulated by proinflammatory cytokines and that proinflammatory cytokines secreted during disease may play a role in the development of atherosclerotic lesions *via* modification of PON1 mRNA expression and effects on the antioxidative status of HDL (175). In intestinal Caco-2/15 cells, expression of all three PONs is inhibited by oxidative stress induced by the addition of iron-ascorbate into the culture. However, this inhibition can be markedly attenuated by preincubation of Caco-2/15 cells with strong antioxidants such as butylated hydroxytoluene and Trolox. Lipopolysaccharides (LPS) also dose-dependently affect PON expression in Caco-2/15 cells; 200 μg/ml LPS decreases PON1 and PON3 mRNA but not PON2 mRNA levels compared with control cells. On the other hand, 50 μg/ml LPS increases PON2 mRNA levels without affecting PON1 and PON3 mRNA. When Caco-2/15 cells were treated with tumor necrosis factor-alpha (TNF-α) or interferon-γ, their PON1 and PON3 mRNA were reduced, with a significant increase in PON2 mRNA. Notably, these effects are partially mediated through the NF-κB pathway (267).

PON2 transcription is promoted by urokinase plasminogen activator (uPA) *via* its receptor uPAR through an integrated multistep pathway in macrophages (100). PON2 expression is regulated by cellular cholesterol content (285, 311) and cellular oxidative stress (309), and PPAR-γ, AP-1 activation (310), and NADPH oxidase activation (101, 308) all play important roles in stimulating PON2 mRNA expression. Fuhrman *et al.* identified the signal transduction pathway initiated by uPA that promotes *PON2* transcription in macrophages (100). Following uPAR activation by uPA, PDGF receptor-β is activated through association with uPAR, leading to PI3K activation. NADPH oxidase is then activated, resulting in ROS production. ROS activate ERK1/2, which stimulate sterol regulatory binding protein-2 (SREBP-2) activity. SREBP-2 translocates into the nucleus and binds to transcriptional regulatory elements upstream of the *PON2* gene, promoting its expression (100).

3OC12-HSL, one of the two virulence factors of *P. aeruginosa*, decreases PON2 activity in host cells by down-regulating PON2 mRNA and protein levels and inhibiting PON2's hydrolytic activity, resulting in increased vulnerability of the cells to pyocyanin-induced oxidative stress. The effects are mediated by Ca^{2+}, as the Ca^{2+} chelator 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid etrakis (acetoxymethyl ester) diminishes the ability of 3OC12 to decrease PON2 and 3OC12 causes an increase in cytosolic Ca^{2+} levels. These results not only support a significant role for PON2 in defending against *P. aeruginosa* but also suggest that this bacterium may attenuate the protection presented by PON2 (133).

D. Pharmacological modulators of PONs

Various pharmaceutical drugs, particularly traditional lipid-lowering drugs such as statins and fibrates, have been tested for effects on PON expression and activity. These studies have yielded conflicting results, however (25, 58, 75).

1. Statins. Statins, which inhibit cholesterol synthesis by blocking 3-hydroxy-3-methyl-glutaryl-CoA reductase, are commonly used as hypolipidemic drugs. Short-term simvastatin administration does not improve PON activity; however, treatment of hyperlipoproteinemia patients with atorvastatin increases HDL-associated PON activity and

exerts a favorable effect on the lipid profile (120, 164, 261). Orlistat also has a beneficial effect on the lipid profile and improves antioxidant status by promoting serum PON1 activity after 6 months of treatment in obese populations (19). Tsimihodimos *et al.* reported that atorvastatin does not change the serum PON activity toward paraoxon and phenylacetate but did improve the serum PON1 activity toward LDL cholesterol owing to dramatically lowered LDL cholesterol levels (336).

Simvastatin dose-dependently upregulates the promoter activity of the *PON1* gene in expression cassettes including 1 kb of the upstream sequence transfected into HepG2 cells. This effect was blocked by administration of mevalonate and other intermediates of the cholesterol biosynthetic pathway. Simvastatin increases SREBP-2 levels, and this effect is also blocked by mevalonate. The consistency among the cellular studies and clinical application affirms the effects of simvastatin treatment, which has the potential to influence antiatherogenic mechanisms at the HDL level, thus providing evidence for one molecular mechanism by which PON expression could be regulated (83). In Huh7 cells, Arii *et al.* found that pitavastatin treatment activates *PON1* gene expression and increases PON1 protein expression by increasing the phosphorylation of p44/42 MAP kinases, probably by activating SREBP-2 and Sp1 and increasing their binding to *PON1* DNA (18). Pitavastatin can also increase Sp1 binding to *PON1* DNA by activating PKC, especially the PKCzeta isoform (17). In apoE-deficient mice, pravastatin treatment increases the plasma PON activity by up to 80%; treatment of mice with the A-002 A secretory phospholipase A2 inhibitor also results in a profound increase in plasma PON activity. These compounds synergize with pravastatin to inhibit atherogenesis (300). These experimental animal results support the clinical evidence regarding the stimulatory effects of lipid-lowering medications on PON activity.

2. Fibrates. Durrington *et al.* investigated the effects of two fibric acid derivatives, bezafibrate mono and gemfibrozil, on serum PON activity and found no significant effects (91). Gemfibrozil administration has been shown to increase serum PON activity, improving antioxidant status and lowering lipid levels in type 2 diabetic patients with associated hypertriglyceridemia (30). Three months of treatment of CHD patients with micronized fenofibrate not only normalized the lipid profile but also improved antioxidant status by increasing serum PON activity (259). Fenofibrate increases PON activity, decreases the expression of inflammatory markers, and improves lipid and lipoprotein levels in patients with combined hyperlipidemia (360). Ciprofibrate has also been reported to favorably affect the lipid profile and increase the resistance of LDL to oxidation by increasing serum PON activity, thus improving the antioxidant status in patients with metabolic syndrome (260).

3. Other cardiovascular drugs. Traditionally used as a platelet aggregation inhibitor, low-dose aspirin (acetylsalicylic acid) was reported to increase serum PON1 activity (by $\sim$13%) in coronary patients (44), although administration of aspirin did not alter the serum PON1 activity of healthy volunteers (180). Aspirin was also found to increase PON1 activity in HuH7 cells (6). Some derivatives of aspirin, such as nitro-aspirin and the aspirin metabolite salicylic acid, were also able to increase PON1 expression and activity (292). Administration of aspirin (2 mg/day for 6 days) to atherogenic diet-fed LDLr$^{-/-}$ or C57BL6 mice resulted in a sevenfold increase in liver PON1 gene expression and a twofold increase in serum PON1 activity in an Ah receptor-dependent manner. Similar results were observed in HepG2 cells (144). Interestingly, PON1 had been reported to hydrolyze aspirin and nitro-aspirin (292).

Probucol, a drug used to lower cholesterol, was reported to increase serum PON activity and PON1 expression in the hepatocytes of hypercholesterolemic rabbits when given at 500 mg/kg/day for 14 days (132). The cholesterol absorption-inhibiting drug ezetimibe was also reported to increase serum PON1 activity when given to hyperlipidemic patients for 12 weeks (10 mg/day) (337).

4. Diabetic drugs. Rosiglitazone, a PPAR-γ agonist, is used in the treatment of type 2 diabetes. Recently, its effects on PON1 have been investigated. Administration of rosiglitazone (4 mg twice daily for 8 weeks) to diabetic patients caused an increase (9%–13%) in fasting and postprandial serum PON1 activity without changing PON1 expression (345). Rosiglitazone was also reported to increase serum PON1 activity by promoting the synthesis of apo AI and smaller HDL particles in rabbits (61). Similarly, treatment of rats with rosiglitazone was also found to reverse the decrease in hepatic PON1 activity induced by administration of a high-fructose diet (3). Eplerenone is a selective aldosterone blocker used as an antidiabetic drug. It was recently found that eplerenone increases hepatic PON1 activity in control and streptozotocin-treated diabetic mice (249). Oral hypoglycemic sulfonylureas used in the treatment of diabetes, such as glimepiride and glibenclamide, were reported to increase PON1 activity in the livers of control and streptozotocin-treated diabetic rats, although the plasma PON1 activity was decreased in control rats and unchanged in diabetic rats (356).

5. Other drugs. In addition to the aforementioned cardiovascular and antidiabetic drugs, many other pharmaceutical drugs have been investigated for effects on PON1 activity and/or expression.

The commonly used antibiotics clarithromycin and chloramphenicol were reported to inhibit the enzyme activity of purified human serum PON (serum PON1) and PON purified from human hepatoma (HepG2) cells (liver hPON1) (315). In addition, gentamycin sulfate and cefazolin sodium have been shown to decrease the activity of HepG2 cell PON, purified human serum PON1, and mouse serum PON1 (313). Other antibiotics, such as sodium ampicillin, ciprofloxacin, and clindamycin sulfate (but not rifamycin), also inhibit the PON activity of purified human PON1 (314). When tested in mice, these antibiotics induced increases or decreases in serum PON1 activity at various time points, with similar results observed in the liver (314).

The liver X receptor (LXR) receptor has been suggested to be involved in the modulation of PON activity (114), and the LXR agonist T0901317 has been documented to rescue the decrease in rat serum PON1 activity resulting from leptin administration (37). Bile acids, however, activate another nuclear hormone receptor, the farnesoid X receptor, and

induce a decrease in serum PON1 activity and liver PON1 expression in mice (304).

Oral contraceptives (desogestrel or levonorgestrel in combination with ethinyl estradiol) are reported to increase serum PON1 activity in mice, although they significantly decrease mouse liver PON1 activity (168). Estrogen replacement therapy (ERT) with conjugated equine estrogen and medroxyprogesterone acetate increases the serum PON1 activity in postmenopausal women with type 2 diabetes. This effect is more evident in women with lower PON1 baseline levels and in those who have a concomitant increase in HDL cholesterol (326). In normal postmenopausal women, however, no significant effect on serum PON1 activity was observed after intranasal administration of estradiol (95). ERT can also rescue the reduction in PON1 and increase in plasma MDA resulting from estrogen ablation by surgical menopause (176). The anabolic steroid nandrolone decanoate, which is used as an adjuvant to erythropoietin in the treatment of anemia, has been reported to induce a mild but significant decrease in serum PON1 activity (104). The cholinergic muscarinic antagonist atropine has been documented to inhibit human plasma PON activity *in vitro* at high concentrations (76).

Several protein drugs have also been investigated with respect to their effects on PON. In predialysis patients with chronic renal disease and anemia, serum PON1 activity was increased by ~20% by treatment with erythropoietin beta (221). The ApoA-I mimetic peptide D-4F, which was developed for use in CHD patients, has been shown to be able to slightly increase plasma PON1 activity in mice and nonhuman primates (58).

E. Dietary factors and lifestyle factors

It is believed that atherosclerosis is to some extent a "lifestyle disease," although the extent to which it is affected by lifestyle depends on genetic background. Some lifestyle factors affect the development of atherosclerosis by modifying PONs.

1. Vitamin C and vitamin E. Administration of the dietary antioxidants vitamin C (ascorbic acid) and E (α-tocopherol) was reported to be associated with elevated PON activity in male Caucasian subjects (155). An *in vitro* study in human plasma showed that vitamin C administration reverses the decrease in serum PON activity induced by hypochlorite (177). In rats, it was reported that the decrease in serum PON activity caused by propylthiouracil-induced experimental hypothyroidism was reversed by vitamin E administration and that vitamin E also induced a small increase in serum PON1 activity in control animals (294). In high-cholesterol diet-fed rabbits, vitamin E was shown to increase serum and liver PON activity without affecting hepatic PON1 mRNA levels (156). A recent small study in humans showed that vitamin E supplementation prevented an exercise-induced reduction in serum PON1 activity (335).

2. Natural plant extracts. Eucommia ulmoides Oliver (Du-zhong) leaf extract significantly elevated plasma PON activity and lowered blood glucose concentrations when administered to type 2 diabetic (C57BL/6J-db/db) mice (262). When rats were fed with a diet containing 20% pistachio for 10 weeks, their HDL levels were increased, whereas their total cholesterol, LDL-cholesterol, and triglyceride levels were unaffected. Further, consumption of pistachio as 20% of the daily caloric intake also increased serum PON activity (35%) and arylesterase activity (60%). However, this effect was blunted when the pistachio intake was increased to 40% of daily caloric intake, for unknown reasons (9). When juice from wonderful variety pomegranates (WPJ) and pomegranate polyphenol extract (WPOMxl) were given to diabetic patients at doses of 50 ml/day for 4 weeks (WPJ) or 5 ml/day for 6 weeks (WPOMxl), there were no significant effects on fasting blood serum glucose or hemoglobin A1c levels. However, basal serum oxidative stress levels were significantly decreased, and HDL-associated PON1 arylesterase, PON, and lactonase activities were significantly increased by WPJ and WPOMxl consumption. PON1 binding to HDL was also significantly increased. Therefore, pomegranate consumption may retard the development of atherosclerosis *via* promoting serum PON1 stability and activity in diabetic patients (281).

3. Dietary polyphenol compounds. Some dietary polyphenol compounds, such as naringenin, flavones, quercetin, and catechin, increase PON-1 mRNA transcription when administered to HuH7 human hepatoma cells, although the effect of catechin is mild. These polyphenols must bind to and activate aryl hydrocarbon receptor (AhR) to exert their effects, because these effects are inhibited by specific AhR 7-ketocholesterol and AhR-directed short interfering RNA. Upon ligand binding and activation, AhR translocates into the nucleus and forms a heterodimer with the AhR nuclear translocator (ARNT). The AhR/ARNT heterodimer complex then binds to xenobiotic responsive elements located in the *PON1* promoter regions to promote *PON1* transcription (113).

Low-dose red wine polyphenolic extract (PE) supplementation significantly attenuates the decreases in hepatic and plasma PON1 activity induced by chronic hyperhomocysteinemia in mice. PE treatment also resulted in a drastic reduction in plasma Hcy levels and in aortic expression of proinflammatory cytokines and adhesion molecules. (248). The phytoalexin resveratrol, which is considered to be the main mediator of the beneficial effect of wine (117, 265), has been shown to display antioxidant, antiplatelet, and anti-inflammatory effects, inhibit lipid peroxidation, and decrease serum triglycerides and LDL levels *in vivo* (233, 265). In addition to modulating gene expression by affecting NFκB, AP-1 (60), and estrogen receptor (ER) (51, 117), resveratrol has also been shown to bind to the AhR (62). Gouedard *et al.* found that resveratrol treatment increased PON-1 expression in both human hepatocytes and the HuH7 hepatoma cell line. Like other plant polyphenols, this effect of resveratrol was mediated by the AhR pathway rather than the ERalpha signal (112).

4. Dietary flavonoids. Quercetin, a flavonol frequently present in fruits and vegetables, has been testified to be able to induce moderate but significant upregulation of *PON1* gene expression in mice but not in human (45, 46). To evaluate the effect of dietary flavonoids on PON2 expression, Boesch-Saadatmandi *et al.* treated cultured murine macrophages and overweight subjects with a high cardiovascular risk phenotype with quercetin. They found that treatment of murine

RAW264.7 macrophages with quercetin or its methylated derivative isorhamnetin dose-dependently increased PON2 mRNA and protein levels compared with untreated controls. Quercetin's glucuronidated metabolite quercetin-3-glucuronide, however, did not affect *PON2* gene expression in cultured macrophages. However, the investigation failed to find any effect of dietary quercetin supplementation on mRNA levels in human monocytes *in vivo*. (47) The discrepancy between *in vitro* and *in vivo* effect of dietary factors regarding the PON1 inducing activity may be mainly due to that most polyphenols shown to induce PON in cultured cells have a low bioavailability *in vivo* (296).

5. Dietary lipids. In rats, 8 weeks of high omega-3 PUFA and ethanol feed decreased liver PON1 mRNA expression, serum PON1 levels, and Hcy thiolactonase activity compared with a low omega-3 PUFA-fed group. However, betaine attenuated these effects when administered simultaneously. These results suggest that dietary betaine protects against atherosclerosis *via* promoting PON1 activity and quenching free radicals (346). Diets with a high trans-unsaturated fat content reduce PON1 activity, whereas consumption of olive acid (from olive oil) is associated with increased PON1 activity (332, 350).

6. Alcohol. In an early *in vitro* study, several aliphatic alcohols, including ethanol, were reported to inhibit human serum PON1 activity (76). Subsequent studies in humans, however, showed that moderate alcohol consumption increases serum PON1 activity (274, 312, 342), although one study found that a similar level of exposure did not alter serum PON activity (293). Similarly, moderate alcohol consumption for 8 weeks in rats also caused 20% and 25% increases in serum and liver PON1 activity, respectively, accompanied by an increase in hepatic PON1 mRNA levels (274). $LDL^{-/-}$ mice given a diet containing 18% ethanol for 4 weeks experienced a 31% increase in liver PON1 mRNA levels and a 64% increase in plasma PON1 activity (186). Heavy alcohol consumption in humans, in contrast to moderate alcohol consumption, leads to a significant decrease in PON1 activity (220, 274). In rats, heavy alcohol consumption also decreases serum and liver PON1 activity (186, 274).

7. Cigarettes. Cigarette smoke has been shown to inhibit PON1 activity *in vitro* (247) and *in vivo* (151), probably because of the acrolein present in cigarette smoke. It has been shown that acrolein inhibits HDL PON-1 activity in a time- and concentration-dependent fashion in studies where human HDL was incubated with 0–10 m*M* acrolein for 2 h (116).

8. Fasting. Fasting increases serum PON1 activity; effects can be seen within 6 h in rats. However, this is only a short-term adaptation designed to attenuate blood lipid peroxidation caused by fasting and cannot be sustained long term (331).

These results show that lifestyle factors, which can be readily modified, affect atherogenesis, partially through effects on PON1.

VIII. PONs Mimetics

In addition to upregulating or protecting endogenous PONs, PON activity can be modified by adding exogenous PONs or PON mimetics to the appropriate subjects. Reliable artificial molecules that can mimic PON's atheroprotective function would be extremely useful for such purposes. Using DNA family shuffling, investigators managed to express the first PON variants in a soluble and active form in *Escherichia coli*. These PON1 variants have 40-fold higher organic phosphate–hydrolyzing activities than wild-type PONs and a specificity switch of >2000-fold (5). These molecules open new prospects for improving PON catalytic activities and preventing atherosclerosis. For example, Mackness *et al.* used adenovirus-mediated *PON1* gene transfer (AdPON1) to overexpress human PON1 in mice with metabolic syndrome induced by a combined deficiency of leptin and the LDL receptor. AdPON1 expression led to a 4.4-fold increase in serum PON1 activity in AdPON1 mice compared with mice injected with control virus. Overexpressing human PON1 also significantly reduced the total plaque volume and altered plaque composition by reducing the plaque macrophage volume and enhancing the SMC content. Accordingly, the levels of plaque-associated oxLDL and the titer of autoantibodies against MDA-modified LDL were also lowered upon AdPON1 overexpression. No effects on total plasma cholesterol or triglycerides were detected after adenovirus transfection. This report suggests that AdPON1 could be a useful strategy to prevent or retard atherosclerosis in humans by reducing oxLDL levels (208). Using a recombinant *Escherichia coli* gene expression system, Stevens *et al.* successfully produced a highly purified engineered recombinant human PON1 ($rHuPON1_{K192}$) that protects against DZO. When injected into PON1-deficient mice, the nontoxic engineered PON1 molecules remain in the serum for at least 2 days and protected against at least three times the median lethal dose of DZO (321). Expression of active rHuPON1 in *Escherichia coli* or other recombinant gene expression systems could lead to the generation of PONs with high oxLDL catalytic efficiency that can protect against or treat atherosclerosis.

IX. Conclusions and Future Directions

Atherosclerosis has become the most prevalent cardiovascular cause of disabling illness and death in developed societies, and the current prevention and treatment strategies are unsatisfactory. As a multiple-factor chronic inflammatory disease, atherosclerosis results from interactions among blood flow, modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular components of the vessel walls, such as endothelial cells and SMCs. OxLDL and Hcy play important roles in promoting initiation, progression, and complications of atherosclerosis by affecting almost all of the components of atherosclerotic plaques. HDL protects against atherosclerosis; one of the important mechanisms mediating this protection is HDL's ability to detoxify oxLDL and Hcy-thiolactone by virtue of its binding partner PON1. PON1 and its family members PON2 and PON3 have been shown to protect against atherosclerosis in mouse models and clinical studies. The PON family also provides protection against many other diseases. The expression and activity of the PONs are modulated by many factors, allowing for multiple strategies to target PON activity in atherosclerotic patients. Most of the currently available literature supports the notion of using PONs in the prevention

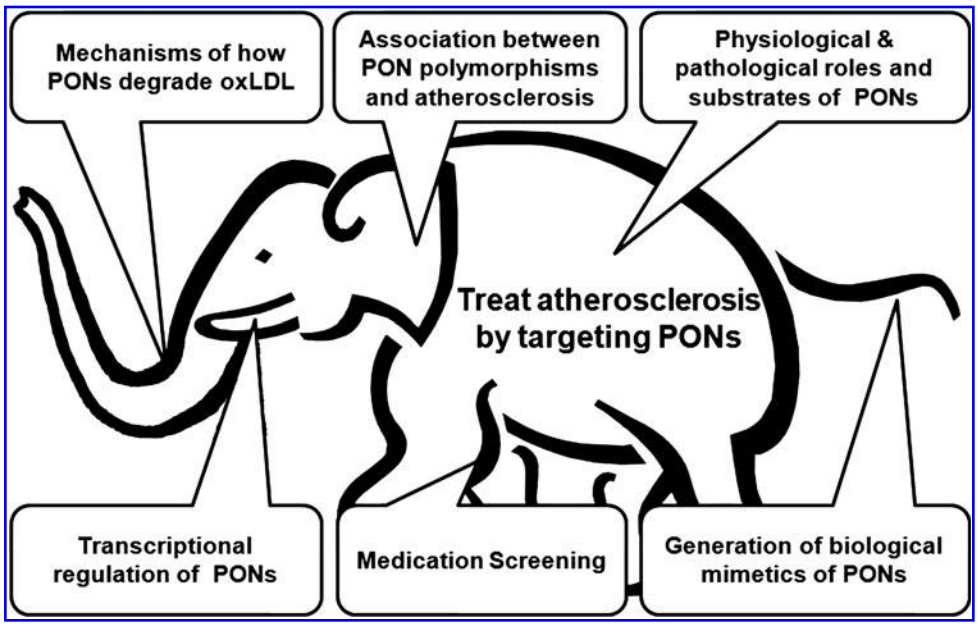

FIG. 11. Future directions required for targeting PONs to treat atherosclerosis. The indicated issues of PONs have to be addressed in future PON-related studies to facilitate clinical interventions of PONs activities in atherosclerosis treatment.

and treatment of atherosclerosis. However, to pave the road for clinical interventions based on PON activity, the following concerns need to be addressed in future studies (Fig. 11).

More convincing investigations need to be conducted to examine the association between *PON* polymorphisms and atherosclerosis-related CHD, because to date the results have not been consistent across different studies. In addition, several of the association studies conducted thus far suffer from several potential problems. For example, when the target populations are composed of multiple ethnic groups, a positive association could exist only due to differences in allelic frequency among ethnic groups. The appropriate analytical procedures, such as transmission-distortion analysis, should be used to minimize this problem. Also, probable associations have sometimes been dwarfed by insufficiently stringent thresholds when significant findings were established. Some results have been taken from separate studies in separate populations. Attempting to analyze these studies together is not reliable because of the conflicting nature of the documents, although the most convincing evidence for a PON–atherosclerosis association is the significant reproducibility among separate studies and separate populations (129).

The physiological roles and physiological substrates of PONs have to be yet confirmed. PONs are now considered

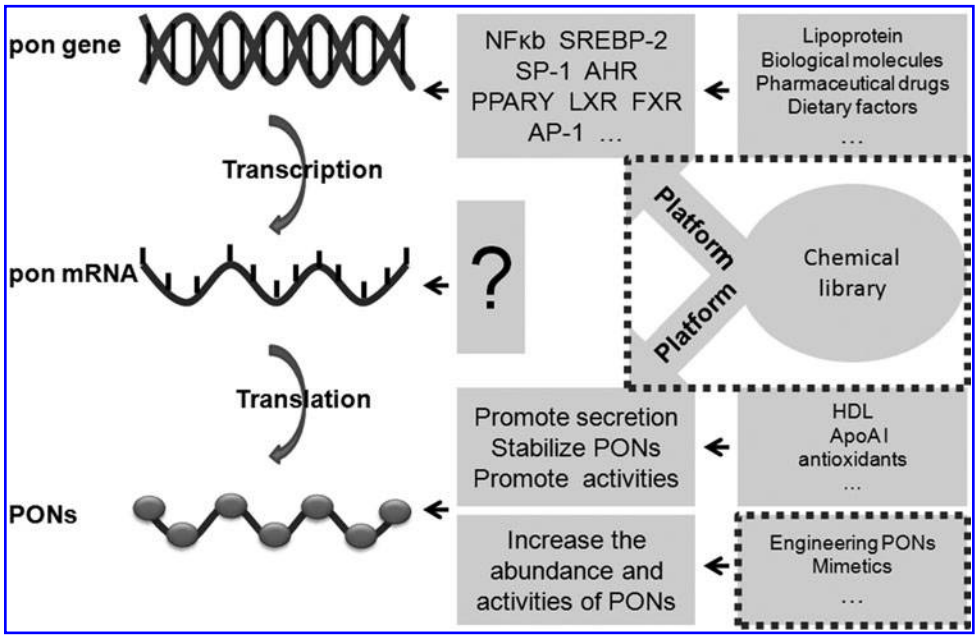

FIG. 12. Strategies to target PONs. Biological and chemical molecules have been reported to be able to, on the one hand, promote expression of PONs *via* corresponding transcription factors at DNA level; on the other hand, improve PON status at protein level. More effective and safer chemicals can be screened using probable platform to increase PONs expression, to promote secretion and activities, or to stabilize PONs. Engineering PON mimetics are also prosperous to this end. AhR, aryl hydrocarbon receptor; AP-1, activator protein 1; apo, apolipoprotein; FXR, farnesoid X receptor; PPAR, peroxisome proliferator-activated receptors; SREBP, sterol regulatory element binding protein Sp1, specificity protein 1; LXR, liver X receptor.

lactonases based on their catalytic activity toward their common substrate lactone. However, the fact that the best PON substrates are artificial chemicals instead of natural materials is still difficult to understand. Why would such an evolutionarily well-conserved cluster of proteins lack an important physiological function? What survival advantages does the PON gene cluster confer? What kind of evolutionary pressure led to the duplication of the PON gene and preserved the three genes through evolution? Ablation of PON1 or PON2 alone resulted in increased susceptibility to several stresses (organic phosphate toxicity and atherosclerosis, among others); however, no lethality has been reported from PON1 or PON2 deletion. The effects of PON3 deletion are as yet unknown, as is the phenotype of the triple knockout. Answers to these questions will lead to an increased understanding of the physiological roles of the PON gene family. At the same time, there is an urgent need to explore the physiological substrates of the PONs. Elucidating the physiological substrates and functions of PONs may also facilitate the prediction of probable side effects of atherosclerosis treatments targeting PONs.

The biochemical mechanisms by which PONs degrade oxidized lipids, especially oxLDL, have not been clearly identified. As early as 1995, Watson *et al.* reported that PON1 bound to HDL could degrade the biologically active lipids present in mildly oxLDL, thus exerting an anti-inflammatory effect in the cells of the artery wall (351). Even purified PON1 significantly reduces the ability of MM-LDL to induce monocyte–endothelial cell interactions (26, 27, 209, 351). This function of PON1 is supported by other studies in PON1 knockout and transgenic mice, in which HDL from PON1 knockout mice was observed to lack most of its capability to degrade oxLDL (303, 305), and HDL from PON1 transgenic mice exhibited a much higher oxLDL detoxification activity (338). PON2 and PON3 also act to decrease LDL oxidation (87, 245). However, Draganov *et al.* found that baculovirus-mediated recombinant and purified PONs (PON1, PON2, and PON3) failed to protect LDL against copper-induced oxidation *in vitro*, although these enzymes hydrolyze many overlapping substrates and distinctive substrates (88). A possible explanation for these results is that the system used is not appropriate for examining physiologically relevant PON activity (88). It is also possible that HDL or other associating proteins are indispensable for the PONs, especially PON1, to protect lipoproteins against oxidation. Dissecting the specific mechanisms of PON-mediated protection against oxLDL toxicity will provide strong evidence as to the mechanism of the PONs' protection against atherosclerosis.

To control the expression of the PONs, we first need to fully understand their transcriptional regulation. The three *PON* member genes exist in the genome as an end-to-end cluster. Thus far, no other genes have been reported to localize to this cluster. As a gene cluster, are their expressions controlled in a spatial-temporal manner, like the hemoglobin gene cluster? Are there any coregulatory elements in this cluster that coordinate their expression? If these three members are not governed by a common transcriptional regulation module, are there any insulators or other cis-elements that interrupt the continuity of the genomic structure? If the three members are expressed independently, how are they individually regulated? Thus far, only the human *PON1* promoter has been characterized. Characterization of the *PON2* and *PON3* promoters is necessary for a full understanding of the regulation of this gene cluster and its use for treatment.

Screening effective and safe medications that increase the protective effect of PONs on atherosclerosis is another leading direction for future PON research. Because the status (quantity and quality) of the enzyme in the serum is significantly related to an individual's risk for developing atherosclerosis-related diseases, it is important to elucidate the factors influencing the serum levels of PON1 and use them therapeutically to increase its activity. The concentration and activity of the PONs are highly variable among individuals. Genetic polymorphisms, gene expression levels, protein stability, protein secretion, association with HDL, and environmental factors all contribute to affecting the status of the PONs. Therefore, molecules that are able to improve these processes and/or increase these factors are potential therapeutic candidates. Setting up feasible and reliable high-throughput screening platforms will be very important for screening PON activators. Genetic engineering and DNA shuffle techniques could also be used to synthesize and optimize artificial *PON* gene, leading to expression of novel PON or PON-like proteins with higher catalytic activities toward oxLDL than natural PONs. These techniques will enable us to create and apply extrinsic PON mimetics to treat patients suffering from atherosclerosis (Fig. 12).

In conclusion, previous results solidly support the protective roles of the PONs in atherosclerosis; if groundbreaking progress in the directions mentioned earlier is made, the *PON* gene cluster will be used as a prospective target for the treatment of atherosclerosis.

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Abbreviations Used

3-OC12-HSL = *N*-(3-oxododecanoyl)-L-homoserine lactone
5-HETEL = 5-hydroxy-6*E*,8*Z*,11*Z*,14*Z* eicosatetraenoic acid lactone
AdPON1 = PON1-expressing adenovirus
AhR = aryl hydrocarbon receptor
AP-1 = activator protein 1
apo = apolipoprotein
ARNT = AhR nuclear translocator
BAC = bacterial artificial chromosome
CAD = coronary artery disease
CHD = coronary heart disease
CVD = cardiovascular disease
Cys = cysteine
eNOS = endothelial nitric oxide synthase
ER = estrogen receptor
ERK = extracellular signal-regulated kinase
ERT = estrogen replacement therapy
FXR = farnesoid X receptor
Hcy = homocysteine
HDL = high-density lipoprotein
H_2O_2 = hydrogen peroxide
ICAM-1 = intercellular adhesion molecule 1
IL-1 = interleukin 1
LDL = low-density lipoprotein
LPS = lipopolysaccharides
LXR = liver X receptor
MAPK = mitogen-activated protein kinases
MCP-1 = monocyte chemotactic protein-1
MDA = malondialdehyde
mm-LDL = minimally modified oxidized low-density lipoprotein
MS = methionine synthase
NADPH = nicotinamide adenine dinucleotide phosphate
NFκB = nuclear factor kappa B
oxLDL = oxidized low-density lipoprotein
PDGF = platelet-derived growth factor
PE = polyphenolic extract
PI3K = phosphatidylinositol 3-kinase
PKB = protein kinase B
PKC = protein kinase C
PON = paraoxonase
PPAR = peroxisome proliferator-activated receptors
PUFAs = polyunsaturated fatty acids
RCT = reverse cholesterol transportation
rHuPON1 = recombined human PON1
ROS = reactive oxygen species
SAM = *S*-adenosyl methionine
SMC = smooth muscle cell
SNP = single-nucleotide polymorphism
Sp1 = specificity protein 1
SRs = scavenger receptors
SREBP = sterol regulatory element binding protein
TNF-α = tumor necrosis factor α
uPA = urokinase plasminogen activator
uPAR = urokinase plasminogen activator receptor
VLDL = very low-density lipoprotein
VSMC = vascular smooth muscle cell
WPJ = wonderful variety pomegranate juice

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