Metals and Paraoxonases

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Abstract The paraoxonases (PONs) are a three-gene family which includes PON1, PON2, and PON3. PON1 and PON3 are synthesized primarily in the liver and a portion is secreted in the plasma, where they are associated with high-density lipoproteins (HDLs), while PON2 is an intracellular enzyme, expressed in most tissues and organs, including the brain. PON1 received its name from its ability to hydrolyze paraoxon, the active metabolite of the organophosphorus (OP) insecticide parathion, and also more efficiently hydrolyzes the active metabolites of several other OPs. PON2 and PON3 do not have OP-esterase activity, but all PONs are lactonases and are capable of hydrolyzing a variety of lactones, including certain drugs, endogenous compounds, and quorum-sensing signals of pathogenic bacteria. In addition, all PONs exert potent antioxidant effects. PONs play important roles in cardiovascular diseases and other oxidative stress-related diseases, modulate susceptibility to infection, and may provide neuroprotection (PON2). Hence, significant attention

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has been devoted to their modulation by a variety of dietary, pharmacological, lifestyle, or environmental factors. A number of metals have been shown in in vitro, animal, and human studies to mostly negatively modulate expression of PONs, particularly PON1, the most studied in this regard. In addition, different levels of expression of PONs may affect susceptibility to toxicity and neurotoxicity of metals due to their aforementioned antioxidant properties.

Keywords Paraoxonases • Metals • Lead • Mercury • Cadmium • Manganese • Oxidative stress

Abbreviations

Ag	Silver
Al	Aluminum
As	Arsenic
Ba	Barium
Cd	Cadmium
Ce	Cesium
Co	Cobalt
Cr	Chromium
Cu	Copper
Fe	Iron
Gd	Gadolinium
HDL	High-density lipoprotein
Hg	Mercury
L	Leucine
La	Lanthanum
LDL	Low-density lipoprotein
M	Methionine
MeHg	Methylmercury
Mn	Manganese
Ni	Nickel
OP	Organophosphate
Pb	Lead
PCR	Polymerase chain reaction
PON	Paraoxonase
ppb	Parts per billion
ppm	Parts per million
Q	Glutamine
R	Arginine
Sm	Samarium
Y	Yttrium
Zn	Zinc

Introduction

The paraoxonases (PONs) are a three-gene family which includes PON1, PON2, and PON3, all clustered in tandem on the long arm of human chromosome 7 (7q21.22). PON1 and PON3 are synthesized primarily in the liver and a portion is secreted in the plasma, where they are associated with high-density lipoproteins (HDLs); low levels of PON1 and PON3 may be expressed in a number of other tissues, primarily in epithelia (Primo-Parmo et al. 1996; Marsillach et al. 2008). In contrast, PON2 is an intracellular enzyme, expressed in most tissues and organs, including the brain. PON1 received its name from its ability to hydrolyze paraoxon, the active metabolite of the organophosphorus (OP) insecticide parathion, which is its first and most studied substrate. PON1 more efficiently hydrolyzes the active metabolites of several other OP insecticides (e.g., chlorpyrifos oxon, diazoxon) and less efficiently nerve agents such as sarin and soman (Costa et al. 2003, 2013a). PON2 and PON3 do not have OP-esterase activity, but all PONs are lactonases and are capable of hydrolyzing a variety of lactones, including certain drugs (bioactivating some, e.g., the antibacterial prodrug prulifloxacin, or inactivating others, e.g., glucocorticoids), endogenous compounds (e.g., lactone metabolites of arachidonic acid), and N-acyl homoserine lactones, which are quorum-sensing signals of pathogenic bacteria (Draganov et al. 2005; Teiber et al. 2008). All PONs have potent antioxidant effects: PON1 and PON3 protect low-density lipoproteins (LDLs) (Mackness et al. 1991), as well as HDL from oxidation (Aviram et al. 1998; reviewed in Costa et al. 2003), while PON2 exerts intracellular antioxidant effects (Costa et al. 2014). PON1 is the most studied of the PONs, because of its important roles in modulating susceptibility to OP neurotoxicity and in cardiovascular disease and other diseases (Costa and Furlong 2002; Costa et al. 2003; Furlong et al. 2010). PON2 has received more attention recently, and novel important roles in the central nervous system and in tumor cells are emerging (Costa et al. 2014; Witte et al. 2011). PON3 is the least studied of the three PONs, but there is evidence that it plays important roles in cardiovascular disease, in susceptibility to infection, and in tumor cells (Shih et al. 2007; Schweikert et al. 2012a, b; Marsillach et al. 2015).

PON1

Human Polymorphisms of PON1 and Definition of PON1 Status

Earlier observations on the polymorphic distribution of serum paraoxonase activity in human populations led to the purification, cloning, and sequencing of human (and rabbit) PON1, as well as in the molecular characterization of its polymorphisms (Furlong et al. 1993; Humbert et al. 1993). Of the two polymorphisms observed in the PON1 coding sequence (Q192R and L55M), the former significantly affects the catalytic efficiency of PON1 for some substrates (Humbert et al. 1993).

The $PON1_{R192}$ allozyme hydrolyzes paraoxon or chlorpyrifos oxon more readily than $PON1_{Q192}$, while the opposite is true in the case of sarin or soman (Davies et al. 1996). In the case of diazoxon, both PON1 alloforms hydrolyze this compound with the same efficiency, and both alloforms are able to provide in vivo protection against exposure (Li et al. 2000). Lactones are hydrolyzed preferentially by either $PON1_{R192}$ or $PON1_{Q192}$, depending on their structure (Draganov et al. 2005). For example, $PON1_{R192}$ is more efficient at hydrolyzing homocysteine thiolactone (HCL), while gamma-valerolactone and 2-coumaranone are more rapidly hydrolyzed by $PON1_{Q192}$. However, it is important to note that hydrolysis of HCL by PON1 is orders of magnitude less efficient than by bleomycin hydrolase and especially by biphenyl hydrolase-like protein (Marsillach et al. 2014). Furthermore, $PON1_{Q192}$ has also a higher efficiency in protecting against LDL oxidation than the $PON1_{R192}$ allozyme (Mackness et al. 1998).

The L/M polymorphism at position 55 does not appear to affect catalytic activity, but has been associated with plasma PON1 protein levels, with $PON1_{M55}$ being associated with low plasma PON1 activity (Mackness et al. 1998). However, this appears to result primarily from linkage disequilibrium with the low-efficiency –108T allele of the −108 promoter region polymorphism (Brophy et al. 2002). Of the several additional polymorphisms found in the noncoding region of the PON1 gene, one of the most significant is this polymorphism at position −108, with the –108C allele providing levels of PON1 about twice as high on average as those seen with the –108T allele (Brophy et al. 2001).

Most studies investigating the association of PON1 with various diseases have examined nucleotide polymorphisms (mainly Q192R, L55M, C-108T) with PCR-based assays. A functional genomic activity analysis, however, provides a much more informative approach, as measurement of an individual's PON1 function (serum activity) takes into account all polymorphisms and other factors that might affect PON1 activity or expression. This is accomplished through the use of high-throughput enzyme assays involving two PON1 substrates (traditionally diazoxon and paraoxon at high salt concentration, but more recently the nontoxic phenyl acetate at high salt, and 4-(chloromethyl)phenyl acetate at low salt; Richter and Furlong 1999; Richter et al. 2008, 2009). Both the earlier assay with the two OP substrates and the new assays using the safer non-OP substrates provide a clear separation of the three $PON1_{192}$ functional genotypes (QQ, QR, RR), as well as information on enzyme activity within each genotype (Richter and Furlong 1999). This approach, which provides a functional assessment of the plasma $PON1_{192}$ alloforms, including information on the plasma level of PON1 for each individual, has been referred to as the determination of PON1 "status" for an individual (Richter and Furlong 1999). In a given population, plasma PON1 activity can vary up to 40–50-fold, and differences in PON1 protein levels up to 13–15-fold are also present within a single *PON1*$_{192}$ genotype in adults (Richter and Furlong 1999). The use of PON1 substrates that are not affected by the Q192R polymorphism (e.g., phenyl acetate hydrolysis at low salt to measure arylesterase activity) provides a surrogate measure of PON1 plasma protein level as does direct analysis of PON1 protein concentration (e.g., by ELISA or mass spectrometry). In contrast, given that PON1

activity is strongly determined by enzyme genotype, assays using paraoxon as a substrate would provide equivocal results, if each group is not matched for genotype, since each $PON1_{192}$ alloform hydrolyzes paraoxon with different efficiencies. A good example of analyzing individuals within each $PON1_{192}$ functional genotype is provided in the study of PON1 status and stroke (Jarvik et al. 2000).

The importance of PON1 status in modulating susceptibility to the acute toxicity of a number of OP insecticides has been shown by several studies (Shih et al. 1998; Li et al. 2000; Cole et al. 2005). Studies with transgenic animal models have shown that PON1-deficient mice are highly susceptible to the toxicity of specific OPs (Shih et al. 1998; Li et al. 2000). Depending on the OP, PON1 levels alone (as in the case of diazoxon) or $PON1_{192}$ functional genotype as well as activity level (as in the case of chlorpyrifos oxon) may determine the degree of protection against a specific OP (Li et al. 2000). Alterations in circulating PON1 levels have been found in a variety of diseases involving oxidative stress, including cardiovascular disease, diabetes, Alzheimer's disease, chronic renal failure, and chronic liver impairment (Costa and Furlong 2002; Costa et al. 2003; Marsillach et al. 2007a, b; Furlong et al. 2010; Androutsopoulos et al. 2011). Studies investigating the role of PON1 in cardiovascular disease have provided evidence that PON1 status (encompassing genotype and activity levels) is a much better predictor of disease than PON1 genotype alone (Mackness et al. 2001; Jarvik et al. 2003).

Modulation of PON1 Activity and Expression

Given the role of PON1 in protecting against toxic pesticide exposures and cardiovascular disease, and its decreased activity levels in a number of pathological conditions, it is not surprising that particular attention has been devoted to factors that may positively modulate PON1, i.e., increase its activity or expression (reviewed in Costa et al. 2005, 2011; Camps et al. 2009). While a major determinant of PON1 activity is represented by genetic polymorphisms, age also plays an important role, as PON1 activity is very low before birth and gradually increases during the first year or two of life in humans (Cole et al. 2003). PON1 activity may also decline with aging, possibly because of the development of oxidative stress conditions (reviewed in Costa et al. 2005). An influence of gender has also been suggested, with female mice displaying higher PON1 activity (reviewed in Costa et al. 2005). Several studies investigating modulation of PON1 have involved pharmaceutical drugs, particularly lipid-lowering compounds such as statins and fibrates, as well as other drugs (reviewed in Costa et al. 2005, 2011). As PON1 is easily inactivated by exogenous or endogenous oxidants, several strategies to increase PON1 have focused on the administration of dietary antioxidants such as vitamin C (ascorbic acid), vitamin E (alpha-tocopherol), and several dietary polyphenols, particularly quercetin and pomegranate juice and extract, which contain several polyphenolic compounds such as punicalagin, gallic acid, and ellagic acid (reviewed in Costa et al. 2011). Dietary lipids (e.g., olive oil or omega-3 fatty acids) and moderate

doses of alcohol also increase PON1 activity and expression (reviewed in Costa et al. 2005, 2011).

While most attention has been devoted to identifying pharmacological or dietary factors that may increase PON1 activity, other factors that, in contrast, may negatively impact PON1 should also be considered, as they may increase susceptibility to diseases and/or toxic effects. High alcohol consumption, smoking, and consumption of certain high-fat diets have been shown to decrease PON1 expression (reviewed in Costa et al. 2005, 2011). Several studies have shown that metals can also negatively modulate PON1 (see section "Interactions of metals with PONs").

PON2

PON2 as an Intracellular Antioxidant Enzyme

PON2, a PON isozyme less studied than PON1, is nevertheless emerging as an important defense system toward oxidative stress and inflammation. In contrast to PON1 and PON3, PON2 is a ubiquitously expressed intracellular enzyme, but is not present in plasma (Mochizuki et al. 1998; Ng et al. 2001; Marsillach et al. 2008; Giordano et al. 2011). In peripheral tissues, PON2 is considered important in modulating sensitivity to bacterial infections because of its high acyl-HSL hydrolytic activity, and also plays a significant role in atherosclerosis (Ng et al. 2006), and in antagonizing oxidative and inflammatory processes that may affect mucosal integrity in the gastrointestinal tract (Levy et al. 2007). Subcellular distribution studies have shown that PON2 is localized primarily in the mitochondria, endoplasmic reticulum, and perinuclear region (Devarajan et al. 2011; Giordano et al. 2011), a major source of free radical-related oxidative stress. More recently it was reported that PON2 translocates its catalytic domain to the outside of the cell under certain conditions of oxidative stress, to protect membrane lipids from oxidation (Hagmann et al. 2014).

Two common coding region polymorphisms in strong disequilibrium (A147G and S311C) have been found in human PON2 (Primo-Parmo et al. 1996; Mochizuki et al. 1998). The PON2 S311C polymorphism has been shown to affect lactonase activity, but does not appear to influence antioxidant activity of PON2 (Altenhofer et al. 2010). PON2 mRNA and protein have been found in the central nervous system (CNS) of several species including mouse, rat, nonhuman primate, and human (Costa et al. 2014). In mouse brain, the highest levels of PON2 are in the dopaminergic regions (substantia nigra, striatum, nucleus accumbens), with lower levels in other brain areas (Giordano et al. 2011). In every brain region, as well as in peripheral tissues, PON2 levels are higher in female mice than in male mice. PON2 exerts a protective effect toward oxidative stress, for example, the cytotoxicity of the oxidants hydrogen peroxide (H_2O_2) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) is much greater in brain cells from PON2 knockout mice (Giordano et al. 2011). The

different levels of expression of PON2 protein between male and female mice are also reflected in a differential susceptibility to neurotoxicity (Giordano et al. 2011, 2013). While the apparent anti-apoptotic properties of PON2 may underlie neuroprotection, the same characteristic in cancer cells makes them more resistant to chemotherapy-induced apoptosis (Witte et al. 2011; Krüger et al. 2015).

Modulation of PON2

The higher levels of PON2 in tissues from female mice appear to be related to a positive modulatory effect by estrogens, as suggested by various lines of evidence (Giordano et al. 2013). For example, 17-beta estradiol increases the levels of PON2 in striatal astrocytes from male mice; the effect is due to transcriptional activation of the PON2 gene and appears to be mediated by activation of estrogen receptor alpha (Giordano et al. 2013). In addition, PON2 levels (protein and mRNA) in ovariectomized female mice are significantly reduced in brain regions and in the liver, approaching the levels found in male mice (Giordano et al. 2013).

Activation of dopamine D2 receptors in the kidney positively modulates PON2 expression through activation of NADPH oxidase, leading to a decrease in ROS production (Yang et al. 2012). In the CNS, the highest levels of dopamine D2 receptors are found in the same areas (e.g., striatum, nucleus accumbens, substantia nigra) that also have the highest level of PON2 expression (Giordano et al. 2011). If a similar mechanism as observed in kidneys also occurs in the CNS, the loss of dopamine associated with Parkinson's disease would lead to decreased PON2 levels, thus fostering a spiral of events further aggravating neurodegeneration. The functional consequences of a higher expression of PON2 in females may have important ramifications. For example, oxidative stress plays a highly relevant role in the etiopathology of Parkinson's disease, whose incidence is 90% higher in males (Surmeier et al. 2011; Wirdefeld et al. 2011). Furthermore, as PON2 is expressed in most tissues and levels appear to be higher in females in each tissue examined (Giordano et al. 2011), the reported higher sensitivity of males to oxidative stress in the heart, to atherosclerosis, and to infections may all be related to a differential expression of PON2 (Klein 2000; Kardys et al. 2007; Wang et al. 2010).

In contrast to PON1 and PON3, PON2 expression is increased by oxidative stress (Rosenblat et al. 2003). Additionally, arachidonic acid, unesterified cholesterol, the licorice phytoestrogen glabridin, extracts of yerba mate (*Ilex paraguariensis*), and the hypocholesterolemic drug atorvastatin also upregulate PON2 expression in various cell types (Rosenblat et al. 2004; Fernandes et al. 2012; Yehuda et al. 2016). A recent study found that quercetin increases PON2 protein expression in the brain, thereby providing neuroprotection (Costa et al. 2013b). In contrast to studies on PON1 regulation, no studies on negative modulation of PON2, other than by metals (see section "Interactions of metals with PONs"), have been identified.

PON3: Activity, Polymorphisms, Physiological Functions, and Modulation

PON3 is synthesized mainly by the liver and is found in circulation in HDLs (Reddy et al. 2001) and intracellularly in endoplasmic reticulum (Rothem et al. 2007) and mitochondria (Schweikert et al. 2012a). In mice, PON3 is undetectable in serum or HDL (Ng et al. 2007), but its protein expression has been identified in multiple tissues (Marsillach et al. 2008). PON3 is the least characterized of the PON family of enzymes. It does not hydrolyze OPs, but possesses lipo-lactonase and N-acyl homoserine lactone activities (Draganov et al. 2005). Compared to PON1, PON3 has a higher catalytic activity for statin lactones (such as lovastatin), which are commonly used to monitor PON3 activity (Draganov et al. 2005).

There are only few studies on polymorphisms in the PON3 gene (reviewed in Furlong et al. 2016). Two missense mutations (S311T, G324D) in exons III, IV, and IX of *PON3* were identified in healthy subjects from Southern Italy (Campo et al. 2004), and later in children with diagnosed inflammatory bowel disease (Sanchez et al. 2006), but no relationship between the *PON3* genetic variants and disease was observed.

Human PON3 concentration in serum is about two orders of magnitude lower than PON1 (Aragones et al. 2011). However, recombinant rabbit PON3 seems to be more potent than recombinant rabbit PON1 in protecting LDL from copper-induced oxidative modifications in vitro (Draganov et al. 2000). PON3 and PON2 protect murine macrophages against oxidative damage, with cellular PON3 activity being decreased under oxidative stress (Rosenblat et al. 2003). In vivo, mice overexpressing PON3 are more resistant to atherosclerosis and obesity (Shih et al. 2007; Ng et al. 2007). Interestingly, these effects were only seen in male mice although a protective role of PON3 in obesity has also been reported in female mice with the PON3 gene knocked out (Shih et al. 2015). As previously reported for PON1, human serum PON3 concentration significantly increases in some disease states such as chronic liver disease, coronary and peripheral artery disease, and HIV infection (Garcia-Heredia et al. 2011; Rull et al. 2012; Aragones et al. 2012), while another study has recently reported a significant decrease in PON3 in HDL from patients with autoimmune disease (type 1 diabetes or systemic lupus erythematosus) and subclinical atherosclerosis (Marsillach et al. 2015).

Despite PON3's beneficial role in protecting against a variety of oxidative stress-related diseases, an unexpected finding is a role for PON3 (and PON2), in cancer where PON3 is upregulated (PON3 being much more overexpressed in cancer cells than PON2) and protects tumor cells against mitochondrial superoxide-mediated apoptosis. Also similarly to PON2, PON3 has an important role in the defense against *P. aeruginosa* virulence (Schweikert et al. 2012b).

With the exception of being negatively affected by oxidative stress (Rosenblat et al. 2003), no other information, except for the interaction with metals (see section "Interactions of metals with PONs"), is available on positive or negative modulation of PON3.

Interactions of Metals with PONs

Metals are often defined by their physical properties of the element in the solid state (e.g., high electrical and thermal conductivity, mechanical ductility), but their toxicological relevance is linked to their ability to lose one or more electrons to form cations (Tokar et al. 2013). In addition, metals often exhibit variable oxidation states. Over 75% of elements in the periodic table are regarded as metals or metalloids. Metals are found naturally in the Earth's crust, and their level varies across the continents; they are redistributed naturally in the environment by geologic and biologic cycles. However, human intervention can shorten the residence of metals in ore, may form new compounds, and may increase worldwide distribution. Due to their wide and early use, the toxicity of metals has been known for centuries. Initially, concerns were primarily related to acute effects, though later metal toxicology has shifted to more subtle, chronic, low-dose effects. Some metals (e.g., lithium, bismuth, platinum compounds) can also have beneficial effects and are used as pharmaceutical drugs. Exposure of humans to metals can occur through multiple pathways (mostly inhalation and oral) and in a variety of settings. Occupational exposure to a number of metals (e.g., manganese, lead, cadmium, mercury) is quite common. Exposure to metals through food (e.g., methylmercury in fish) or water (e.g., arsenic, lead, manganese) is also common.

Various metal ions have been studied in relationship to their interactions with PONs. Most studies have examined interactions of metals with PON1, while very few have also included PON2 and PON3. Several in vitro studies have been carried out, but only a handful of in vivo studies in experimental animals are available. A few human studies investigating associations between serum PON1 and blood metal levels in various populations are also available. Below, we review the current information on the interactions of metals with PONs.

In Vitro Effects of Metals on PONs

A number of studies have examined the in vitro effects of metals on PONs, all with one exception on PON1. The first indication that PON1 activity may be affected by metals can be found in an abstract from the 1950s in which Aldridge (1951) reported that E 600 (paraoxon) hydrolase (later identified as PON1) was "inhibited by mercury, copper and nickel (10^{-6} M)". A few years later, Erdös et al. (1959, 1960) investigated the effects of several metals on blood arylesterase activity (identifiable as PON1 by calcium dependence and inhibition by EDTA). In addition to heavy metals (e.g., cadmium, lead, mercury), these investigators also tested rare earth metals such as cerium, samarium, gadolinium, lanthanum, as well as yttrium. The latter metals were actually more potent than other metals in inhibiting arylesterase activity, as the IC_{50} values were in the sub-micromolar range (Erdös et al. 1960; Table 1).

Table 1 In vitro effects of metals on PON1 activity

Study/metal	1	2	3	4	5	6	7	8	9	10
Ag	0.7									
Al								>1000/>1000		
Ba	60	5580/290	930							
Cd	0.3			3.3		150/250		0.05/0.08	730	2840/980
Ce	0.04									
Co	3.0	2330/90		64	80	800/4200				2050/1810
Cr							1991			
Cu	8.0	170/850	63	178	317	50/300			290	747/530
Fe							3960	100/200		
Gd	0.04									
Hg	0.7	520/320	21	4.7	4.0	120/1100		0.2/0.5	490	1810/2840
La	0.06	1116/280	310	85						
Mn	10	3740/170		151	199	300/700				5140/1980
Ni	3.0			21.3		1000/1200			2000	3390/4220
Pb	2.0						838	0.2/1.0		
Sm	0.2									
Zn	1.0	1060/130	920	6.2			7410	0.05/0.1		0.08/0.2
Y	0.2									

Shown are values of IC_{50} (μM). Studies are (1) Erdös et al. 1960; (2) Gil et al. 1994 [plasma/liver]; (3) Gonzalvo et al. 1997; (4) Debord et al. 2003; (5) Pla et al. 2007; (6) Gencer and Arslan 2009 [R/Q]; (7) Ekinci and Beydemir 2010; (8) Cole et al. 2002; Figs. 1 and 2 [R/Q]; (9) Sayin et al. 2012; (10) Erol et al. 2013

Gil et al. (1994) reported that rat plasma paraoxonase activity was inhibited by a number of metals with IC_{50} values ranging from 170 μM (copper) to 5580 μM (barium) (Table 1). Kinetic studies indicated that inhibition was either competitive (Cu, La, Zn, Co) or noncompetitive (Hg, Mn, Ba). Results were somewhat different when the same metals were tested on the paraoxonase activity of the rat hepatic microsomal fraction. In particular, cobalt was the most potent metal, followed by zinc and manganese (Table 1). All metals appeared to be more potent (by up to 25-fold) in inhibiting liver paraoxonase activity compared to plasma, with the exception of copper, for which the opposite was found. In addition, kinetic characteristics of inhibition in the liver were different from plasma (Gil et al. 1994). In a follow-up study, Gonzalvo et al. (1997) studied the ability of a number of metals to inhibit paraoxon hydrolysis in human liver microsomes (Table 1). They found that mercury was the most potent inhibitor (IC_{50} = 21 μM), followed by copper and lanthanum.

Debord et al. (2003) reported that several metals inhibited arylesterase activity of human serum, with copper being the most potent (Table 1). The paraoxonase activity of PON1 purified from rat liver was inhibited by various heavy metals, including mercury, manganese, copper, and cobalt (Pla et al. 2007; Table 1). Similarly, paraoxonase activity of purified human PON1 (no indication of genotype) was found to be inhibited by metals, and lead was the most potent in this regard (Ekinci and Beydemir 2010; Table 1). Kinetic studies indicated that inhibition was of the competitive type for lead and iron and noncompetitive for chromium and zinc. Sayin et al. (2012) and Erol et al. (2013) describe the purification of PON1 from blood of sharks and of two breeds of sheep (merino and kivircik) and the effect of various metals on paraoxonase activity (Table 1). Of the metals tested, cadmium and copper appeared to be the most potent. Kinetic analysis of the type of inhibition provided results similar to those of Gil et al. (1994); for example, inhibition by copper and cobalt was of the competitive type, while that of mercury was noncompetitive. A study by Sukketsiri et al. (2013) examined the effects of lead acetate on PON1 arylesterase activity in HepG2 (human hepatoma) cells. In contrast to other studies, at concentrations of up to 100 μg/ml (263.6 μM), lead had no effect on PON1 arylesterase activity.

Gencer and Arslan (2009) were the first to investigate the relative effects of metals on $PON1_{R192}$ and $PON1_{Q192}$ allozymes. For all metals tested (Cd, Co, Cu, Hg, Mn, Ni), the $PON1_{R192}$ allozyme displayed a higher sensitivity to inhibition, ranging from less than two- to ninefold. In our laboratory, we tested the ability of cadmium, mercury, iron, zinc, lead, and aluminum (all chloride salts) to inhibit plasma PON1 activity. Metals were incubated with PON1 for various lengths of time (15 min, 4 h, 24 h), followed by measurement of PON1 arylesterase activity. Initial experiments used human PON1 purified from individuals expressing either the $PON1_{R192}$ or $PON1_{Q192}$ alloform (Fig. 1). All metals tested inhibited PON1 arylesterase activity to some degree. Inhibition was similar at the three incubation times tested (15 min, 4 h, and 24 h), and results for 4 h are shown in Fig. 1. Cadmium, mercury, lead, and zinc were potent inhibitors of PON1, with nearly complete inhibition at 0.75 μM and significant inhibition in some cases at less than 0.1 μM. For these four metals,

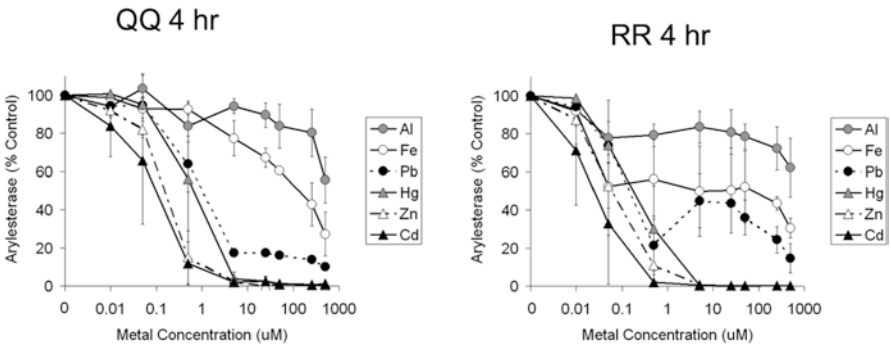

Fig. 1 Effect of various metals on the activity of purified human $PON1_{Q192}$ and $PON1_{R192}$ after 4 h incubation. Results are expressed as means (± SE) with n = 4

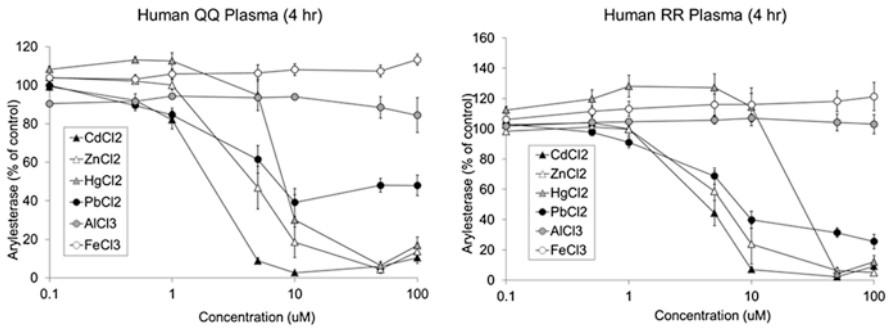

Fig. 2 Effects of various metals on arylesterase activity in plasma from individual homozygotes for the 192Q or 192R alleles of PON1, after 4 h incubation. Results are expressed as means (± SE) with n = 4

the $PON1_{R192}$ alloform was more susceptible to inhibition by cadmium than the $PON1_{Q192}$ alloform (Table 1; Fig. 1), in agreement with the findings of Gencer and Arslan (2009). Iron also inhibited PON1, with significant inhibition at 0.75 μM; inhibition was mainly observed with the $PON1_{R192}$ alloform, while the $PON1_{Q192}$ alloform was relatively resistant to inhibition. Aluminum was the weakest inhibitor of PON1, with <50% inhibition in both PON1 allozymes.

In a second series of experiments, the ability of the same metals to inhibit PON1 arylesterase activity was measured in plasma of individuals homozygous for the $PON1_{R192}$ or the $PON1_{Q192}$ allele. Results of these experiments for the 4 h time point are shown in Fig. 2, and similar results were obtained for the shorter (15 min) and longer (24 h) incubation times. In general, the concentration-response curves for metal inhibition of PON1 were shifted to the right compared to those obtained with purified PON1, suggesting that factors present in plasma provide some protection against metal inhibition of PON1 (compare Fig. 1 and Fig. 2). For example, cadmium

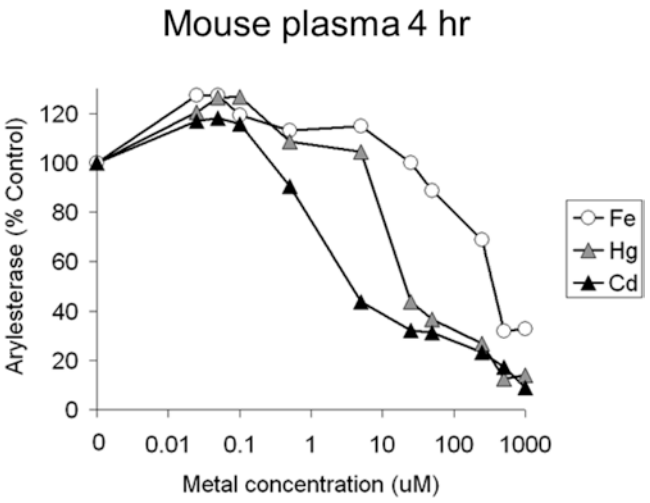

Fig. 3 Effects of cadmium, mercury, and iron (all chlorides) on arylesterase activity in mouse plasma after 4 h incubation. Results are expressed as means (± SE) with n = 4

was still the most potent inhibitor of PON1, but its potency was decreased by >10-fold compared to the purified enzyme. Pb, Zn, and Hg had an intermediate potency, while Al and Fe were devoid of any inhibitory activity (Fig. 2). For all metals, the differences in inhibitory potency between the RR and the QQ genotype were minimal (Fig. 2).

The ability of three metals (Cd, Hg, and Fe) to inhibit serum PON1 arylesterase activity in vitro was also assessed in mouse plasma. As shown in Fig. 3, cadmium was the most potent inhibitor of PON1, followed by mercury and by iron. No significant differences were found when comparing results obtained after 4 h incubation (Fig. 3) with 15 min or 24 h incubations (not shown).

In contrast to PON1, information on the possible effects of metals on PON2 and/or PON3 is scarce. In HepG2 human hepatoma cells, lead acetate significantly inhibited lactonase activity (dihydrocoumarin hydrolysis) at concentrations as low as 0.13 μM (Sukketsiri et al. 2013). Inhibition appeared to increase with the length of incubation (4–72 h), particularly at the lower concentrations. However, lactonase activity was attributed solely to PON2, while all PONs are expressed in the liver and all have lactonase activity. Levels of PON2 protein were not affected by lead, but PON2 mRNA levels were increased, though not in a concentration-dependent manner. Interestingly, addition of calcium (1 mM) prevented the inhibitory effect of lead on PON2 activity. With regard to PON3, there is only one study by Pla et al. (2007) who purified this enzyme from rat liver. Various metals inhibited PON3 lactonase activity (dihydrocoumarin hydrolysis) including (IC_{50}, μM) mercury (2), copper (36), manganese (318), and cobalt (1898). EDTA also inhibited PON3 activity, confirming that activity of this enzyme, like PON1, is also calcium dependent.

Effects of Metals on PONs: Animal Studies

Very few studies have investigated in experimental animals the effects of in vivo exposure to metals on PONs, and all, to our knowledge, have focused on PON1. Tas et al. (2006) examined the effects of treatment of rats rendered diabetic with streptozotocin with vanadyl sulfate (a vanadium salt) on serum paraoxonase and arylesterase activities. Vanadyl sulfate had no effect on PON1 activity in control rats; however, it was capable of partially reversing the decrease in paraoxonase and arylesterase activities induced by streptozotocin, in virtue, according to the authors, of its antioxidant properties (Tas et al. 2006). This represents the only animal study in which a metal derivative was found to "increase" PON1 activity.

Rats given cadmium chloride in drinking water at the levels of 15 or 100 ppm for 2 months had serum levels of Cd of ~6 and ~15 ppb, respectively, compared to ~0.5 ppb in controls (Ferramola et al. 2012). At the highest dose, Cd caused an increased in oxidative stress in serum and a 40% decrease of serum PON1 paraoxonase activity. In another study, female C57Bl/6 mice were given cadmium chloride in drinking water (5 mg/L) for 1 month (Ramambason et al. 2016), yielding levels of Cd in the liver of 0.33 μg/g (Thijssen et al. 2007). A significant 30% decrease in liver PON1 arylesterase activity was found in Cd-treated animals (Ramambason et al. 2016). In contrast, in a study in which cadmium chloride was given by i.p. injections for 2 weeks at doses of 0.1 to 0.5 mg/kg, no changes in serum PON1 activity were found (Cole et al. 2002). Ebabe Elle et al. (2013) fed rats a standard diet supplemented with 500 mg/kg silver nanoparticles for 81 days. Silver caused oxidative stress and inflammation in the liver and decreased plasma PON1 paraoxonase activity by 15%. In contrast, administration of aluminum by intraperitoneal injections to Wistar rats for 2 weeks did not alter PON1 activity in plasma (Maghraoui et al. 2014).

As Hg was a potent in vitro inhibitor of human and mouse PON1 arylesterase activity (Figs. 1, 2 and 3), we examined the effects of Hg exposure in vivo on plasma and liver PON1 activity. Male C57/B6 mice were exposed by subcutaneous (s.c.) injections of methylmercury hydroxide (10, 20, or 30 μmol/kg/d, equivalent to 2.33, 4.65, and 6.98 mg/kg/d) for 14 days. Unfortunately, Hg tissue levels were not measured in this study, though based on other similar studies they are expected to be in the sub-micromolar to low nanomolar range. Somewhat unexpectedly, neither plasma nor liver PON1 activity (diazoxonase and paraoxonase) was decreased by treatment (not shown). To investigate the potential effect of iron on PON1 activity, we took advantage of an ongoing Fe overload study in female *apoE*$^{-/-}$ mice 6–8 weeks of age. Groups of mice were fed a low-iron (0.02% Fe) or a high-iron (2%) diet for 12 weeks. Serum nonheme iron and liver iron levels were determined together with diazoxonase and paraoxonase activity in plasma. Figure 4a shows that serum Fe levels increased significantly in mice fed the high-iron diet, and liver iron levels increased to a much greater extent. Activity of diazoxonase in plasma was decreased only by a nonsignificant 12%, while plasma paraoxonase activity was decreased by ~20% (Fig. 4b and c). In this case the minimal effect of Fe on plasma PON1 activity in vivo is not surprising, given the limited effectiveness of this metal

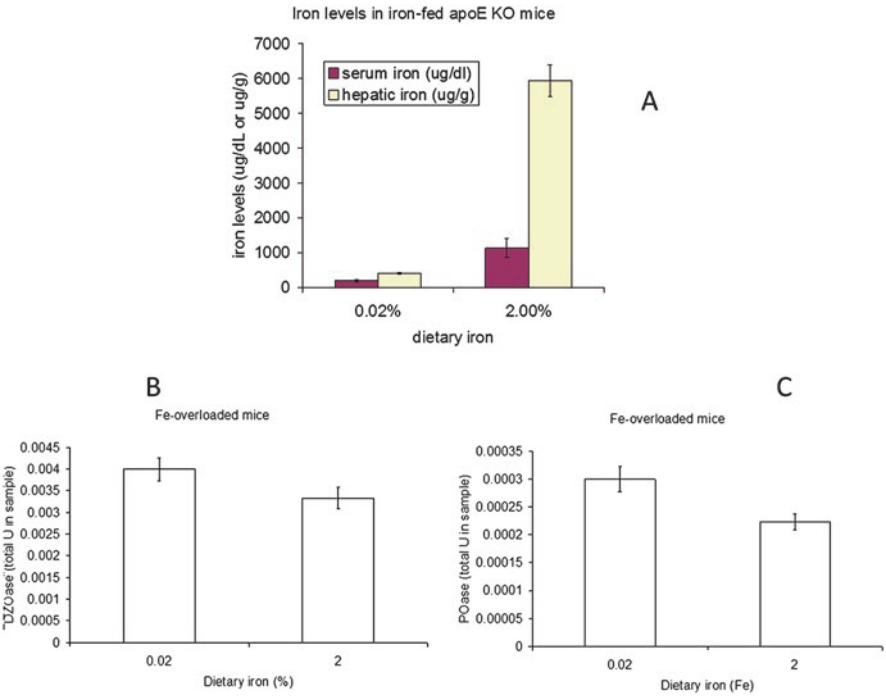

Fig. 4 Effect of dietary iron overload on plasma PON1 activity in apoE knockout mice. (**a**) Levels of Fe in serum and liver. (**b**) Diazoxonase activity in plasma; (**c**) paraoxonase activity in plasma. Results represent the mean (± SE) of four mice

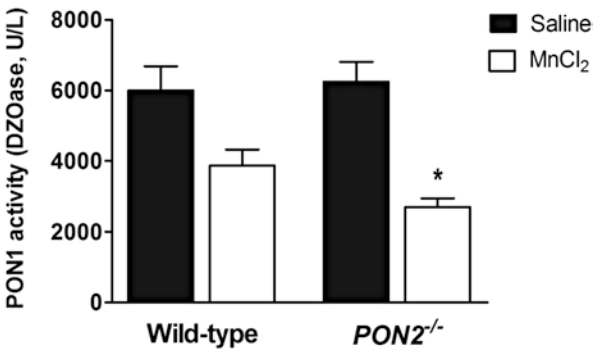

Fig. 5 Effect of manganese chloride on serum PON1 activity (diazoxonase) in male wild-type and PON2$^{-/-}$ mice. Results represent the mean (± SE) of four mice

in inhibiting PON1 in human and mouse plasma (Figs. 2 and 3). In an additional preliminary experiment, male wild-type and PON2 knockout (PON2$^{-/-}$) mice were given three doses of manganese ($MnCl_2$, 100 mg/kg, s.c.) and sacrificed 1 week later. As shown in Fig. 5, Mn decreased serum PON1 activity (measured as

diazoxonase), and its effects were more pronounced in PON2$^{-/-}$ mice, suggesting that PON2 may protect PON1 from oxidative stress related to metal exposure (Marsillach et al., unpublished observations).

Overall, it is evident that animal studies investigating the effects of metals on PONs are limited and almost all focus on PON1, with little attention paid so far to PON2 and PON3. Given the widespread exposure to metals and the relevance of all three PONs in a variety of diseases, further animal studies of the effects of metals on PONs activity and expression are certainly warranted.

Metals and PONs: Human Studies

A number of studies have examined the association between blood metal levels and PON1 activity and/or expression in humans. While the in vitro studies described in section "In vitro effects of metals on PONs" were presented in chronological order of publication, as numerous metals were tested in each study, human studies are described below by grouping them for each metal investigated.

Lead

Li et al. (2006) examined the associations between blood Pb levels and PON1 activity (measured as paraoxonase, arylesterase, and diazoxonase activities) in about 600 workers in Taiwan. Workers were divided into three groups on the basis of blood Pb levels (μg/dL): ≤ 10, 10 ≤ 40, and ≥40. There was a small (10–13%) but significant decrease in PON1 activity with increasing blood Pb concentrations. The three most relevant PON1 polymorphisms (Q192R, L55M, and C-108T) were also determined and found to be similar to those reported for the Chinese population. The strongest inverse association between Pb and PON1 was found in PON1$_{R192}$ homozygotes (RR), while the results in heterozygotes (QR) and QQ homozygotes were not statistically significant (Li et al. 2006). Levels of Pb in blood were in the low micromolar range, a concentration that had been shown in some studies to inhibit PON1 activity in vitro (Erdös et al. 1960; Cole et al. 2002; Figs. 1 and 2). In agreement with these in vivo findings, Pb had been found to be more potent in inhibiting arylesterase activity of the PON1$_{R192}$ genotype in vitro (Figs. 1 and 2).

A study by Pollack et al. (2014) examined the association between blood Pb and PON1 activity in a group of 250 women, and their findings are in agreement with those of Li et al. (2006) in that a decreased PON1 activity was associated with Pb, but only in individuals homozygous for the PON1$_{R192}$ allele. Levels of blood Pb in this cohort were very low, about 1 μg/dL (Pollack et al. 2014). Further support for an in vivo effect of Pb on PON1 activity has been provided by another study which investigated the association between blood Pb and PON1 in a group of 100 workers of a lead battery factory (Kamal et al. 2011). Blood Pb levels in all workers averaged 45.7 μg/dL (vs. 12.5 μg/dL in controls), and PON1 was decreased by an average of

60%. When stratifying workers based on blood Pb levels (< 40, 40–59, ≥ 60 μg/dL), PON1 activity was decreased by 36, 63, and 69%, respectively. These investigators also reported that PON1 activity was lowest in the Pb-exposed workers homozygous for the $PON1_{R192}$ allele, thus substantiating previous findings in humans (Li et al. 2006; Pollack et al. 2014) and in vitro (Figs. 1 and 2). An additional study has reported associations between blood Pb levels and PON1 activity. In a group of Pb-exposed earthenware factory workers in Thailand (n = 65; mean blood Pb level 31.4 μg/dL), PON1 arylesterase activity was decreased by 24% (Permpongpaiboon et al. 2011). However, PON1 paraoxonase activity did not differ between Pb-exposed workers and controls; this may be related to a differential $PON1_{192}$ genotype distribution between Pb-exposed workers and controls, though this was not determined. An increase in blood parameters of oxidative stress was also found in this study, and the authors attributed the decrease in PON1 arylesterase activity to oxidative stress, which is known to negatively affect PON1 (Nguyen and Sok 2003).

Arsenic

A single study examined the influence of arsenic (As) exposure on PON1 activity in 196 residents from an arseniasis-endemic area in Southwestern Taiwan (Li et al. 2009). However, consumption of As-contaminated well water had ceased for several years, and indeed only urinary excretion of inorganic As was higher in the endemic group. Overall, plasma PON1 activity was similar between controls and the endemic group and was actually higher in $PON1_{Q192}$ homozygotes with As exposure compared to controls. However, high As exposure together with low PON1 activity increased the risk for developing atherosclerosis by 5.7-fold (Li et al. 2009). When analyzing *PON1* (Q192R and C-108T) and *PON2* (A148G, C311S) polymorphism distribution in the control and As-exposed populations, some differences were found, whose significance is unclear. Hernandez et al. (2009) also examined the association between urinary levels of As and plasma PON1 activity in a population of healthy individuals (n = 536). They found no associations between As levels and PON1 activity, though carriers of the $PON1_{R192}$ alloform had higher levels of urinary As (Hernandez et al. 2009).

Methylmercury and Selenium

Various human studies have also explored possible association between exposure to methylmercury (MeHg) and PON1 activity. A study in Nunavik, Canada, of 896 Inuit adults found a significant inverse correlation between plasma Hg levels and PON1 activity (Ayotte et al. 2011). With increasing blood Hg levels (from ≤30 to >100 nmol/L; geometric mean = 53.2 nmol/L), PON1 activity decreased to a maximum of 14%; however, there was no association with any specific $PON1_{192}$ genotype. Interestingly, as found in other studies (Cayir et al. 2014; Laird et al. 2015), blood concentrations of selenium (Se) appeared to counteract the effect of Hg on

PON1. Selenium is present in the active site of several enzymes, many of which are involved in modulation of oxidative stress (e.g., thioredoxin reductase, glutathione peroxidase), and this may explain its "protective" effect toward PON1. The study by Ayotte et al. (2011) was utilized by Ginsberg et al. (2014) to mechanistically address the issue of a possible association of MeHg exposure with cardiovascular disease suggested by some, but not all epidemiological studies (Virtanen et al. 2007; Mozaffarian et al. 2011). Ginsberg et al. (2014) calculated that a dose of 0.3 mg/kg/day of MeHg would cause an average 6.1% decrease in PON1 level, and this would increase the risk of cardiovascular disease by 9.7%.

In another Canadian population (Cree people in Eastern James Bay) with lower MeHg exposure (blood Hg geometric mean = 16.7 nmol/L; n = 369), no association between blood Hg levels and PON1 activity was found (Drescher et al. 2014). However, a negative correlation was found between Hg and PON1 in carriers of the (rare) TT allele at position −108 (the low PON1 activity allele). This polymorphism disrupts a recognition site for Sp1, a zinc-finger transcription factor whose DNA-binding activity can be inhibited by Hg ions through interaction with Cys_2His_2 zinc-binding domains (Rodgers et al. 2001; Deakin et al. 2003). People with the -108T allele, who have a compromised interaction between Sp1 and the promoter sequence ($PON1_{-108T}$), may thus be more susceptible to further disruption by Hg (Drescher et al. 2014).

Additional information on potential associations between Hg and PON1 were provided by the studies of Hernandez et al. (2009), Pollack et al. (2014), and Laird et al. (2015). In contrast to most other studies, Hernandez et al. (2009) found a positive association between urinary levels of Hg and PON1 in a population of healthy subjects in Spain, i.e., higher PON1 activity with increasing blood Hg levels. On the other hand, Pollack et al. (2014) found that women exposed to Hg and homozygous for the $PON1_{R192}$ genotype had 23% lower PON1 activity in plasma. Finally, a recent study examined PON1 activity in plasma in relationship to blood levels of Hg, Pb, Cd, and Se in a population of over 2000 Inuit in Canada (Laird et al. 2015). PON1 activity was measured with a commercially available kit which utilizes 7-diethylphospho-6,8-difluor-methylumbelliferyl as a substrate, and no assessment of PON1 genotype was done. As expected, PON1 activity in the population varied by 27-fold. No correlations were found between Pb and PON1, in contrast to previous studies (Li et al. 2006; Hernandez et al. 2009). Somewhat in agreement with the findings of Hernandez et al. (2009), a positive correlation between blood Hg and PON1 activity was found. Blood Se levels were also positively associated with PON1 activity, as also reported by others (Ayotte et al. 2011). Similarly, Cayir et al. (2014) found that in obese children from Turkey (who already have ~40% lower serum PON1 activity than normal weight children) blood Se levels are positively associated with plasma PON1 activity.

Cadmium

Three studies examined the associations between cadmium (Cd) and PON1. In the recent one by Laird et al. (2015), Cd was the only metal negatively associated with PON1 activity after adjustment for a number of co-variables. This result confirmed

previous findings by Hernandez et al. (2009) and Pollack et al. (2014). In the former study, blood Cd was negatively associated with PON1 paraoxonase, arylesterase, and diazoxonase activities (Hernandez et al. 2009), while Pollack et al. (2014) found a similar association but only in $PON1_{R/R192}$ and $PON1_{Q/R192}$ individuals.

Other Metals

Limited or no information exists with regard to other metals. Levels of blood copper were negatively associated with PON1 activity in obese children (Cayir et al. 2014). No association was found between manganese blood levels and PON1 in two studies (Hernandez et al. 2009; Cayir et al. 2014). Finally, with regard to zinc, one study reported no association with PON1 (Cayir et al. 2014), while another found a decreased PON1 activity (Hernandez et al. 2009), in agreement with in vitro results.

Overall, human studies indicate, for the most part, an association between metal exposure and decreased PON1 activity, especially in individuals homozygous for the $PON1_{R192}$ allele. However, most studies are incomplete and lack important information on exposure to metals and to other potential confounding factors (e.g., smoking, alcohol, drugs), duration of exposure, blood levels of metals, PON1 genotype, accurate PON1 activity measurements, and levels of PON1 protein.

Potential Mechanisms of PON1 Modulation by Metals

Metals may reduce PON1 activity and/or expression by generating oxidative stress, by directly interacting with the enzyme, by interfering with its transcription/translation, or by combinations of these mechanisms. Most metals cause oxidative stress (Jaishankar et al. 2014; Matovic et al. 2015; Valko et al. 2016), and PON1 is known to be inactivated by oxidative stress (Nguyen and Sok 2003). The same is true for PON3 (Rosenblat et al. 2003), while PON2 expression is increased by oxidative stress (Aviram and Rosenblat 2004). Certain antioxidants may increase PON1 activity by preventing its oxidative inactivation (Aviram et al. 1999), and this may explain the results observed with selenium. A direct interaction between metals and the PON1 protein is also likely, as suggested by the findings of the in vitro studies detailed above. Some metals (e.g., zinc, nickel) may bind to histidine (His) in positions 115, 134, 155, and 243, which are essential for PON1 activity (Josse et al. 2002). Other metals (e.g., mercury, lead) may bind to free sulfhydryl groups on the enzyme. PON1 has three cysteine (Cys) residues (in positions 42, 284, and 353), with a disulfide bond between Cys-42 and Cys-353, while Cys-284 is a free thiol (Josse et al. 2002). The disulfide-linked Cys-42 and Cys-353 are essential for PON1 esterase activity, while Cys-284 is not; however, Cys-284 is close to the active site for catalytic activity of PON1, and its covalent modification interferes with PON1 activity (Sorenson et al. 1995). Most importantly, the free Cys-284 is essential for PON1 to be protective against LDL oxidation (Aviram et al. 1998; Josse et al. 2002).

However, this Cys is buried, and thus it is unclear if it may represent a target for some metals (Harel et al. 2004; Hernandez et al. 2009; Laird et al. 2015).

Calcium-binding sites on PON1 are also most relevant. The crystal structure for a recombinant PON1 has indicated that PON1 is a six-bladed β-propeller, which in the central tunnel contains two calcium ions, one of which is essential for enzyme activity and the other for stability of the protein (Harel et al. 2004). Removal of calcium ions from PON1 by the chelating agents EDTA or EGTA leads to inhibition of its esterase activity, but not of its ability to protect against LDL oxidation (Kuo and La Du 1998; Aviram et al. 1998). Lead is known to mimic calcium, causing stimulation or inhibition of calcium-dependent enzymes depending on its concentration (Simons 1993). This may explain the observed decrease in PON1 activity attributed to Pb exposure in several studies (Li et al. 2006; Kamal et al. 2011; Pollack et al. 2014). However, alternative mechanisms have also been proposed to explain the effect of Pb on PON1. For example, Pb may interfere with copper utilization, and Cu deficiency has been suggested to decrease PON1 activity (Klevay 2006; Laird et al. 2015). However, this hypothesis appears unlikely, as in vitro studies have consistently shown that copper is a relatively potent inhibitor of PON1 activity (Table 1).

The GGCGGG consensus sequence in the binding site for transcription factor Sp1 in the 5′ regulatory region of PON1 has been shown to be the site of the C-108T mutation which affects PON1 expression (Brophy et al. 2001; Deakin et al. 2003). This site has been shown to be the target for the positive modulation of PON1 by statins and by low alcohol consumption (reviewed in Costa et al. 2005, 2011) and has been suggested as a possible target of mercury (Drescher et al. 2014). As more information on the interactions of metals with PONs emerges, further mechanistic investigations are also warranted.

Summary and Conclusions

Humans are exposed to a several different metals and organometallic compounds in a variety of settings. Occupational exposure to certain metals (e.g., manganese, lead, cadmium, mercury) is quite common. In addition, exposure of large populations to metals through the diet (e.g., methylmercury in fish) or through contaminated drinking water (e.g., arsenic, manganese) also occurs. It is known that metals can exert a number of adverse health effects involving multiple mechanisms, with oxidative stress being a major one (Jaishankar et al. 2014; Matovic et al. 2015; Valko et al. 2016).

The three PONs exert significant roles as antioxidant and anti-inflammatory proteins, and several studies indicate their involvement in a variety of diseases. Evidence summarized in this chapter from available in vitro, animal, and human studies indicates that metals can modulate PON activity and expression, and this in turn may relate to some of their adverse health effects. The majority of studies have focused so far on the most studied of the PONs, PON1; however, as knowledge on the other

PONs increases, so should information on the interactions of metals with PON2 and PON3. Given the important role of PON1 in cardiovascular disease (reviewed in Costa and Furlong 2002; Furlong et al. 2008), effects of metals on this enzyme activity and/or expression should be further investigated, also considering the existence of possible differential effects linked to PON1 polymorphisms. Similar considerations also apply to PON2 and PON3, which also play a role in cardiovascular disease. Two other areas that deserve investigation are related to the role of PONs in modulating bacterial infection or in protecting tumor cells (PON2 and PON3 in particular) and the possibility that metal exposure may alter important homeostatic mechanisms by affecting their activity. Furthermore, in the case of PON2, a negative modulation of this enzyme by metals may affect neuroprotective mechanisms, and in the case of PON3, it may induce obesity and related metabolic syndromes.

In addition to the multiple potential effects of metals on PONs and the possible involvement of these in adverse effects of metals, another toxicological aspect involving the reverse effect should also be considered. The level of expression of PONs, determined by genetic background or by other factors, may be relevant in modulating metal toxicity. For example, results shown in Fig. 5 show that the effects of Mn on PON1 are more pronounced in *PON2*$^{-/-}$ mice, suggesting that levels of PON2 (e.g., in males vs. females) may affect Mn toxicity. Low level of PON2 may increase susceptibility to neurotoxic metals, and low levels of any of the three PONs may increase susceptibility to metal-induced cardiovascular effects and microbial infections.

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