Paraoxonase-2 Deficiency Aggravates Atherosclerosis in Mice Despite Lower Apolipoprotein-B-containing Lipoproteins

ANTI-ATHEROGENIC ROLE FOR PARAOXONASE-2*

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Paraoxonases (PONs) are a family of proteins that may play a significant role in providing relief from both toxic environmental chemicals as well as physiological oxidative stress. Although the physiological roles of the PON family of proteins, PON1, PON2, and PON3, remain unknown, epidemiological, biochemical, and mouse genetic studies of PON1 suggest an anti-atherogenic function for paraoxonases. To determine whether PON2 plays a role in the development of atherosclerosis in vivo, we generated PON2-deficient mice. When challenged with a high fat, high cholesterol diet for 15 weeks, serum levels of high density lipoprotein cholesterol, triglycerides, and glucose were not significantly different between wild-type and PON2-deficient mice. In contrast, serum levels of very low density lipoprotein (VLDL)/low density lipoprotein (LDL) cholesterol were significantly lower (−32%) in PON2-deficient mice compared with wild-type mice. However, despite lower levels of VLDL/LDL cholesterol, mice deficient in PON2 developed significantly larger (2.7-fold) atherosclerotic lesions compared with their wild-type counterparts. Enhanced inflammatory properties of LDL, attenuated anti-atherogenic capacity of high density lipoprotein, and a heightened state of oxidative stress coupled with an exacerbated inflammatory response from PON2-deficient macrophages appear to be the main mechanisms behind the larger atherosclerotic lesions in PON2-deficient mice. These results demonstrate that PON2 plays a protective role in atherosclerosis.

Atherosclerosis is a disease of chronic inflammation and lipid accumulation. Increasing evidence suggests that oxidative stress plays a key role in the pathogenesis of this disease. More specifically, the oxidation of LDL has been shown to trigger a number of pro-inflammatory events that initiate and exacerbate the atherogenic process (1). HDL, on the other hand, normally plays an anti-atherogenic role, and its protective capacities have been ascribed primarily to its ability to remove excess cholesterol from peripheral tissues in the cholesterol transport pathway and to its ability to protect against LDL oxidation (2–4). These protective effects of HDL have been attributed to the various proteins HDL associates with in the circulation. Paraoxonase 1 (PON1) is one such HDL-associated protein that has been reported to possess antioxidant/anti-inflammatory properties and to protect against atherogenesis (5–7).

As suggested by its name, PON1 was initially discovered for its ability to hydrolyze paraoxon, the metabolite of the organophosphate parathion. Increasing evidence, however, suggests that PON1 may have other functions, including a role in coronary heart disease (CHD). In vitro, PON1 has been shown to protect against LDL oxidation, an important step in atherogenesis. Mice deficient in PON1 develop significantly larger atherosclerotic lesions in their aortas compared with their wild-type counterparts (5,6), and mice overexpressing PON1 exhibit atheroprotective properties, developing significantly lower levels of aortic lesions and decreased levels of inflammatory chemokines like monocyte chemotactic protein-1 relative to their control counterparts (7). In humans, PON1 activity has been shown to be an independent risk factor for CHD (8).

PON1, however, is just one member of a multigene family that includes PON2 and PON3 (9), both of which surprisingly lack paraoxonase activity. PON1, PON2, and PON3 do, however, possess the ability to hydrolyze lactones with the three PON proteins exhibiting overlapping but distinct substrate specificities (10). Recent studies have shown that the PON proteins can hydrolyze a number of acylhomoserine lactones (AHLS), molecules that mediate bacterial quorum-sensing signals, which are important in regulating expression of virulence factors and in inducing a host inflammatory response (10–12). Although all three PON proteins possess the ability to hydrolyze AHLS, PON2 exhibits the highest activity (10,12). Another distinguishing feature between the members of this gene family is that PON2, unlike PON1 or PON3, does not associate with HDL fractions in the circulation but remains...
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intracellular, associated with the membrane fractions (13). Furthermore, whereas human PON1 and PON3 expression is limited primarily to the liver, PON2 is widely expressed in a number of tissues and cell types, including the cells of the artery wall (9, 13). These unique properties of PON2 make it an interesting candidate to study the physiological or pathophysiological function of paraoxonases.

The results of population studies on PON2 genetic polymorphisms implicate PON2 in a number of diseases, including Alzheimer disease, type 2 diabetes mellitus, and CHD (14–18). However, to date, little is known about the functional role of PON2 in the pathogenesis of these diseases. In vitro studies suggest that PON2 may have an anti-atherogenic function (10, 16). Using cells that overexpress PON2 protein, we have previously demonstrated that PON2 possesses antioxidant and anti-inflammatory capacities, capable of preventing LDL oxidation and the ability of oxidized LDL to induce monocyte chemotaxis (13). Using purified recombinant PON2 protein, Rosenblat et al. (19) have shown that PON2 can inhibit the oxidative modification of LDL and reduce cellular lipid hydroperoxides. The purpose of the present study was to determine whether PON2 could protect against the development of atherosclerosis in vivo.

Using PON2-deficient mice, we demonstrate, for the first time, that PON2 protects against atherogenesis in vivo by modulating lipoprotein properties, reducing cellular oxidative stress, and attenuating the inflammatory response. Furthermore, the PON2-deficient mouse is one of a few unique animal models described to date in which levels of VLDL/LDL cholesterol correlate inversely with atherosclerotic lesion development, making this a unique animal model to delineate the mechanisms of atherogenesis.

EXPERIMENTAL PROCEDURES

Generation of PON2-deficient Mice—A mouse embryonic stem cell line (cell line XE661, strain 129/Ola) containing an insertional mutation in PON2 was identified using BayGenomics, a gene-trapping resource (20). The gene-trap vector used (pGT1Lxf) contained a splice-acceptor sequence upstream of a reporter gene, βgeo (a fusion of β-galactosidase and neomycin phosphotransferase II) (20). As determined by 5′ rapid amplification of cDNA ends (21), the insertional mutation in XE661 phosphotransferase II) (20). As determined by 5′ rapid amplification of cDNA ends (21), the insertional mutation in XE661 was identified using BayGenom

AHL Inactivation Bioassay—Reactions were carried out at room temperature in a 50-μl volume of 25 mM Tris-HCl, pH 7.4, 1 mM CaCl2 containing 10 μg of crude membrane extracts (10) prepared from mice and 0.5 mM 3-oxo-C12-homoserine lactone (3OC12-HSL). Reactions were stopped with an equal

Northern and Quantitative Real-time PCR—Total RNA was isolated from mouse tissues using TRizol reagent (Invitrogen) according to the manufacturer’s instructions, and mRNA was prepared using a PolyATtract mRNA isolation system (Promega, Madison, WI). Northern blot analyses were performed using 2 μg of mRNA per sample. A 32P-labeled 650-bp DNA fragment from exons 4 to 9 of the mouse PON2 cDNA was used as the probe. For real-time quantitative RT-PCR, 1 μg of mRNA from each sample was reverse-transcribed into first strand cDNA using the ThermoScript RT-PCR system (Invitrogen) according to the manufacturer’s protocol. Real-time RT-PCR was then performed using the QuantiTect SYBR Green PCR kit (Qiagen) in an ABI Prism 7700 cycler. Serial dilutions of cloned cDNA plasmids were used as standards. Both samples and standards were assayed in duplicate. The primer pairs used for real-time RT-PCR were: mPON1–350U (AAC AAG AAG GAG CCA GCA GTG TCA GA) and mPON1–474L (AGT GGA CGA GGA GTC TGG ATG GTT TA), mouse PON2–390U (GAG TCA GCT GGG GCT TTG ATC TGG CT) and mouse PON2–607L (ATT GGT GCC GTA GAA GTG GGT GGG C), mPON3–265U (GAT CTG AAT GAG CAA AAC CCA GAG GC) and mPON3–386L (GAG TCC ATG TTG GGG TGA TTC ACG AC), and mouse GAPDH-103U (TGC CAT TTG CAG TGG CAA AGT GG) and mouse GAPDH-517L (TTG TCA TGG ATG ACC TTG GCC AGG). The PCR conditions were 95 °C for 15 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 1 min, and 4 °C hold.

Antibodies and Western Blots—Mouse PON2 antibody was generated as previously described for human PON2 antibody (13), except mouse PON2-specific peptide CDERPPSLEEL-RVSWGFDL was used as the antigen. Rabbit anti-mouse PON2 antiserum was affinity-purified using purified recombinant mouse PON2 protein (generously provided by D. Draganov) immobilized onto nitrocellulose membrane. Mouse apolipoprotein-B antibody was purchased from Biodesign International. Mouse PON2 and apolipoprotein-B-100 (apoB) proteins were measured by Western blot analysis. Briefly, crude membrane extracts (10) prepared from livers (15, 30, and 60 μg) were prepared for 10 μg from wild-type and PON2-deficient mice were fractionated by SDS-PAGE, transferred onto nitrocellulose membranes, and blocked in 0.1% Tween-TBS (T-TBS) plus 5% nonfat dried milk for 1 h at room temperature. Mouse PON2 antibody was used at 1:500, whereas mouse apoB antibody was used at 1:2000. Primary antibodies were diluted in 1% bovine serum albumin plus T-TBS and incubated overnight at 4 °C. Anti-rabbit horseradish peroxidase-coupled secondary antibody was used at 1:5000 in 1% bovine serum albumin plus T-TBS for both PON2 and apoB immunoblots and incubated for 1 h at room temperature. Proteins were illuminated using ECL plus (GE Healthcare-Amersham Biosciences) and quantitated with ImageQuant software (Molecular Dynamics).

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volume of acetonitrile, and 0.01 ml of a 1:100 dilution was used to measure 3OC12-HSL by quantitative bioassay using Escherichia coli MG4 (pKDT17) as described by Pearson et al. (22). 3OC12-HSL and E. coli MG4 (pKDT17) were kindly provided by K. Janda (The Scripps Research Institute, San Diego, CA) and E. Greenberg (University of Iowa), respectively.

**Diet-induced Atherogenesis**—At 8 weeks of age, female PON2-deficient mice and their wild-type and heterozygous littermates were switched from a chow diet to an atherogenic diet consisting of 15.8% fat, 1.25% cholesterol, and 0.5% cholic acid (TD 90221, Harlan Teklad, Madison, WI). Fifteen weeks later, mice were sacrificed, and the blood and heart were collected for analysis. This protocol was approved by the Animal Research Committee at UCLA.

**Serum Lipid Analysis and PON1 Activity**—Serum was isolated from overnight-fasted mice. Total cholesterol, HDL cholesterol, triglycerides, and glucose were measured with enzymatic kits. VLDL/LDL cholesterol was deduced by subtracting HDL cholesterol from total cholesterol. Serum PON1 activity was measured spectrophotometrically using paraoxon as the substrate.

**VLDL Production**—Fasted mice were injected intraperitoneally with 1 g/kg Poloxamer 407 (P-407) (23) (graciously provided by BASF) in saline. Mice were bled at 0, 1, 3, and 6 h after injection, serum was isolated, and triglyceride concentrations were determined. VLDL production rate was calculated by measuring the change in triglyceride levels following P-407 injection.

**LDL Clearance**—Mice were injected intravenously with 1 mg of human LDL in phosphate-buffered saline and bled 15 min and 2, 6, and 24 h post-injection. Human apoB levels in the serum of these mice were measured by enzyme-linked immunosorbent assay as previously described (24) using MB47, a monoclonal antibody specific to human apoB-100 (25) as the capture antibody and biotin-labeled goat anti-human apoB-100 (Biodesign) as the detection antibody. Serum samples were diluted 1:500. To control for any differences in the amount of LDL injected, the concentration of human apoB detected at 15 min post-injection was used as the starting point.

**Aortic Lesion Analysis**—Atheroma formation in the aortic sinus of mice was analyzed as previously described (6). Briefly, the heart, including the proximal aorta, was isolated and embedded in OCT compound. Serial 10-μm thick cryosections of aorta were stained with Oil Red O and hematoxylin. The mean area of lipid staining per section from 10 sections was determined for each mouse. Image-Pro Plus software (Media Cybernetics) was used for quantitation. Aortic sections were blocked in 4% bovine serum albumin plus 10% Ringer buffer and treated with either 10 ng/ml of LPS (Sigma), 300 μM H2O2 (Sigma), or 1 μg/ml HPODE (13S-hydroperoxy-9Z,11E-octadecadienoic acid, Biomol) in Krebs-Ringer buffer. Fluorescence was measured at the indicated times using a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Devices) with an excitation filter of 485 nm and an emission filter of 530 nm.

**LPS-induced Inflammatory Response**—Peritoneal macrophages from wild-type or PON2-deficient mice were cultured onto 6-well plates (5 × 10⁶ cells/well) and treated with 10 ng/ml LPS in Dulbecco’s modified Eagle’s medium containing 1% lipoprotein-deficient serum. Eighteen hours later, LDL supernatants were collected, and lipid hydroperoxides were quantitated as described above.

**Oxidation of LDL**—Peritoneal macrophages from wild-type or PON2-deficient mice were cultured onto 96-well plates (5 × 10⁵ cells/well) and loaded with 100 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF) assay was used to quantify intracellular oxidative stress as previously described (13). Briefly, peritoneal macrophages from wild-type or PON2-deficient mice were cultured onto 96-well plates (5 × 10⁵ cells/well) and incubated with 250 μg of LDL in Dulbecco’s modified Eagle’s medium containing 1% lipoprotein-deficient serum. Eighteen hours later, LDL supernatants were collected, and lipid hydroperoxides were quantitated as described above.

**Intracellular Oxidative Stress**—A 2′,7′-dichlorodihydrofluorescein diacetate (DCF) assay was used to quantify intracellular oxidative stress as previously described (13). Briefly, peritoneal macrophages from wild-type or PON2-deficient mice were cultured onto 96-well plates (5 × 10⁵ cells/well) and loaded with 100 μM 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen-Molecular Probes) for 1 h at 37 °C. Cells were then washed with Krebs-Ringer buffer and treated with either 10 ng/ml of LPS (Sigma), 300 μM H2O2 (Sigma), or 1 μg/ml HPODE (13S-hydroperoxy-9Z,11E-octadecadienoic acid, Biomol) in Krebs-Ringer buffer. Fluorescence was measured at the indicated times using a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Devices) with an excitation filter of 485 nm and an emission filter of 530 nm.

**RESULTS**

**PON2-deficient Mice**—PON2-deficient mice were generated using a mouse embryonic stem cell line XE661 (strain 129/Ola) obtained through BayGenomics, a gene-trap resource from...
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A.

NHLBI, National Institutes of Health (20). The integration site of the gene-trap vector was determined by 5′ rapid amplification of cDNA ends and long-distance PCR to be located in intron 2 of the mouse PON2 gene (Fig. 1A). This insertional mutation will thus result in a fusion transcript consisting of exon 1–2 sequences from PON2 and βgeo (Fig. 1A). Following successful germ line transmission, PON2-deficient mice were backcrossed for five generations to the C57Bl/6J background before initiating phenotype analyses.

Previous studies utilizing mutant mice derived from gene-trap methodology noted that the gene-trap approach may in some cases result in a slight leakiness of the targeted gene (28–30). To determine the expression status of the trapped PON2 gene, mRNA was isolated from various tissues of PON2 wild-type (WT) and PON2 homozygous gene-trap (HM) mice and subjected to Northern analysis for PON2 mRNA. Using 2 μg of poly(A)-mRNA (equivalent to 200 μg of total RNA) we were unable to detect PON2 message by Northern blot analysis in various tissues of PON2 HM mice (Fig. 1B). In addition, PON2 protein was virtually undetectable in the livers of PON2 HM mice (Fig. 1C), and lactonase activity was significantly lower in PON2 HM mice as measured by hydrolysis of 3-oxo-C12-homoserine lactone (3OC12-HSL) (Fig. 1D). However, using real-time quantitative RT-PCR, low levels of PON2 mRNA were detected in tissues of PON2 HM mice. The relative PON2 message levels in the brain, liver, lung, and stomach of PON2 HM mice as compared with those of the WT mice were 8%, 8%, 5%, and 12%, respectively. These results indicate that low levels of PON2 message and protein (<10% in most tissues) may be present in PON2 HM mice and will thus be treated as PON2-deficient mice.

**Lipid Parameters**—To assess the protective capacity of PON2 in atherogenesis, PON2-deficient mice and their wild-type counterparts were placed on a high fat, high cholesterol, cholate-containing diet to induce atherosclerosis. After 15 weeks on the diet, serum lipid levels of wild-type and PON2-deficient mice were measured. Although there was no significant difference in fasting levels of HDL cholesterol, triglycerides, free-fatty acids, glucose, and weight between wild-type and PON2-deficient mice,
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Mice deficient in PON2 exhibited significantly lower levels of total (−24%) and VLDL/LDL (−32%) cholesterol compared with their wild-type counterparts (Table 1). In addition, serum apoB levels were also lower in PON2-deficient mice compared with controls (73 ± 27 versus 183 ± 32, units of apoB protein, p < 0.05) (Fig. 2A). Although the concentration of apoB-containing cholesterol particles was lower in PON2-deficient mice, there was no significant difference in lipoprotein particle size between wild-type and PON2-deficient mice (Fig. 2B).

To explore the possible mechanisms behind lower levels of VLDL/LDL cholesterol, we investigated the rate of VLDL production and LDL clearance in these mice. To determine the rate of VLDL production, fasted wild-type and PON2-deficient mice were injected with 1 g/kg of the nonionic detergent, P-407, previously shown to inhibit VLDL clearance via inhibition of lipoprotein lipase (23). The rate of VLDL production, as calculated by measuring the increase in triglycerides over time, was significantly lower in PON2-deficient mice relative to wild-type controls (Fig. 2C). In contrast, there was no significant difference in LDL clearance between PON2-deficient mice and their control counterparts (Fig. 2D).

To investigate the potential mechanism behind attenuated VLDL production, we took a closer look at the regulation of hepatic apoB. Oxidative stress has been reported to increase apoB degradation via stimulation of the PERPP pathway (31). Because PON2 is expressed in the liver and has also been shown to possess antioxidant capacities, we hypothesized that the lower levels of apoB in the serum of PON2-deficient mice may be due to an increase in apoB degradation via up-regulation of the PERPP pathway by oxidative stress. Indeed, the livers of PON2-deficient mice exhibited higher levels of oxidative stress, containing significantly more lipid hydroperoxides relative to their control counterparts (137 ± 17 versus 107 ± 16 ng of lipid hydroperoxides, p < 0.05) (Fig. 2E).

Aortic Lesions—High circulating levels of apoB-containing cholesterol particles are an established risk factor for coronary heart disease. After 15 weeks on an atherogenic diet, PON2-deficient mice exhibited significantly lower levels of VLDL/LDL cholesterol. However, lower levels of VLDL/LDL cholesterol did not translate into lower levels of atheromatous lesions in the aortas of these mice. In fact, mice deficient in PON2 developed significantly larger lesions over time, was significantly lower in PON2-deficient mice relative to wild-type controls (Fig. 2C). In contrast, there was no significant difference in LDL clearance between PON2-deficient mice and their control counterparts (Fig. 2D).

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Lipoproteins—Increasing evidence suggests that it is not just the quality but also the quantity of lipoproteins that is important in determining the risk of atherosclerosis (32, 33). To determine whether PON2 protects against atherosclerosis by modulating the properties of circulating lipoproteins, LDL oxidizability and the capacity of HDL to protect against LDL oxidation were assessed. LDL isolated from PON2-deficient mice...
was more susceptible to oxidation, inducing significantly more monocyte chemotaxis relative to LDL from their wild-type counterparts (35.3 ± 8 versus 18.5 ± 7 monocytes, p < 0.05) (Fig. 4A, left panel). Moreover, HDL isolated from PON2-deficient mice was significantly more inflammatory and less able to protect against LDL-induced monocyte chemotactic activity compared with HDL from their wild-type littermates (22.2 ± 2 versus 13.5 ± 3 monocytes, p < 0.05) (Fig. 4A, right panel). On the atherogenic diet, serum PON1 activity was ~26% lower in PON2-deficient mice compared with wild-type mice (Table 1). When compared with PON1 activity in wild-type mice on a chow diet, PON1 activity in wild-type and PON2-deficient mice on the atherogenic diet was 32.6% ± 1.8% and 23.7% ± 1.4%, respectively (Fig. 4B). To determine whether the differences in PON1 activity account for the reduced protective capacity of HDL from PON2-deficient mice, HDL samples from wild-type (~100% PON1 activity), heterozygous (~50% PON1 activity), and homozygous (~0% PON1 activity) mice were tested along with HDL samples from PON2 wild-type and -deficient mice for their ability to protect against LDL-induced monocyte chemotactic activity (Fig. 4C). The protective capacity of HDL from PON2-deficient mice (~24% PON1 activity compared with wild-type HDL) was similar to HDL from PON1 null mice (0% PON1 activity), suggesting that other factors, in addition to PON1 activity, may also be responsible for the decreased protective capacity of HDL in PON2-deficient mice. The ability of PON2 to alter the properties of LDL and HDL may be mediated through the interactions these lipoproteins have with a number of cell types, including macrophages. Indeed, LDL incubated with peritoneal macrophages deficient in PON2 developed significantly higher levels of lipid hydroperoxides than LDL incubated with macrophages from wild-type mice (1292 ± 35 versus 902 ± 64 ng of lipid hydroperoxides, p < 0.05) (Fig. 4D).

Macrophages—PON2 and to a lesser extent, PON3, have been previously reported to be expressed in mouse macrophages (19). In the PON2-deficient mouse, mRNA levels of PON2 were ~11% of wild-type controls (12 ± 3 versus 106 ± 4 units of PON2 mRNA, p < 0.05), whereas mRNA levels of PON3 were not significantly different (15 ± 0.4 versus 20 ± 4 units of PON3 mRNA, p = 0.35). As previously reported, PON1 is undetectable in mouse macrophages (19).

In an in vitro assay, LDL from PON2-deficient mice induced significantly more monocyte chemo-
taxis than LDL from their control littermates. To determine whether this translated into enhanced mac-
phage trafficking into the artery wall in vivo, aortic sections from wild-type and PON2-deficient mice were stained for macrophages using CD68 as the marker. Indeed, there were higher levels of macrophage immunoreactivity in the aortic sections of PON2-deficient mice (Fig. 5, C and D) compared with their wild-type controls (Fig. 5, A and B). Not only were there more macrophages in the artery wall of PON2-deficient mice, macrophages deficient in PON2 exhibited higher levels of oxidative stress and enhanced pro-inflammatory properties. Peritoneal macrophages isolated from PON2-deficient mice exhibited higher levels of oxidative stress when treated with oxidative stress inducing agents like hydrogen peroxide, HPODE, and LPS, compared with their control counterparts (Fig. 6, A–C). Moreover, when treated with LPS, PON2-deficient macrophages exhibited an exacerbated inflammatory response, inducing higher levels of the pro-inflammatory cytokines TNF-α and IL-1β than their wild-type counterparts (Fig. 7, A and B).

**DISCUSSION**

Although the physiological functions of the PON proteins are unknown, mounting evidence suggests that these proteins may all play an anti-atherogenic role. PON1 is by far the most studied member of this gene family. In vitro, PON1 has been shown to protect against copper-induced LDL oxidation (34). Supernatants from cells overexpressing PON1 were able to protect against LDL-induced monocyte chemotaxis (35). PON1 KO mice have increased aortic lesions (~70% more than the control group), whereas transgenic mice overexpressing PON1 are less susceptible to lesion formation (5, 7). In the present study, whereas PON1 expression in the livers of PON2-deficient mice was not significantly different (data not shown), serum PON1 activity was ~26% lower in PON2-deficient mice relative to their wild-type counterparts. It is possible that the phenotype in the PON2-deficient mice might be resulting from a combination of reduced PON1 and PON2 activities (e.g. pro-inflammatory PON1-deficient HDL interacting with PON2-deficient macrophages). However, this difference in PON1 activity alone cannot explain the larger atheromatous lesions exhibited by PON2-deficient mice. PON1-heterozygous KO mice and PON1-homozygous KO mice had 50 and 0%, respectively, of the PON1 activity seen in their wild-type littermates (5). PON1 homozygous KO mice developed lesions that were ~70% larger than their wild-type littermates, whereas there was no significant difference in lesion formation between PON1 heterozygous KO mice and their wild-type counterparts (5, 38). In other words, a 50% reduction in PON1 activity did not result in larger aortic lesions. In the current study, PON1 activity was ~26% lower in PON2-deficient mice, but these mice developed lesions that were significantly larger (~270%) than their wild-type littermates. The lower levels of PON1 activity exhibited by PON2-deficient mice were most likely a result of higher levels of oxidative stress in these mice. Reports of PON1 activity being suppressed by oxidative stress and preserved by antioxidants support this claim (39). Moreover, Forte and colleagues (40) have also demonstrated that anti-oxidant enzymes associated with HDL, including PON1, are inhibited by oxidized phospholipids and lipid hydroperoxides.

The results of large epidemiological studies and clinical trials using statins have left little doubt as to the importance of LDL cholesterol in CHD. A number of studies have demonstrated that lowering LDL cholesterol levels significantly decreases one’s risk of developing CHD (1, 41). In the present study, after 15 weeks on an atherogenic diet, PON2-deficient mice exhib-
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FIGURE 6. PON2-deficient macrophages have higher levels of oxidative stress. Peritoneal macrophages isolated from PON2 wild-type (WT) or PON2-deficient mice (HM) were treated with either 300 μM H2O2 (A), 1 μg/ml HPODE (B), or 10 ng/ml LPS (C). Oxidative stress was measured at the indicated times by quantifying the fluorescence emitted when the non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate is oxidized by reactive species to the highly fluorescent DCF.

Aortic sections from PON2-deficient mice exhibited higher levels of macrophage chemotaxis relative to their wild-type littermates. However, despite significantly lower levels of VLDL/LDL cholesterol, PON2-deficient mice developed significantly larger atheromatous lesions (~270%) relative to their wild-type counterparts (Fig. 3). Although there was significantly less VLDL/LDL cholesterol in PON2-deficient mice, cholesterol particles from these mice were significantly more inflammatory than VLDL/LDL cholesterol isolated from their wild-type counterparts. When tested in a tissue culture model of the artery wall, LDLs isolated from PON2-deficient mice were more inflammatory, inducing significantly more monocyte chemotaxis than LDL isolated from their wild-type littermates, whereas HDLs from PON2-deficient mice were less able to protect against LDL-induced monocyte chemotaxis relative to their control counterparts. This decrease in HDL protection may have been in part due to lower PON1 activity in PON2-deficient mice, because HDLs from PON1 KO and PON1 heterozygotes were less able to protect against LDL-induced monocyte migration compared with HDLs from wild-type mice (Fig. 4C) (5). PON2, however, has been shown to modulate lipoprotein properties in the absence of PON1. We have previously reported that LDL incubated with HeLa cells overexpressing PON2 contained significantly less lipid hydroperoxides (13). In the current study, LDL incubated with macrophages deficient in PON2 had significantly more lipid hydroperoxides compared with control macrophages.

Aortic sections from PON2-deficient mice exhibited higher levels of macrophage immunoreactivity compared with aortic sections from their wild-type counterparts (Fig. 5). In addition, PON2-deficient macrophages exhibited higher levels of intracellular oxidative stress and exhibited an exacerbated inflammatory response (Figs. 6 and 7). Taken together, these observations may help explain why PON2-deficient mice developed significantly larger atheromatous lesions despite having lower levels of VLDL/LDL cholesterol. PON2 deficiency may be creating a milieu of enhanced oxidative stress and inflammation,
which can accelerate the atherogenic process, overriding any benefit that may come with lower LDL cholesterol levels. The results of this study underscore the importance of assessing not only the quantity but also the quality of cholesterol carrying lipoproteins.

VLDL/LDL and apoB levels were significantly lower in PON2-deficient mice compared with wild-type controls (Table 1 and Fig. 2A). The results of the current study suggest that this difference in VLDL/LDL cholesterol may be due to differences in the production of VLDL (Fig. 2C) rather than the clearance of these lipoprotein particles (Fig. 2D). To explore the possible mechanisms behind lower VLDL production, we took a closer look at apoB. ApoB secretion is regulated primarily at the co- and post-translational levels via the ER-associated degradation and the re-uptake pathways. More recently, Fisher and colleagues (42) have reported that apoB secretion may be regulated by a third pathway: the post-ER pre-secretory proteolysis or PERPP pathway. In addition, these same authors have reported that the PERPP pathway is stimulated by lipid peroxidation and oxidant stress, i.e. oxidant stress can increase apoB degradation via stimulation of the PERPP pathway (31). Because PON2 is expressed in the liver and has also been shown to possess antioxidant capacities, we hypothesized that the lower levels of apoB in PON2-deficient mice were due to an increase in apoB degradation via up-regulation of the PERPP pathway by oxidant stress. To test this hypothesis, we measured lipid hydroperoxides from the livers of wild-type and PON2-deficient mice. Indeed, the livers of PON2-deficient mice exhibited higher levels of oxidative stress, containing significantly more lipid hydroperoxides than their control counterparts (Fig. 2E). These results suggest that the lower levels of VLDL/LDL cholesterol in PON2-deficient mice may be an indirect result of the heightened state of oxidative stress seen in these mice. We cannot, however, rule out the possibility that other pathways may also be playing a role in the regulation of apoB and/or VLDL/LDL cholesterol levels in PON2-deficient mice.

In the context of these results, it is important to compare the PON1 KO mice, which also develop larger aortic lesions on an atherogenic diet but without differences in VLDL/LDL levels. Similar to PON2, PON1 is expressed in the liver and has been shown to possess antioxidant properties. If enhanced oxidative stress in the liver is responsible for the increase in apoB degradation and thus a reduction in VLDL, then why was there no difference in VLDL/LDL levels in PON1 KO mice compared with their wild-type controls? Although all three PON proteins have been reported to possess antioxidant capacities, studies suggest that their potency, in terms of protecting against LDL oxidation, may differ. Draganov et al. (43) have reported that rabbit PON3 is a 100 times more potent than rabbit PON1 in protecting against LDL oxidation, and data from a study by Rosenblat et al. (19) suggests that human PON2 and rabbit PON3 attenuate lipid peroxidation equally well. These studies suggest that PON2 possesses more potent antioxidant capacities than PON1. Therefore, oxidant stress may be higher in the PON2-deficient mouse than the PON1 KO mouse relative to their respective controls. In other words, the level of oxidative stress in the livers of PON1 KO mice may not be enough to induce an increase in apoB degradation via the PERPP pathway. In addition, although both PON1 and PON2 have been shown to be expressed in the liver, the two PON proteins may differ in the type of liver cell in which they are expressed and in their cellular localization. The fact that PON1 is secreted while PON2 remains intracellular suggests they may function at different levels, with PON1 acting in the circulation, leaving PON2 to function at the cellular level. These differences in antioxidant capacity and localization at both the cellular and tissue level may explain the differences in hepatic regulation of VLDL between PON1 KO and PON2-deficient mice.

The purpose of this study was to assess in vivo the role of PON2 in atherosclerosis. Using PON2-deficient mice, we demonstrate, for the first time, that PON2 effectively protects against atherogenesis. The primary mechanism behind the anti-atherogenic effects of PON2 includes its ability to protect against the oxidation of lipoprotein particles, to reduce intracellular oxidative stress, and to attenuate the inflammatory response. The antioxidant and anti-inflammatory properties of PON2, along with its intracellular localization, ubiquitous expression, and up-regulated expression by oxidative stress, suggest an important physiological role for PON2 in host defense. In fact, recent studies have shown that the paraoxonases may be involved in the inactivation of AHLs, bacterial quorum sensing molecules that are not only important in activating virulence factors but also in inducing a host inflammatory response. This is supported in the present study, where tissue extracts from PON2-deficient mice ineffective hydrolyzed 3OC12-HSL. We therefore propose that PON2 protects the host from excessive oxidative stress and inflammatory insults, ultimately protecting the host from diseases such as atherosclerosis.

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