Combined Serum Paraoxonase Knockout/Apolipoprotein E Knockout Mice Exhibit Increased Lipoprotein Oxidation and Atherosclerosis*

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Serum paraoxonase (PON1), present on high density lipoprotein, may inhibit low density lipoprotein (LDL) oxidation and protect against atherosclerosis. We generated combined PON1 knockout (KO)/apolipoprotein E (apoE) KO and apoE KO control mice to compare atherogenesis and lipoprotein oxidation. Early lesions were examined in 3-month-old mice fed a chow diet, and advanced lesions were examined in 6-month-old mice fed a high fat diet. In both cases, the PON1 KO/apoE KO mice exhibited significantly more atherogenesis (50–71% increase) than controls. We examined LDL oxidation and clearance in vivo by injecting human LDL into the mice and following its turnover. LDL clearance was faster in the double KO mice as compared with controls. There was a greater rate of accumulation of oxidized phospholipid epitopes and a greater accumulation of LDL-immunoglobulin complexes in the double KO mice than in controls. Furthermore, the amounts of three bioactive oxidized phospholipids were elevated in the endogenous intermediate density lipoprotein/LDL of double KO mice as compared with controls. Finally, the expression of heme oxygenase-1, peroxisome proliferator-activated receptor γ, and oxidized LDL receptors were elevated in the livers of double KO mice as compared with the controls. These data demonstrate that PON1 deficiency promotes LDL oxidation and atherogenesis in apoE KO mice.

Low density lipoprotein (LDL) oxidation has been proposed
to play a key role in initiating atherosclerosis (1–3). In the artery wall, LDL is believed to undergo oxidative modification via the actions of enzymes such as lipoxygenases (4, 5) and myeloperoxidase (6). In cell culture, minimally oxidized LDL stimulates the expression of monocyte chemoattractant protein-1 (MCP-1) (7) and macrophage colony-stimulating factor (M-CSF) (8), and increases monocyte binding to endothelial cells (9), all of which promote the entry of monocytes into the subendothelial space and differentiation into macrophages. Macrophages express high levels of scavenger receptors, such as scavenger receptor type A (SRA) (10), CD36 (11), and macrophilin (12, 13) that take up oxidized LDL (ox-LDL) but not native LDL and eventually become lipid-laden foam cells, the main constituents of the fatty streak. Recent studies using genetically modified or naturally occurring mutant mouse models have confirmed the roles of many genes involved in the process of LDL oxidation, inflammation, macrophage function, and atherogenesis. For instance, MCP-1 deficiency (14) or deficiency of an MCP-1 receptor, CCR2 (15), leads to marked decreases in atherosclerotic lesion sizes in mice. The naturally occurring osteopetrotic (op) mice that lack M-CSF exhibit much less atherosclerosis lesion formation (16, 17), and mice heterozygous for the op mutation have reduced lesion sizes as well (17, 18). The SRA type I and type II gene-targeted mice also develop smaller atherosclerotic lesions than their wild-type control mice (19). The 12/15-lipoxygenase-deficient mice on the apoE-deficient mouse background exhibit reduced levels of autoantibodies against ox-LDL and diminished atherosclerotic lesion sizes as compared with wild-type mice, consistent with an important role of 12/15-lipoxygenase in LDL oxidation and atherogenesis (20).

There is increasing evidence that HDL exerts its antiatherogenic effects in part by preventing LDL oxidation (21–23). There are at least two enzymes on HDL, PON1 (24, 25) and platelet-activating factor acetylhydrolase (26), that have been shown to prevent the formation of ox-LDL in vitro. PON1 is a 45-kDa protein associated with HDL (27, 28). It was first iden-

SRA, scavenger receptor type A; SR-BI, scavenger receptor BI; PCR, polymerase chain reaction; bp, base pair; DMPC, dimyrystylophosphatidylcholine; LPC, lysophosphatidylcholine; POVPc, 1-palmityl-2-(5) oxovaleroyl-sn-glycero-3-phosphorylcholine; PGPC, 1-palmityl-2-glutaroyl-sn-glycero-3-phosphorylcholine; EUPc, 1-palmityl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; FPLC, fast protein liquid chromatography.

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1 The abbreviations used are: LDL, low density lipoprotein; ox-LDL, oxidized low density lipoprotein; MDA-LDL, malondialdehyde-modified low density lipoprotein; VLDL, very low density lipoprotein; LDL, intermediate density lipoprotein; HDL, high density lipoprotein; PON1, serum paraoxonase; KO, knockout; apoE, apolipoprotein E; apoB, apolipoprotein B; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; op, osteopetrotic; HO-1, heme oxygenase-1; PPARγ, peroxisome proliferator-activated receptor γ;
titified by its ability to hydrolyze and detoxify organophosphate insecticides (29–31), and PON1 may play an important role in organophosphate detoxification in vivo (32, 33). In recent years, PON1 has also been shown to inhibit LDL oxidation in vitro (24, 25). Among the oxidized lipids that PON1 can destroy are 1-palmitoyl-2-(5)-oxovaleryl-sn-glycero-3-phosphorylcholine (POPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC) (34), 1-palmitoyl-2-(5,6)-epoxyisoprostane E2-sn-glycero-3-phosphorylcholine (PEIPC) (35), and cholesteryl linolate hydroperoxides (36). PON1 can also destroy hydrogen peroxide (H2O2), a major reactive oxygen species produced under oxidative stress during atherogenesis (36), suggesting that it has peroxidase activity as well. Polymorphisms in the human PON1 gene have been associated with risk for coronary artery disease, providing evidence for a protective function of the enzyme (37–40).

To investigate the physiological role of PON1, we have created PON1 gene-targeted mice (41). The PON1 KO mice were more susceptible to organophosphate toxicity, and the HDL isolated from the PON1 KO mice failed to prevent LDL oxidation induced by a co-culture model of artery wall cells. When PON1 KO mice were bred onto the C57BL/6J mouse background and fed an atherogenic diet, they developed significantly larger fatty streak lesions in the aortic sinus as compared with the PON1 wild-type littermates (41).

We have now extended our studies of PON1 deficiency to the apoE KO mouse model that exhibits advanced atherosclerosis and increased lipoprotein oxidation. ApoE mediates the uptake and removal of chylomicron and VLDL remnants via hepatic lipoprotein receptors. ApoE KO mice exhibit severalfold higher levels of plasma total cholesterol as compared with wild-type littermates and develop advanced atherosclerotic lesions even when maintained on low fat chow diets (42, 43). The atherosclerosis that develops in apoE KO mice appears to be oxidation-dependent, as their lesions contain oxidation-specific epitopes by immunostaining, and there are high plasma titers of autoantibodies against varying epitopes of ox-LDL (44). Lipid peroxidation products are detected in the circulating lipoproteins of apoE KO mice as well (45). Consistent with the importance of oxidation in the apoE KO mice, their atherosclerosis can be inhibited by vitamin E despite the presence of marked hypercholesterolemia (46). In the present study, we crossed the PON1 null mutation onto the apoE KO mouse background and examined the effects of PON1 deficiency on atherosclerotic lesion formation at two different time points and using two different diets. We also compared lipoprotein oxidation and expression of genes involved in the metabolism of oxidized lipoproteins in these mice.

**EXPERIMENTAL PROCEDURES**

**Mice and Diet**—PON1 KO mice were generated as described (41). ApoE KO mice on the C57BL/6J background were purchased from The Jackson Laboratory (Bar Harbor, ME). To obtain PON1 KO/apoE KO and apo E KO littermates, the crosses were set up as follows. PON1 KO mice that were of the 87.5% C57BL/6J and 12.5% 129/SvJ genetic background were crossed to apoE KO mice on the C57BL/6J background to obtain PON1 heterozygous/apoE heterozygous (het) mice. The PON1 het/apoE het mice were backcrossed to the apoE KO mice again to obtain PON het/apoE KO mice. PON1 het/apoE KO mice were then intercrossed to produce PON KO/apoE KO and apo E KO littermates with a genetic makeup of 97% C57BL/6J and 3% 129/SvJ. Only female mice were included in the experiments. Mice were maintained on a 6% fat chow diet after weaning. For studies of atherosclerotic lesion formation in the whole aortic tree, mice were switched to a 42% fat, 0.15% cholesterol “Western” diet (diet number TDI88137, Harlan Teklad, Madison, WI) at 6–8 weeks of age and were maintained on this diet for 16 weeks.

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2 A. Wagner and M. Navab, personal communication.
then bled 15 min, 2, 6, and 24 h after injection. Plasma samples were stored with 1 mM EDTA at −80 °C before assay. Each plasma sample was then assayed by chemiluminescent immunoassay technique for the relative amount of human LDL in plasma, the amount of murine IgG or IgM bound per human LDL particle, and the amount of oxidized phospholipid epitopes present on each human LDL particle, as detected by antibody EO6. For these assays, murine plasma was added to wells of a microtiter plate that had previously been coated with a monoclonal antibody, MB47, specific for human apoB (53). This was achieved by plating 10 μg/ml MB47 in phosphate-buffered saline overnight. This antibody does not detect murine LDL (53). Then a 1:50 dilution of murine plasma was added. Preliminary experiments demonstrated that at this dilution the content of human LDL did not lead to saturation of binding to the coated MB47. To determine the content of human apoB, biotinylated anti-human apoB monoclonal antibody, MB24 (54), was added. This antibody binds to a distinct site on apoB separate from that recognized by MB47 (54). Because there is only one MB24-binding site per LDL, the amount of MB24 bound represents the number of apoB particles bound. To determine the amount of oxidized phospholipid epitope present on LDL, biotinylated monoclonal antibody EO6 (55) was added in a separate set of wells. EO6 binds to the oxidized phospholipid, POVP (56). Finally, to detect the amount of murine immunoglobulin bound to the captured human LDL, alkaline phosphatase-labeled anti-mouse IgG or IgM antibodies were added. For each of these assays, the amount of EO6 binding or the content of bound IgG or IgM was expressed per MB24, e.g. normalizing each value per LDL particle. Each plasma sample was assayed in triplicate. Each data point represents the average value of assays from five mice.

**RNA Isolation, Northern Blot, and RT-PCR Analyses—**Liver total RNA was isolated using Trizol reagent (Life Technologies, Inc.) according to manufacturer’s protocol. Northern blot was performed as described (57). The probes used in hybridization were a 588-bp PCR product from the exon 5 of mouse heme oxygenase-1 (HO-1) gene, an KpnI 500-bp fragment from a mouse macroinial cDNA clone, a ClaI–Apol 757-bp fragment from mouse F4/80 cDNA clone, and a HindIII/Perl1 280-bp fragment from mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA clone. Quantitation of mRNA levels on the Northern blots was done using a PhosphorImager 445SI (Molecular Dynamics). For analysis of mRNA of other genes by RT-PCR, first strand cDNA was reverse-transcribed from total RNA using the SuperScript Preamplification System (Life Technologies, Inc.). The first strand cDNA was then used as template in RT-PCR. The program for RT-PCR of scavenger receptor BI (SR-BI) was 94 °C for 4 min, 25 cycles of 94 °C for 30 s, and 66 °C for 5 min, followed by 72 °C for 7 min. The program for all other RT-PCRs was 94 °C for 4 min, 25 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 7 min. The primer pairs for RT-PCR were as follows: for peroxisome proliferated receptor-α (PPARα) (product size = 775 bp), 5′-GGC ATT GAG TGC CCA GTG TGT G-3′ and 5′-GGC ATT GAG TGC CCA GTG TGT G-3′; for SR-A (product size = 328 bp), 5′-CCG AGT CCT TGC AGA GTC TG-3′ and 5′-AGC CTC TCCACC TTC TC-3′; for CD56 (product size = 491 bp), 5′-CAT CCA GGC AGA GCC TTC ATC-3′ and 5′-GAA ACA CGC TAG ATG AAC GAG TCT-3′; for SR-BI (product size = 1767 bp), 5′-CTG CTC AGC CCC GGC AGC-3′ and 5′-CTG CTC AGC CCC GGC AGC-3′; for SR-BII (product size = 438 bp), 5′-CTG CAT TCG TCG TCA TCT CTG-3′ and 5′-CTG CAT TCG TCG TCA TCT CTG-3′; for GAPDH (product size = 328 bp), 5′-GAA ACA CGC TAG ATG AAC GAG TCT-3′ and 5′-GAA ACA CGC TAG ATG AAC GAG TCT-3′. PCR products were fractionated by 1.5% agarose gel, stained with SYBR Green (Molecular Probes, Eugene, OR), and scanned and quantitated using a Hitachi FBMO II Multi-View scanner. Known amounts of DNA standards were included on the gels for construction of standard curve for quantitation of the PCR products.

**RESULTS**

**Combined PON1 KO/ApoE KO Mice—**PON1 KO mice were intercrossed with apoE KO mice to produce mice lacking both PON1 and apoE. The study was designed to maintain a largely inbred (97%) genetic background of strain C57BL/6J to avoid possible background effects on lesion development and related traits. The weight and general health of the PON1 KO/apoE KO mice were indistinguishable from the apoE KO mice.

**PON1 KO/ApoE KO Mice Have Increased Atherosclerosis as Compared with ApoE KO Mice—**Aortic atherosclerotic lesion development was determined both at relatively early and advanced stages of lesion development. To examine the effect of PON1 on early lesion development in the apoE KO mice, mice were maintained on a 6% fat chow diet and sacrificed at 3 months of age. The size of lesions in these mice was determined by examining frozen cross-sections of the proximal aorta, from the aortic valves to the arch. The PON1 KO/apoE KO mice had significantly larger lesion areas in the aortic valve (18,420 ± 2,950 μm²/section) as compared with the apoE KO littermates (10,780 ± 1,430 μm²/section, p = 0.01) (Fig. 1). Advanced atherosclerotic lesions were also examined in mice that had been maintained on a high fat Western diet for 16 weeks. These lesions were examined in the whole aortic tree by the en face technique. Again, the PON1 KO/apoE KO mice had significantly larger lesion areas in the aortic tree as compared with the apoE KO mice (Fig. 2, A and B). Therefore, atherosclerosis was enhanced in PON1 KO/apoE KO mice in both the aortic sinus and distal aorta as compared with the apoE KO littermates.

**PON1 Deficiency Alters Plasma Lipid Levels—**Plasma lipid levels and lipoprotein profiles were examined in 3-month-old male PON1 KO/apoE KO mice fed a chow diet. The plasma total cholesterol and non–HDL cholesterol levels of the PON1 KO/apoE KO mice were significantly lower than those of the apoE KO littermates, whereas there was no difference in plasma HDL or triglyceride levels (Table I). FPLC analysis showed that the PON1/apoE KO mice exhibited the same VLDL cholesterol levels and lower IDL/LDL levels as compared with the apoE KO mice (Fig. 3). However, plasma lipid levels of 6-month-old mice fed a Western diet, which resulted in much higher plasma cholesterol levels, did not exhibit significant differences in lipid levels between the PON1 KO/apoE KO and apoE KO mice (Table II).

**ApoB-containing Lipoproteins of PON1 KO Mice Exhibit Increased Levels of Oxidized Lipids—**Since PON1 may exert a protective effect on atherosclerosis by destroying oxidized lipids in apoB-containing lipoproteins, we tested whether the PON1 KO/apoE KO mice had increased oxidized lipids in these particles. By using mass spectrometry, we examined VLDL, IDL, and LDL isolated from 4- to 6-month-old mice fed a chow diet for the presence of bioactive oxidized phospholipids, including POVP, PGPC, and mixed isomers of PEIIPC. Also,
Increased atherosclerotic lesion formation in the PON1 KO/apoE KO mice fed a high fat diet. Female PON1 KO/apoE KO mice (n = 8) and apoE KO mice that were wild-type or heterozygous for the PON1 null mutation (n = 20) between 6 and 8 weeks of age were fed a high fat Western diet for 16 weeks. The mice were then sacrificed and their aortae, from the aortic arch to the iliac arteries, were dissected out and examined for atherosclerotic lesion formation using the en face technique, as described under “Experimental Procedures.” A, two representative aortae each from the apoE KO mice and the PON1 KO/apoE KO mice. B, the extent of atherosclerotic lesion formation in each mouse, expressed as percentage of aortic surface area covered by atherosclerotic lesions. The mean atherosclerotic lesion area of each group of mice is indicated by a horizontal bar with mean value shown next to it.

**TABLE I**

|                  | ApoE KO (n = 24) | PON1 KO/apoE KO (n = 15) | p value |
|------------------|------------------|--------------------------|---------|
| Total cholesterol| 528 ± 23         | 448 ± 29                 | <0.05   |
| VLDL/LDL cholesterol | 507 ± 23         | 425 ± 29                 | <0.05   |
| HDL cholesterol  | 21 ± 2           | 24 ± 2                   | NS      |
| Triglycerides    | 56 ± 5           | 48 ± 4                   | NS      |

* NS, not significant.

LPC levels were measured. We observed that the VLDL isolated from the PON1 KO/apoE KO mice exhibited a significant 20% increase in LPC as compared with the apoE KO VLDL, whereas the levels of POVPC, PGPC, and mixed isomers of PEIPC were similar between the two VLDLs (Fig. 4A). As shown in Fig. 4B, the PON1 KO/apoE KO IDL exhibited significant 14, 73, 85, and 19% increases in LPC, POVPC, PGPC, and mixed isomers of PEIPC levels, respectively, as compared with the apoE KO IDL. We found that LDL isolated from the PON1 KO/apoE KO also exhibited significant 42, 39, 41, and 107% increases in LPC, POVPC, PGPC, and mixed isomers of PEIPC levels, respectively, as compared with the apoE KO LDL (Fig. 4C). Therefore, PON1 deficiency resulted in higher levels of oxidized phospholipids in circulating apoB-containing lipoproteins, especially in IDL and LDL.

High titers of autoantibodies against epitopes of ox-LDL are prevalent in animal models of atherosclerosis and in patients with coronary heart disease (44, 58, 59). In addition, autoantibodies against ox-LDL and ox-LDL-immunoglobulin complexes are detected in atherosclerotic lesions (60). We determined the titers of autoantibodies against ox-LDL and MDA-LDL in the plasma of PON1 KO/apoE KO mice and the apoE KO mice, and we found no differences between these two groups of mice (Fig. 5).

**FIG. 3.** FPLC profiles of plasma collected from mice on a chow diet. 400 μl of plasma pooled from 5 apoE KO mice (open circles) or 5 PON1 KO/apoE KO mice (filled circles) was used in FPLC analysis. Starting at 20 min (flow rate = 0.5 ml/min) after the application of sample and initiation of elution, fractions were collected in 0.5-ml aliquots, and cholesterol content of each fraction was measured and plotted. The elution positions of VLDL, IDL/LDL, and HDL were previously determined (49) by applying density-isolated lipoproteins to the FPLC column.

**TABLE II**

|                  | ApoE KO (n = 9) | PON1 KO/apoE KO (n = 9) | p value |
|------------------|----------------|-------------------------|---------|
| Total cholesterol| 1442 ± 113     | 1547 ± 146               | NS      |
| VLDL/LDL cholesterol | 1415 ± 111     | 1520 ± 143               | NS      |
| HDL cholesterol  | 27 ± 2         | 27 ± 3                   | NS      |
| Triglycerides    | 47 ± 14        | 43 ± 11                  | NS      |

* NS, not significant.
circulation after the indicated periods. To follow clearance of the injected human LDL, plasma samples were collected at various time points after injection. Plasma titer of autoantibodies (IgG and IgM) against ox-LDL and MDA-LDL were determined separately as described under “Experimental Procedures.” Anti-oxLDL titers from five PON1 KO/apoE KO mice (filled bars) and five apoE KO mice (open bars) are shown (values are mean ± S.E.).

Fig. 6. Accelerated LDL clearance in the PON1 KO/apoE KO mice as compared with the apoE KO mice. Four-month-old, chow-fed PON1 KO/apoE KO mice and apoE KO mice were bled at time 0 and injected with 1 mg of human LDL. The mice were then bled at 2, 6, and 24 h after injection for collection of plasma. The human LDL content in plasma, as measured by relative human apoB level, was determined by ELISA as described under “Experimental Procedures.” The means from five PON1 KO/apoE KO mice (filled circles) and five apoE KO mice (open squares), and the standard errors (bars) are shown. *, p < 0.05, PON1 KO/apoE KO versus apoE KO, as analyzed by unpaired Student’s t test.
FIG. 7. Accelerated LDL oxidation in the PON1 KO/apoE KO mice as compared with the apoE KO mice. The same plasma samples analyzed in Fig. 4 were assayed for the content of oxidation epitopes per human LDL by ELISAs as described under “Experimental Procedures.” The means from five PON1 KO/apoE KO mice (filled circles) and five apoE KO mice (open squares) and the standard errors (bars) are shown.

Interestingly, we found that the expression of HO-1 in the double KO mice was 169% that of the apoE KO mice (Table III). The increased expression of HO-1 in the PON1 KO/apoE KO mice suggests that the double KO mice are under higher oxidative stress as compared with the apoE KO mice.

PPARγ is a transcription factor that plays important roles in the differentiation of both adipocytes (62, 63) and macrophages (64). Expression of PPARγ in monocytes and macrophages is also greatly induced by ox-LDL (64–66). We observed that the expression of PPARγ in the livers of PON1 KO/apoE KO mice was 184% that of the apoE KO mice (Table III), a finding that could be relevant to macrophage functions and atherosclerosis.

We then examined the expression of ox-LDL receptors including SRA (10), CD36 (11), and macrosialin (12, 13), all of which are expressed in macrophages and have been shown to be induced by ox-LDL (64, 65, 67). The expression levels of SRA, CD36, and macrosialin in PON1 KO/apoE KO mice were 254, 207, and 171% those of the apoE KO mice (Table III). The increased expression of the scavenger receptors may enhance the clearance of ox-LDL in these mice. The expression of a macrophage cell surface marker, F4/80, was also higher in the livers of PON1 KO/apoE KO mice as compared with the apoE KO mice, whereas hepatic expression of an HDL receptor, SR-BI, was the same in both groups of mice (Table III).

**DISCUSSION**

A variety of *in vitro* studies and epidemiologic data originally suggested that PON1 may protect against LDL oxidation (24, 25) and atherosclerosis (37–40). To test this hypothesis, we constructed PON1 KO mice and examined them for HDL functions and diet-induced fatty streak formation (41). The results were consistent with a role for PON1 in protecting LDL from oxidation and inhibiting the progression of atherosclerosis. We have now extended our studies using an apoE KO mouse model which develops high levels of atherogenic lipoproteins as well as advanced atherosclerosis (42, 43). Our present studies show that even in this severe model of atherosclerosis, PON1 exhibited a protective effect on advanced lesions as well as fatty streaks and that the effect was observed throughout the aortic tree. In mice fed a chow diet there was a significantly lower level of apoB-containing lipoproteins (including IDL and LDL) in the double KO mice, but no such difference was found between the double KO and the apoE KO mice when fed a Western diet. This could be due to smaller sample sizes and/or to the extreme hypercholesterolemia that Western diets caused. We also observed that PON1 deficiency significantly increased the levels of oxidized lipids in circulating IDL and LDL (Fig. 4). Furthermore, the increased levels of HO-1 in the liver suggest an increase in oxidative stress in double KO animals. Finally, using a human LDL tracer that allowed us to distinguish it from the endogenous lipoproteins, we observed an increased rate of clearance of LDL, an increased rate of accumulation of oxidized phospholipids on the injected LDL, and an increased

| Gene expression | ApoE KO | PON1 KO/apoE KO | p value |
|-----------------|--------|----------------|--------|
| HO-1            | 1.00 ± 0.11 | 1.69 ± 0.28 | p < 0.05 |
| PPARγ           | 1.00 ± 0.15 | 1.84 ± 0.15 | p < 0.01 |
| SRA              | 1.00 ± 0.13  | 2.54 ± 0.38  | p < 0.01 |
| CD36            | 1.00 ± 0.24  | 2.07 ± 0.16  | p < 0.01 |
| Macrosialin     | 1.00 ± 0.15  | 1.71 ± 0.13  | p < 0.01 |
| SR-BI           | 1.00 ± 0.17  | 0.97 ± 0.11  | p = 0.90 |
| F4/80           | 1.00 ± 0.15  | 2.67 ± 0.45  | p = 0.01 |
rate of formation of LDL-immunoglobulin complexes in PON1 KO mice. These latter results indicate that LDL oxidation contributes to its metabolism in the circulation.

We found that IDL and LDL isolated from the PON1 KO/apoE KO mice, as compared with the apoE KO mouse, contained higher levels of the oxidized phospholipids POVPc, PGPC, and mixed isomers of PEIPC, all of which are destroyed by PON1 in vitro.2 These three molecules, at concentrations of about $10^{-8}$ M, activate endothelial cells to produce both monocyte-hind ing molecules and MCP-1. Interestingly, we did not observe significant difference in POVPc, PGPC, and mixed isomers of PEIPC levels between the circulating VLDLs of double KO and apoE KO mice. This could be due the following. 1) VLDL is the precursor for IDL and LDL; thus, VLDL has less time to accumulate oxidized species. 2) The large size of VLDL would hinder its entrance into the subendothelial space and peripheral tissues where the oxidation most likely occurs, thus making VLDL less likely to be oxidized. LPCs are generated in oxidatively modified LDL by two sequential events, the oxidation and fragmentation of the sn-2 residues of phosphatidylcholine, followed by the hydrolysis of the shortened fatty acid residues by LDL-associated platelet-activating factor acetylhydrolase (68, 69). The increase in LPC in the PON1 KO/apoE KO VLDL, IDL, and LDL, as compared with those of the apoE KO, also suggests an enhanced rate of oxidation in the PON1-deficient mice. LPC is proinflammatory (70–75), and its increase in the apoB-containing lipoproteins of PON1 KO/apoE KO mice may also play a role in promoting atherogenesis. However, LPC is active at about $10^{-5}$ M, 1000 times higher than the active concentrations of POVPc, PGPC, and PEIPC. This and previous studies (24, 25, 36) strongly suggest that PON1 is important in 1) preventing initiation of oxidation by destroying reactive oxygen species (such as H$_2$O$_2$), 2) blocking propagation of oxidation by its peroxidase activity, and 3) destroying biologically active oxidized phospholipids, such as POVPc, PGPC, and PEIPC, via mechanism(s) yet to be determined.

No significant differences in autoantibody titers against ox-LDL and MDA-LDL were observed between the PON1 KO/apoE KO mice and the apoE KO mice. These results are surprising since we did observe more atherosclerosis in the double KO mice and since higher levels of bioactive oxidized phospholipids were present in the circulating IDL and LDL of double KO mice. One possible explanation for lack of an increase in autoantibody titers in the double KO mouse is the fact that autoantibody titers are already considerably elevated in apoE KO mice (44, 56). In addition, the increased levels of oxidized lipids in the PON1 KO/apoE KO could result in increased formation of complexes with the antibodies in the plasma, leading to an underestimate of the content of antibodies actually present (76, 77).

In this study, we used the human LDL as a marker for studying lipoprotein oxidation and clearance for the following reasons. First, we could not do the experiment with mouse VLDL as we could not distinguish the injected VLDL from the endogenous VLDL, and the whole point of the experiment was to use the human LDL as a "tracer," as we could distinguish it from endogenous lipoproteins. Second, it would be difficult to use radiiodinated murine VLDL as the labeling would likely influence oxidation rapidly. Besides, the use of labeled mouse VLDL will only provide an estimate of turnover, not the demonstration of accumulation of oxidized epitopes and immune complexes over time. Woolfett et al. (78) have shown that LDL (from both mouse and human) is cleared faster in the apoE-deficient mice as compared with the wild-type mice, due to lack of competition from apoE-containing lipoproteins at the LDL receptor. Our data in the PON1-deficient animals cannot be explained solely on that basis, however, as both the PON1 wild-type and PON1 knockout mice were on apoE KO background and, thus, difference in clearance must be due to an alternative mechanism (e.g., immune-mediated clearance). Third, if one is to use the mouse VLDL, the source of the mouse VLDL is also a problematic area. If one is to use the apoE KO VLDL, then a vast majority of it contains apoB48, which will not be bound by low density lipoprotein receptor-related protein or LDL receptors in the absence of apoE, thus making it extremely slow to be cleared. Furthermore, we have observed that the apoE KO VLDL contains more than 3-fold higher levels of LPC as compared with the wild-type VLDL, indicating that some level of oxidation is already present in the apoE KO VLDL. Since the main point of our experiment is to study the accumulation of oxidation of the injected lipoproteins during the course of the experiment, we feel that unoxidized, fresh lipoproteins from normal human subjects will be a better source than the apoE KO VLDL. The use of wild-type VLDL would be difficult given the very low levels in wild-type mice, and, moreover, they will be cleared differently as compared with the endogenous VLDL. Therefore, we believe that human LDL provides the best tracer for studying oxidative modification and clearance of lipoprotein in our mice.

As shown in Fig. 7, the rate of acquisition of oxidized phospholipids by human LDL was greater in the double KO mice, and this in turn presumably led to the greater rate of complex formation with both IgG and IgM (Fig. 8). Assuming that a similar process occurs with the endogenous murine lipoproteins, it would be expected that such immune complex formation would lead to enhanced clearance (79, 80). We have previously shown that enhanced uptake of such LDL-immune complexes is mediated by macrophages present in liver, spleen, and bone marrow. The clearance of such complexes in rabbits is a function of the extent of lipoprotein modification and the autoantibody titer (80). Because the absolute titers of anti-ox-LDL antibodies were the same in the apoE KO and PON1 KO/apoE KO mice, this suggests that the enhanced rate of formation of LDL immune complexes in the double KO mice almost certainly occurred because of the enhanced generation of oxidation-specific epitopes on the injected LDL. Thus, the increased levels of IDL/LDL-Ig complexes caused by increased oxidized lipid levels may be one of the pathways that lead to faster clearance of endogenous IDL/LDL in the PON1 KO/apoE KO mice and could explain in part the lower levels of IDL/LDL seen in the double KO mice fed a chow diet. The VLDL of the double KO mice, on the other hand, was only marginally more oxidized than that of apoE KO mice, and not surprisingly, no difference in its level was observed. Additionally, it has been shown that ox-LDL is taken up by liver, mainly via the scavenger receptors of Kupffer cells (81), at a much faster rate (within minutes) as compared with the native LDL (81–83). Although the extent of modification of LDL noted here is more likely to put these particles into the "minimally oxidized" LDL category, which do not react with scavenger receptors, it is conceivable that a small fraction are sufficiently modified so as to engage scavenger receptors. Thus, it is possible that, in the PON1 KO/apoE KO mice, increased IDL/LDL oxidation may also lead to increased clearance via the scavenger receptors of Kupffer cells as compared with the apoE KO mice.

To address possible mechanisms by which PON1 might influence lipoprotein metabolism and atherosclerosis, we examined expression of several genes known to be induced by ox-LDL. We found that the PON1 KO/apoE KO mice expressed significantly higher hepatic levels of HO-1, PPARγ, SRA, CD36, and macro sia lin, as compared with the apoE KO mice.
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These data provide indirect evidence that the PON1 deficiency leads to higher LDL oxidation in mice. The biological consequences of increased expression of SRA, CD36, and macroisalin would lead to increased uptake and clearance of oxidatively modified LDL, if such sufficiently oxidized LDL particles are present in circulation as discussed above. Recent studies have shown that PPARγ plays an important role in macrophage differentiation and function (64, 66). In vitro, constituents of ox-LDL are known to up-regulate and activate PPARγ, which in turn up-regulates the expression of CD36 in macrophages (64, 66, 84). In the mice of oxidation-prone PON1-deficient mice, we found elevated expression of both PPARα and CD36, suggesting a similar regulatory cascade occurring in Kupffer cells in vivo as well. We did not observe significant difference in SR-BI expression in liver total RNA between the two groups of mice. However, the regulation of SR-BI gene expression in liver is complex. One study (SS) showed that, in rat liver, oxidative stress stimulated SR-BI expression in Kupffer cells, while at the same time inhibited SR-BI expression in parenchymal cells. Our data were derived from liver total RNA and could not address whether there were significant differences in SR-BI expression in parenchymal and Kupffer cells, respectively, between the double KO and apoE KO mice. We speculated that as a result of greater SRA expression in liver total RNA between the two groups of mice, increased expression of genes responsive to ox-LDL stimulation could also account in part for the increased expression of scavenger receptors noted in the liver.

In conclusion, our results have demonstrated that, in apoE-deficient mice, lack of PON1 leads to increased levels of oxidized phospholipids in circulating apoB lipoproteins, an increased rate of accumulation of oxidized phospholipids in LDL, a consequent increased rate of LDL-immunoglobulin complex formation, and increased LDL clearance. These mice exhibit increased expression of genes responsive to ox-LDL stimulation and develop significantly larger atherosclerotic lesions. In aggregate, these data strongly support the notion that PON1 plays an important role in preventing atherosclerosis by decreasing the accumulation of oxidized lipoproteins.

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