Human Paraoxonase-1 Overexpression Inhibits Atherosclerosis in a Mouse Model of Metabolic Syndrome

Bharti Mackness, Rozenn Quarck, Wim Verreth, Mike Mackness, Paul Holvoet

**Background**—The metabolic syndrome is typified by obesity, dyslipidemia, diabetes, hypertension, increased oxidative stress, and accelerated atherosclerosis. Paraoxonase1 (PON1), a high-density lipoprotein (HDL)-associated antioxidant enzyme that prevents the oxidation of low-density lipoprotein (LDL), is low in the metabolic syndrome.

**Methods and Results**—We used adenovirus-mediated PON1 gene transfer (AdPON1) to overexpress human PON1 in mice with combined leptin and LDL receptor deficiency, a model of metabolic syndrome. PON1 activity, plasma lipids, the titer of autoantibodies against malondialdehyde (MDA)-modified LDL, and atherosclerosis in AdPON1 mice were compared with those in mice that received a control recombinant adenovirus (AdRR5). PON1 activity was increased 4.4-fold \((P<0.001)\) in AdPON1 mice \((N=12)\), whereas in AdRR5 mice \((N=11)\) activity did not change. Expressing human PON1 significantly reduced the total plaque volume, the volume of plaque macrophages, and of plaque-associated oxidized LDL. It increased the percentage of smooth muscle cells in the plaques. Expressing human PON1 lowered the titer of autoantibodies against MDA-modified LDL, a proxy for oxidized LDL in mice. It had no overall effect on plasma total cholesterol and triglycerides, as evidenced by the similar area under the curves, and on the HDL distribution profile.

**Conclusion**—Our data suggest that in this mouse model of metabolic syndrome, expressing human PON1 inhibited the development of atherosclerosis, probably by reducing the amount of oxidized LDL in plasma and in the plaque, thereby preventing its proatherogenic effects. Adenovirus-mediated gene transfer of human PON1 may be a potential and useful tool to prevent/retard atherosclerosis in humans. (*Arterioscler Thromb Vasc Biol. 2006;26:1545-1550.*)

**Key Words:** atherosclerosis ■ metabolic syndrome ■ oxidized LDL ■ paraoxonase-1
Mice with combined leptin and LDL receptor deficiency (DKO) feature obesity, dyslipidemia, hypertension, insulin resistance, and impaired glucose tolerance and/or diabetes. These metabolic syndrome attributes are associated with increased oxidative stress, low PON1 activity, and accelerated atherosclerosis.\(^4,10\) Therefore, we chose these mice to study the effect of overexpression of human PON1 on the development of atherosclerosis.

### Methods

#### Experimental Protocol

Homozygous LDL receptor knockout mice (LDLR\(^{-/-}\)), heterozygous ob\(^{+/-}\), and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Me) LDLR\(^{-/-}\) mice were backcrossed into a C57BL6 to the 10\(^{th}\) generation. To obtain DKO mice with combined leptin deficiency (ob/ob) and LDL receptor, LDLR\(^{-/-}\) and ob\(^{+/-}\) mice were crossed as previously described.\(^{10}\) All offspring were genotyped by polymerase chain reaction techniques. All mice were housed at 22\(^\circ\)C on a fixed 12/12-hour light/dark cycle. Experimental procedures in animals were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee of the Katholieke Universiteit Leuven.

All mice were fed standard chow containing 4% of fat (Pavan Service, Belgium). Food intake of free-fed DKO mice was \(\approx 5.7 \text{ g/d from weaning.} \)

### Adenovirus-Mediated Gene Transfer of Human PON1

Human PON1 cDNA containing the Q/M polymorphism was subcloned in the shuttle plasmid pShuttle-CMV (Stratagene) downstream of the cytomegalovirus promoter/enhancer (CMV). PON1 recombinant adenovirus (AdPON1) was generated using the AdEasy\textsuperscript{TM} Adenoviral Vector System, a commercially available system adapted from methods originally developed by He et al.\(^{22}\) All offspring were genotyped by polymerase chain reaction techniques. All mice were housed at 22\(^\circ\)C on a fixed 12/12-hour light/dark cycle. Experimental procedures in animals were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee of the Katholieke Universiteit Leuven.

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Blood of conscious mice was collected by tail bleeding into tubes after an overnight fast at different time intervals (Figure 1). Serum and plasma samples were prepared. Routinely, plasma total cholesterol and triglycerides were measured using standard enzymatic colorimetric assays (Boehringer Mannheim). High-sensitivity lipoprotein profiling was studied using HDL cholesterol fractions separated with the LipoSEARCH system by Skylight Biotech Inc (Tokyo, Japan).\(^{24}\) Serum paraoxonase-1 (PON1) activity was measured using paraoxon as substrate in 0.1 mol/L Tris/HCl buffer pH 8.0 containing 2 mmol/L CaCl\(_2\).

The titers of Ig autoantibodies against malondialdehyde (MDA)-modified LDL were determined in individual plasma samples (1:500 dilutions). The amount of Ig bound to the MDA-LDL antigen was detected with alkaline phosphatase-labeled anti-mouse IgG. Data are expressed as relative absorbance units.\(^{10,26}\)

### Atherosclerosis

The extent of atherosclerosis was determined in control DKO mice at 18 weeks (N = 7) and at 24 weeks (N = 10) and in AdRR5 (N = 11) and AdPON1 (N = 12) DKO mice 42 days after adenovirus injection by analysis of cross-sections from the aortic root (Figure 1). Approximately 10\(\times\)7-\(\mu\)m frozen sections per staining per animal were used for morphometric and immunohistochemical analysis. Lipids were stained with oil-red O, ox-LDL with mAb4E6,\(^{4,10}\) smooth muscle cells with an \(\alpha\)-actin--specific antibody (Dako), macrophages with an antibody against mouse Mac-3 antigen (Pharmingen), and PON1 with a human PON1 antibody.\(^{23}\) Double-blinded analysis of positive immunostained sections was performed with the QuantiRed image analyzer (Leica). Absolute volumes were determined by multiplying the positive immunostained areas by the thickness of the sections (7 \(\mu\)m). The contribution to plaque was determined as the percentage staining, for each parameter, compared with total plaque volume.\(^{4,10,28,29}\)

### Statistical Analysis

Placebo and PON1-treated mice were compared with Mann-Whitney test (Graph Pad Prism version 3.02). Characteristics at 7, 14, 28, and 42 days were compared with these at baseline by paired \(t\) test. Correlations were calculated using the nonparametrical Spearman correlation coefficient. The areas under the curve (AUCs) were calculated using the nonparametrical Spearman correlation coefficient. The areas under the curve (AUCs) were calculated using the nonparametrical Spearman correlation coefficient.

### Results

#### Blood Analysis

PON1 activity is lower in our mouse model of metabolic syndrome than in wild-type mice.\(^{10}\) Overexpression of human PON1 resulted in a 4.4-fold increase of PON1 activity (N = 12; \(P < 0.001\) ) 7 days after adenovirus injection, whereas in AdRR5-treated mice PON1 activity did not change (N = 11) (Figure 2A). PON1 activity decreased thereafter to reach pre-injection levels after 21 days (Figure 2B). The AUC of the PON1 activity in AdPON1 mice was significantly higher than that in AdRR5 mice in the first 3 weeks (Figure 2C). Six weeks after virus injection, PON1 activity was comparable in both groups. The titer of circulating autoantibodies against MDA-modified LDL decreased to 34% of baseline values at 6 weeks after virus injection in AdPON1, whereas in AdRR5 mice the titer remained high (Figure 2D).

In AdPON1 mice the absolute difference in titer of autoantibodies between 6 weeks and baseline correlated with the absolute difference in PON1 activity between day 7 and baseline (R\(_s\) = -0.66, \(P<0.05\)). Overexpression of human PON1 resulted in somewhat lower plasma total cholesterol (Figure 3A) and triglycerides (Figure 3B) at 6 weeks (\(P<0.05\)). However, the AUCs for both cholesterol and triglycerides for the 6-week treatment period were similar in AdPON1 and AdRR5 mice.

The HDL distribution profiles were analyzed with the LipoSEARCH system. Total HDL cholesterol levels measured with this method were similar to those measured in our laboratory by high-performance liquid chromatography.
(56±17 mg/dL versus 46±18 mg/dL in control DKO). In AdRR5 mice (n=5) 57±5% of the HDL cholesterol was in large HDL (15 to 12 nm), 26±3% in medium HDL (12 to 9 nmol) and 17±2% in small HDL (<9 nmol). In AdPON1 mice (n=5) the percentages were similar: 64±3% large HDL, 22±2% medium HDL, and 14±1% small HDL.

**Atherosclerosis**
Plaque volume in 18-week-old DKO mice was 0.010±0.008 mm$^3$. Overexpression of human PON1 (the Q/M isoform) significantly inhibited atherosclerosis (Figure 4) compared with AdRR5 mice. Compared with 18-week-old control DKO mice plaque volume in AdRR5 mice was 11-fold higher, whereas in AdPON1 mice plaque volume was only 3.1-fold higher. Plaque volume in AdRR5 was comparable to that in 24-week-old control DKO mice (0.11±0.06 versus 0.09±0.04).

Overexpression of human PON1 resulted in lower total plaque volume (−71%, P<0.001) and lower absolute volume of macrophages (−69%, P<0.001), ox-LDL (−80%, P<0.001), and SMC (−49%, P<0.05), compared with AdRR5 mice. PON1 gene transfer caused a change in plaque composition as seen by a lower percentage of ox-LDL, a higher percentage of SMC, and a higher percentage SMC to percentage macrophage ratio (P<0.05 for all) (Figure 4). Because the lower absolute volume of macrophages seen in AdPON1-treated mice was associated with a lower plaque volume, expressing human PON1 had no effect on the

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**Figure 2.** A, PON1 activity before and 7 days after expressing RR5 (N=11) or human PON1 (N=12) in DKO mice. B, Change of PON1 activity in AdRR5 and AdPON1 mice. C, Area under the curve (AUC) of PON1 activity from day 0 until day 21 in AdRR5 and AdPON1 mice. D, Change of titer of circulating autoantibodies against MDA-modified LDL in AdRR5 and AdPON1 mice. PON1 activity at day 7 after virus injection was compared with baseline values by paired $t$ test. ***$P<0.001$ and **$P<0.01$.

**Figure 3.** Change and AUC of total cholesterol (A) and triglycerides (B) in AdRR5 and AdPON1 mice.
percentage of macrophages in the plaque. Overexpression of human PON1 resulted in a 1.5-fold higher percentage staining for hPON1 compared with AdRR5 mice (22% vs. 15%, P<0.05). The immunostaining found in the plaques of AdRR5 mice is caused by the significant sequence similarities between mammalian PON1s leading to the cross-reactivity of anti-hPON1 antibodies toward different mammalian PON1s (although the antibody has a lower affinity toward mouse PON1 than human PON1), rather than to cross-reactivity toward other plaque proteins. Using a higher threshold to correct for the cross-reactivity of the anti-hPON1 antibody toward mouse PON1s, we still detected staining for hPON1 in AdPON1 mice, whereas in AdRR5 we did not detect any staining.

**Discussion**

In this experimental model of combined obesity and insulin-resistance, which is susceptible to atherosclerosis development, we have shown unequivocally that the expression of human PON1 significantly inhibits the development of atherosclerosis, reducing total plaque volume, and the amount of ox-LDL and macrophages in the plaques. Expressing human PON1 in this mouse model had no overall effect on plasma total cholesterol and triglycerides and on the HDL distribution profiles. The latter finding is in agreement with previous studies in PON1 overexpressing transgenic mice showing that a 5-fold increase in PON1 had no effect on HDL composition, size, or charge. Our data thus indicate that the anti-atherosclerotic effects of PON1 are not caused by a reduction in lipids and lipoproteins, but by a specific mechanism, i.e., the reduction in LDL oxidation. PON1 transfer was associated with a significantly lower titer of antibodies against oxidatively modified LDL and a lower amount of ox-LDL in the plaques. Thus, the decrease in ox-LDL levels in the plaque could be the result of a decrease in ox-LDL levels in the plasma, or of a decrease in oxidative stress in the plaque due to the increase in PON1.

**Effect of PON1 Gene Transfer on Plasma Ox-LDL Levels**

We, among others, have demonstrated an association between the oxidation of LDL and coronary artery disease. We also have shown that the metabolic syndrome is associated with higher levels of circulating ox-LDL that are associated with a greater disposition to atherothrombotic coronary disease. PON1 is an HDL-associated enzyme that, among many other functions, metabolizes pro-inflammatory lipids formed during the oxidation of LDL. It has also been shown that plasma ox-LDL levels correlate with human serum paraoxonase in patients with type 2 diabetes.

Because the assay for ox-LDL in blood is based on a mouse monoclonal antibody, ox-LDL cannot be measured directly in mouse blood. Previously, the titer of autoantibodies against MDA-modified LDL has been used as a proxy for ox-LDL in mice. Compared with ob/ob and LDLR−/− mice, the DKO mice had higher titers of autoantibodies. We show that the transient increase in PON1 activity in AdPON1-treated mice correlated with a decrease in the titer of autoantibodies against MDA-modified LDL, even until 42 days after the virus injection, supporting a direct effect of human PON1 overexpression on circulating ox-LDL. The decrease in circulating autoantibodies against MDA-modified LDL was paralleled with a decrease in ox-LDL in the plaque. These data support the hypothesis that autoantibody titers to ox-LDL reflect changes in ox-LDL content in atherosclerotic lesions in mice. In summary, our data suggest that an increase in PON1 activity could decrease ox-LDL in the plaque by decreasing circulating ox-LDL in the plasma of DKO mice.

**Effect of PON1 Gene Transfer on Oxidative Stress in the Plaque**

We have previously shown that PON1 increases in the human artery wall during the development of atherosclerosis, probably as a protective response to the oxidative stress. In the present study, overexpression of human PON1 resulted in a 1.5-fold higher percentage staining for hPON1 in the plaque compared with AdRR5 treated mice. Using a higher threshold to correct for the cross-reactivity of the anti-hPON1 antibody toward mouse PON1s we still detected staining for hPON1 in
AdPON1 mice, whereas in AdRR5 we did not detect any staining. It is most likely that the PON1 found in the plaques is derived from HDL as the AdPON1 contained a liverspecific promoter (CMV) and vascular cells do not express PON1.27 It would appear, therefore, that in our model of atherosclerosis, human PON1 also acts by attenuating the oxidative stress directly in the artery wall, thereby decreasing ox-LDL in the plaque.

Previously, a decrease in PON1 activity was associated with an increase in macrophage homing in the plaque of our DKO mice.10 An adenovirus-induced increase in PON1 in the aortic arch decreased the number of macrophages, probably caused by the ability of PON1 to attenuate the oxidation of LDL and thereby the ox-LDL–induced upregulation of MCP-1 and macrophage colony-stimulating factor by vascular endothelial cells.12

Previous studies in apolipoprotein E-deficient mice have also shown that expressing human PON1 inhibits atherosclerosis because of a role of PON1 in decreasing macrophage oxidative stress.37 These authors also indicated that PON1 could stimulate mouse macrophage reverse cholesterol transport, by a mechanism not fully understood but that appeared to involve lysocephospholipid generated by the hydrolysis of LPOs.37,38

Relevance of the Study
Our results are of interest in the context of the relative contributions of the 3 PON family members to protection against atherosclerosis. Because the isofrom of PON1 used in these studies (Q/M) is the isofrom most efficient at inhibiting LDL oxidation in vitro,39 it could be postulated that this isofrom would be the most efficient at inhibiting atherosclerosis in vivo. However, similar studies with the other PON isofroms are required to confirm this. Whether overexpression of human PON1 is also able to regress atherosclerosis is the subject of ongoing work, in which the adenovirus-mediated gene transfer will be used to overexpress human PON1 in the same mice, now starting at 24 weeks, when plaques are already well-developed and cardiac function is impaired compared with control C57BL6 mice. Although it is obvious that overexpressing human PON1 inhibits atherosclerosis development, the roles of PON2 and PON3 are more uncertain. It would appear that PON3 is not present in sufficient quantities in mouse plasma to prevent atherosclerosis.17 Further studies of the roles of PON2 and PON3 in atherosclerosis are warranted.

Conclusion
In conclusion, we have shown for the first time to our knowledge that adenovirus-mediated expression of human PON1 attenuates the development atherosclerosis in a mouse model of the metabolic syndrome probably by inhibiting the oxidation of LDL in the plasma and the artery wall. The use of adenovirus expression of human PON1 may be a useful tool for preventing atherosclerosis in humans.

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References


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