Decreased Macrophage Paraoxonase 2 Expression in Patients With Hypercholesterolemia Is the Result of Their Increased Cellular Cholesterol Content: Effect of Atorvastatin Therapy

Mira Rosenblat, Tony Hayek, Khetam Hussein, and Michael Aviram

Objective—To analyze paraoxonase2 (PON2) expression in human monocyte-derived macrophages (HMDM) from patients with hypercholesterolemia in relation to cellular cholesterol and oxidative stress.

Methods and Results—Ten healthy subjects (controls) and ten patients with hypercholesterolemia who received 20-mg/d atorvastatin participated in the study. The patients’ versus controls’ HMDM demonstrated increased cholesterol content (370%) and oxidative stress (30% to 45%). Atorvastatin therapy reduced these parameters (25% to 59%). The patients’ versus controls’ macrophage-PON2 mRNA expression and PON2 activity were lower (100% and 40%, respectively), and atorvastatin therapy increased these parameters (76% and 200%, respectively). Untreated patient HMDM incubation with atorvastatin (0 to 10 μmol/L) resulted in a dose-dependent reduction in cellular cholesterol content and in cell-mediated low-density lipoprotein (LDL) oxidation up to 79% and 66%, respectively. In parallel, PON2 mRNA expression and PON2 activity increased dose-dependently up to 3.6 and 2.1 fold, respectively. On incubation of control HMDM with acetylated-LDL or aggregated-LDL, cellular cholesterol content increased (77% and 100%), and macrophage-PON2 activity decreased (49% and 22%), respectively. In contrast, oxidized-LDL increased both cellular oxidative stress and PON2 expression.

Conclusions—HMDM-PON2 expression is reduced in patients with hypercholesterolemia as a result of their increased cellular cholesterol content. Atorvastatin therapy reduced both macrophage oxidative stress and cholesterol content, and upregulated PON2 expression thus contributes to attenuation of foam cells formation. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key words: Paraoxonase ■ oxidative stress ■ macrophages ■ cholesterol ■ atorvastatin

The early atherosclerotic lesion is characterized by accumulation of monocyte-derived macrophages loaded with cholesterol and oxidized lipids.1,2 Oxidative stress contributes to atherogenesis,2 and during atherogenesis, lipid peroxidation takes place in lipoproteins as well as in arterial macrophages.1,3 These “oxidized macrophages” possess increased capability to oxidize LDL and to take up Ox-LDL,4,5 leading to foam cell formation. In patients with hypercholesterolemia, increased oxidative stress in serum was demonstrated,6 placing these patients at high risk to develop atherosclerosis. Paraoxonases (PONs) genes 1, 2, and 3 are members of a multigene family.7 Serum PON1, an HDL-associated esterase,7 was shown to be reduced in patients with hypercholesterolemia,8 and studies in vitro, as well as in PON1-knockout mice and in PON1-overexpressing mice, have also demonstrated that serum PON1 activity is inversely associated with oxidative stress9-13 and atherosclerosis progression. PON1 can hydrolyze specific oxidized lipids in lipoproteins,12 in macrophages,5,10 and in atherosclerotic lesions.13 Human and rabbit PON3 are also HDL-associated and they can protect LDL against oxidation.14,15 PON2, in contrast, is not present in serum, but the PON2 gene is expressed in various tissues.16 PON2 overexpression in cells was shown to reduce intracellular oxidative state and the cells’ ability to oxidize LDL.17 We have recently demonstrated that, in human macrophages, only PON2 (but not PON1 and PON3) is expressed, and its expression is increased under oxidative stress.18

Multiple interventional trials demonstrated that HMG-CoA reductase inhibitors (statins) effectively reduce serum cholesterol levels and cardiovascular events in patients with hypercholesterolemia and also exert pleiotropic effects on vascular cells, independent of cholesterol lowering.19 Statins or their metabolites can act as antioxidants, either directly or indirectly, by removing “aged LDL,” which is more prone to oxidation, from the circulation. Indeed, statin therapy resulted in reduced LDL oxidation in the treated patients.20 Further-
more, atorvastatin therapy in patients with hypercholesterolemia suppressed cellular uptake of Ox-LDL by the patients’ monocytes/macrophages. 21 Statin therapy is also associated with increased serum PON1 activity. 21,22 No data, however, is available on cellular PON2 expression as it relates to cellular levels of oxidative stress and/or cholesterol in monocytes/macrophages from patients with hypercholesterolemia.

**Methods**

Please see complete Methods online, available at http://atvb.ahajournals.org.

**Results**

**Serum Lipids and Lipoproteins Pattern, Lipids Peroxidation, CRP, and Paraoxonases in Patients With Hypercholesterolemia: Effect of Atorvastatin Therapy**

Serum total and LDL cholesterol concentrations were increased by 2.0- and 2.6-fold in the patients versus controls, whereas HDL cholesterol levels were similar (Table, available at: http://atvb.ahajournals.org). Atorvastatin therapy resulted in a significant decrease, by 32% and 40%, in serum total and LDL cholesterol levels, respectively (Table), with no significant effect on HDL cholesterol levels. Other serum biochemical parameters were similar in both groups, and were not affected by atorvastatin therapy. Serum CRP levels, however, were significantly higher in the patients versus controls by 4.8-fold, and atorvastatin therapy reduced serum CRP levels by 42% (Table).

Serum aldehyde (TBARS) levels were significantly increased in the patients versus controls (4.3 ± 0.6 versus 2.5 ± 0.2 nmol MDA equivalents/mL) and atorvastatin therapy reduced the level of TBARS (by 14%) in the patients’ serum (to 3.6 ± 0.4 nmol/mL). Serum PON1 and PON3 activities were both significantly lower in the patients versus controls (213 ± 15 versus 274 ± 56 U/mL for PON1 and 18.2 ± 1.9 versus 30.9 ± 2.4 U/mL for PON3, respectively). Atorvastatin therapy increased these activities back toward control levels (to 263 ± 33 U/mL for PON1 and to 22.6 U/mL for PON3).

**Macrophage Cholesterol Content and Oxidative Stress in Patients With Hypercholesterolemia: Effect of Atorvastatin Therapy**

We next questioned whether, as shown in serum, oxidative stress and cholesterol levels are also increased in the patients’ monocytes/macrophages. Monocytes from controls and from patients (before and after therapy) were isolated, and were grown in culture in the presence of 10% autologous serum for 8 days to induce their differentiation into mature macrophages (Figure 1). Unlike control macrophages (Figure 1A), the patients’ macrophages (Figure 1B) were morphologically like foam cells (large and filled with lipid droplets). Atorvastatin therapy resulted in a substantial reduction in the number of foam cells (Figure 1C).

We next questioned whether the conversion of the patient monocytes into macrophage foam cells was the result of the patient serum. Thus, we incubated the control monocytes in the patient serum (Figure 1D) or with autologous control serum containing LDL at a similar cholesterol concentration (180 mg%) as present in the patient serum (Figure 1E). As can be seen from Figures 1D and 1E, in both cases, control macrophages were not converted into foam cells, suggesting that the patient serum was not responsible for the conversion of patient monocytes into foam cells, but it is probably an intrinsic characteristic of the patient’s cells. On incubation of the patient monocytes with control serum, they were still converted into foam cells (Figure 1F). Total cholesterol content in the patient HMDM was 3.4 fold higher than in control HMDM (Table). This was the result of free cholesterol accumulation in the patient HMDM, whereas esterified cholesterol content was similar in both types of macrophages (Table). In the patient HMDM that were differentiated in control serum, total and free cholesterol levels were reduced by ~15%, as compared with patient HMDM which differentiated in their autologous serum. These results suggest that the patient serum cholesterol contributed little to the increased patients macrophage cholesterol content (Table). Differentiation of control HMDM in control serum containing LDL resulted in only a 25% increase in total cholesterol level, which did not convert the control HMDM into foam cells (Table, Figure 1E).

Macrophage total cholesterol levels were significantly increased in the patients versus controls by 270% (109.7 ± 1.2 versus 29.8 ± 3.2 μg/mg cell protein), and atorvastatin therapy significantly reduced (by 59%) the patients’ cellular cholesterol levels to 45.4 ± 4.4 μg/mg cell protein. The patients’ HMDM contained also significant (*P<0.01) increased levels (by 39%) of lipid peroxides compared with controls’ HMDM (16.8 ± 2.0 versus 12.8 ± 1.5 nmol/mg cell protein), and atorvastatin therapy reduced the patients’ cellular lipid

<table>
<thead>
<tr>
<th><strong>TABLE 1. Total Cholesterol, Free Cholesterol, and Cholesteryl Ester Content in HMDM From Patients With Hypercholesterolemia and Control Macrophages</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macrophages (HMDM)</strong></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td><strong>Patient HMDM</strong></td>
</tr>
<tr>
<td>In patient serum</td>
</tr>
<tr>
<td>In control serum</td>
</tr>
<tr>
<td><strong>Control HMDM</strong></td>
</tr>
<tr>
<td>In control serum</td>
</tr>
<tr>
<td>In control serum + LDL</td>
</tr>
</tbody>
</table>

Human monocytes were isolated from the blood of patients with hypercholesterolemia and from healthy subjects (control). The monocytes were grown for 8 days in the presence of 10% serum. Results are given as mean±SEM of triplicate dishes.
peroxides levels by 25% to $12.6 \pm 1.3$ nmol/mg cell protein. We next measured the ability of the controls’ and the patients’ macrophages (before and after atorvastatin therapy) to oxidize LDL. Macrophage-mediated LDL oxidation was 32% higher using the patients’ macrophages versus controls’ macrophages (Figure 2A), and atorvastatin therapy significantly reduced (by 48%) the patient’s macrophage capability to oxidize LDL, compared with the results obtained before therapy (Figure 2A). Similarly, LDL that was incubated with the patient macrophages oxidized faster and to a greater extent than LDL incubated with control macrophages, and this phenomenon was abolished after atorvastatin therapy (Figure 2B).

**Figure 1.** Morphology of monocytes/macrophages (HMDM) in patients with hypercholesterolemia versus controls: the effect of atorvastatin therapy. Monocytes were isolated from the blood of healthy subject (control), and from patients with hypercholesterolemia before and after atorvastatin therapy (20 mg/d, for 2 months). The cells were grown for 8 days in the presence of 10% serum. A, Control monocytes differentiated in autologous serum. B, Patient macrophages (before therapy) differentiated in autologous serum. C, Monocytes from patients after atorvastatin therapy differentiated in autologous serum. D, Control macrophages differentiated in the patients’ serum. E, Control macrophages differentiated in autologous serum plus LDL at a cholesterol concentration similar to the patient (180 mg%). F, Patient macrophages differentiated in control serum.

**Figure 2.** LDL oxidation by macrophages from patients with hypercholesterolemia versus controls: effect of atorvastatin therapy. A, Cells ($2 \times 10^6$/35 mm dish) were incubated with LDL (100 \( \mu \)g of protein/mL) for 20 hours at 37°C and cell-mediated LDL oxidation was determined by the TBARS assay. B, The kinetic of LDL oxidation was also monitored after 4, 7, and 20 hours of incubation. Results are given as mean \( \pm \) SEM (n=10). *P<0.01 versus controls, # P<0.01 after atorvastatin therapy versus before therapy.

PON 2 Expression in Macrophages From Patients With Hypercholesterolemia vs Controls: Effect of Atorvastatin Therapy

Analysis of PON2 mRNA expression in monocytes/macrophages from patients with hypercholesterolemia revealed a significant two-fold decrease in PON2 mRNA levels, compared with the levels found in control macrophages (Figure 3A). Atorvastatin therapy resulted in a significant elevation in macrophage PON2 mRNA levels, by 76%, compared with the levels before therapy (Figure 3A). Similarly, PON2 lactonase activity in the patients’ macrophages was significantly lower, by 44%, compared with the controls’ cells (Figure 3B), and atorvastatin therapy significantly increased...
this activity, by 200%, to a level even greater than that of the control cells (Figure 3B).

**In Vitro Effect of Atorvastatin on HMDM Cholesterol Content, Oxidative Stress, and PON2 Expression**

Monocytes from untreated patients with hypercholesterolemia were grown for 8 days in the presence of 10% autologous serum together with increasing concentrations (0 to 10 µmol/L) of atorvastatin. Atorvastatin resulted in a significant dose-dependent decrease, up to 79%, in cellular cholesterol content (Figure 4A).

The cells that were differentiated in the presence of atorvastatin oxidized LDL to a lesser extent (up to 66%) than that observed in the absence of the drug, an effect that was atorvastatin dose-dependent (Figure 4B).

Cell differentiation in the presence of increasing atorvastatin concentrations resulted in a significant dose-dependent increase up to 2.1-fold, in PON2 mRNA levels (Figure 4C).

---

**Figure 3.** PON2 mRNA expression and PON2 activity in macrophages from patients with hypercholesterolemia patients versus controls: effect of atorvastatin therapy. A, Total RNA was extracted from HMDM and densitometric analysis of the PON2 bands intensity is provided. Macrophage PON2 lactonase activity was determined in the cells (10^7) sonicate. Results are given as mean±SEM (n=10). *P<0.01 versus controls, #P<0.01 after atorvastatin therapy versus before therapy.

**Figure 4.** The in vitro effect of atorvastatin on HMDM cholesterol content, cellular oxidative stress, and macrophage PON2 expression. Monocytes from nontreated patients with hypercholesterolemia were incubated in RPMI with 10% autologous serum in the presence of increasing concentrations of atorvastatin (0 to 10 µmol/L) for 8 days. A, The cells were washed and lipids were extracted (2x10^6 cells/35 mm dish) for cholesterol determination. HMDM (2x10^6/35 mm dish) were also incubated with LDL (100 µg of protein/mL) in RPMI medium containing 5 µmol/L CuSO4 for up to 6 hours at 37°C. B, Cell-mediated LDL oxidation was determined by the TBARS assay. Total RNA was extracted from the cells and densitometric analysis of the PON2 bands intensity is provided. C, insert: PON2 and β-actin bands. D, Cellular PON2 lactonase activity was also determined in cells' (10^7) sonicate. Results are given as mean±SEM from three different experiments. *P<0.01 versus “0” concentration.
Similarly, PON2 lactonase activity atorvastatin dose-dependently increased by up to 3.6 fold (Figure 4D).

Effect of Cellular Cholesterol Content on PON2 Expression in Human Macrophages

As PON2 expression was lower in HMDM from the patients versus controls, in spite of their increased macrophage oxidative stress, we questioned whether this phenomenon could have resulted from the increased cholesterol content in the patients’ cells. For this purpose, HMDM from healthy subjects were incubated with no addition (control), with Ox-LDL (25 μg of protein/mL), or with Ac-LDL (100 μg of protein/mL). Ac-LDL and Ox-LDL increased cellular cholesterol content by 77% and only 15%, respectively (from 34.6 ± 4.2 in control cells to 61.2 ± 4.8 and 39.8 ± 5.1 μg/mg cell protein). Macrophage lipid peroxides levels were increased by 9% and 170% on cell incubation with Ac-LDL or with Ox-LDL, respectively (from 10.5 ± 1.5 in control cells to 11.4 ± 1.1 and 28.4 ± 2.5 nmol/mg cell protein).

On macrophage incubation with Ox-LDL, as expected under oxidative stress, PON2 mRNA levels were increased by 30% (Figure 5A). In contrast, HMDM incubation with Ac-LDL resulted in a significant reduction, by 250%, in PON2 mRNA levels compared with control cells (Figure 5A). Similarly, Ox-LDL resulted in a 37% increase in macrophage PON2 activity, whereas Ac-LDL resulted in a 40% reduction in PON2 activity, compared with control cells (Figure 5B).

Using a fixed concentration of Ox-LDL (25 μg of protein/mL) together with increasing concentrations of Ac-LDL (0 to 100 μg of protein/mL), cellular cholesterol content increased dose-dependently, up to 2.3 fold (Figure 5C). In parallel, HMDM PON2 mRNA levels Ac-LDL dose-dependently decreased, by up to 76%, to levels even below those observed in macrophages that were incubated without lipoproteins (Figure 5D). As different lipoproteins (LDL, Ac-LDL, Ox-LDL), bind to different macrophage receptors and their cellular uptake and transport is also different, we analyzed additional means of macrophage cholesterol accumulation.

Incubation of HMDM with aggregated LDL (Agg-LDL, 100 μg of protein/mL for 20 hours) resulted in a 2-fold increase in macrophage cholesterol content up to 60 ± 5 μg/mg cell protein, and in parallel, cellular PON2 activity was significantly (P<0.01, n=3) reduced by 22% (to 0.071 ± 0.005 U/mg cell protein). Finally, the casual effect of macrophage cholesterol content on cellular PON2 activity was also studied using atorvastatin-treated cells in the presence of mevalonate (to bypass the inhibition of cholesterol biosynthesis): Control monocytes were differentiated for 8 days without or with atorvastatin (5 μmol/L), or with atorvastatin together with mevalonate (50 μmol/L). Atorvastatin addition to the incubation medium resulted in a reduction in cellular cholesterol content by 66% (from 30 ± 2 in untreated macrophages to 10 ± 1 μg/mg cell protein, n=3). In parallel, PON2 lactonase activity increased by 40% (from 0.089 ± 0.010 to 0.125 ± 0.011 U/mg cell protein, n=3). The addition of mevalonate to the above system, however, increased the cellular cholesterol content and PON2 lactonase activity back to control values (25.2 ± 2.1 U/mg cell protein, and 0.093 ± 0.012 U/mg cell protein, n=3, respectively).

Discussion

The present study demonstrated increased cholesterol content and enhanced oxidative stress in association with reduced PON2 expression for the first time in monocytes/macrophages (HMDM) from patients with hypercholesterolemia. The reduced expression of PON2 in the patients’ macrophages was shown to result from cellular cholesterol accumulation and not from the increased oxidative stress. Atorvastatin therapy decreased HMDM cholesterol content in association with an increase in cellular PON2 expression.
reduction in cellular oxidative stress, and inhibition of macrophage foam cell formation. Both serum PON1 and PON3 activities were lower in the patients versus controls and this phenomenon may be the result of their inactivation by oxidized lipids.\textsuperscript{26} Atorvastatin therapy resulted in a decrease in serum lipid peroxidation,\textsuperscript{21} which could have resulted from serum LDL cholesterol lowering, by the drug-induced removal (increased in LDL receptors) of “aged LDL,” which is more prone to oxidation, thus reducing substrate availability for oxidation. The antioxidative effect of atorvastatin might also be a direct effect of the drug metabolites,\textsuperscript{20} and/or the result of the drug-induced increment in serum paraoxonase activity, as was previously demonstrated for PON1 activity.\textsuperscript{21,22}

Inflammation was shown to contribute to the pathogenesis of coronary heart disease,\textsuperscript{27} and elevated serum levels of CRP were shown to be associated with increased cardiovascular risk.\textsuperscript{28} In the present study, atorvastatin therapy significantly reduced the increased levels of serum CRP observed in patients with hypercholesterolemia. Similarly, statin therapy in adults with dyslipidemia resulted in a reduction in serum CRP levels.\textsuperscript{28} As inflammation could be associated with oxidative stress, this finding may suggest a possible role for CRP in oxidative stress-induced macrophage foam cell formation.

As increased cholesterol levels and enhanced oxidative stress were shown in the patients’ serum, we hypothesized that these phenomena will also exist in the patients’ monocytes/macrophages; cells which are the hallmark of early atherogenesis. Indeed, the patients’ monocytes were differentiated into large, cholesterol- and lipid peroxides-loaded macrophage foam cells. This phenomenon could be related mostly to the patients’ cell characteristics and not to the patients’ serum effect.

Control monocytes that were differentiated in the patient’s serum or in autologous control serum containing the same LDL cholesterol concentration as in the patient serum were converted to normal macrophages and not to foam cells. Furthermore, the patient monocytes, when differentiated even in control serum, were converted into foam cells. Although the patient serum enhances macrophage cholesterol accumulation and cellular oxidative stress, these effects of the patients’ serum were minor in comparison to the contribution of the patients’ cells themselves.

The patients’ macrophages oxidize LDL faster and to a greater extent than control macrophages, in accordance with previous evidence that lipid peroxides-rich macrophages can oxidize LDL at an enhanced rate.\textsuperscript{4,5} Furthermore, in the patient cells, increased uptake of Ox-LDL due to upregulation of CD36 was recently demonstrated.\textsuperscript{21} Atorvastatin therapy reduced both cellular cholesterol and lipid peroxides content and decreased the number of macrophage foam cells formed. This may be the result of the drug-induced reduction in serum cholesterol and lipid peroxidation, and also a direct effect of the drug on the cells. Atorvastatin therapy significantly reduced the extent of macrophage-mediated LDL oxidation and this may have resulted from a decrease in the expression of essential NADPH-oxidase subunits, and reduced superoxide anion release.\textsuperscript{4,29,30} Similarly, simvastatin inhibits HMDM-mediated oxidation of LDL at physiological dosages.\textsuperscript{31} Furthermore, atorvastatin therapy was shown to decrease CD36 expression and the uptake of Ox-LDL by the patient HMDM.\textsuperscript{21}

All three paraoxonases can protect against oxidative stress.\textsuperscript{9–15,18} However, in human macrophage, only PON2 but not PON1 and PON3, is expressed. The decreased PON2 expression in HMDM from patients with hypercholesterolemia could have possibly resulted from the enhanced cellular oxidative stress and/or increased macrophage cholesterol content in patients versus control cells. However, it was recently shown\textsuperscript{19} that, unlike serum PON1 and PON3, as well as mouse macrophage PON3, which are all inactivated by oxidative stress,\textsuperscript{18,26} macrophage PON2 expression is increased under oxidative stress.\textsuperscript{18} We thus questioned whether the reduced macrophage PON2 in the patients versus controls HMDM could be associated with the increased cellular cholesterol content observed in these patients. In the present study, macrophage cholesterol content was similarly increased by cell incubation with Ac-LDL or Agg-LDL and cellular PON2 activity was significantly decreased. However, the extent of PON2 attenuation is different, as Agg-LDL and Ac-LDL are internalized by macrophages via different receptors/pathways, leading to different cellular cholesterol localization,\textsuperscript{12,33} suggesting that only total cellular cholesterol content affect PON2 expression, but the specific macrophage compartmentalization of the cholesterol also determines the inhibitory effect on cellular PON2.

Analyses of cellular free and esterified cholesterol in the patients’ versus controls’ HMDM and studies using atorvastatin, in the absence or presence of mevalonate, revealed that macrophage-free cholesterol content determines the extent of the inhibitory effect on PON2 expression. Recently, it was shown that simvastatin upregulated the activity of the promoter of the PON1 gene in HepG2, by increasing SREBP2, and this phenomenon was blocked by mevalonate.\textsuperscript{34} We thus hypothesize that, in the present study, atorvastatin upregulated HMDM PON2 expression in a similar mechanism. PON1 is regulated as it is turned on during development and a polymorphism of the 3’ regulatory region may also affect the levels of PON1.\textsuperscript{35}

Atorvastatin therapy reduced the cellular cholesterol levels resulting in increased cellular PON2 expression, both in vivo and in vitro. The reduction in macrophage lipid peroxides content after atorvastatin therapy may also be the result of the increase in HMDM PON2 activity that can possibly hydrolyze cellular lipid peroxides like PON1.\textsuperscript{12,13}

In conclusion then, in patients with hypercholesterolemia, cholesterol and oxidized lipid accumulation in their monocytes/macrophages, as well as reduced cellular PON2 expression, might be the underlined contributors to their accelerated atherogenesis. The anti-atherosclerotic properties of atorvastatin therapy thus could be related to an initial decrease in macrophage cholesterol content which results in upregulation of cellular PON2 expression, leading to decreased macrophage oxidative stress and, hence, to the inhibition of foam cell formation.
Acknowledgments
Research support was obtained from our internal sources (Technion Rappaport Institute and Technion Faculty of Medicine, Haifa, Israel).

References
Decreased Macrophage Paraoxonase 2 Expression in Patients With Hypercholesterolemia Is the Result of Their Increased Cellular Cholesterol Content: Effect of Atorvastatin Therapy

Mira Rosenblat, Tony Hayek, Khetam Hussein and Michael Aviram

Arterioscler Thromb Vasc Biol. published online October 30, 2003;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2003/10/30/01.ATV.0000104011.88939.06.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2004/01/14/24.1.175.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Methods

**Subjects** - Ten male healthy subjects (controls) and 10 male hypercholesterolemic patients (age 20-50 years old) participated in the study. The patients were non-smokers, with no diabetes, hypertension, or coronary artery disease, and they did not take any medication. Their serum total and LDL cholesterol levels were above 280mg/dl and 170mg/dl respectively, and their serum triglyceride levels were lower than 200mg/dl. The patients received 20mg/day of atorvastatin for 2 months.

**Serum Paraoxonases activities** - Paraoxonase activity towards paraoxon (PON1 specific) and towards lovastatin (statinase activity, PON3 specific) were determined as previously described (15).

**Serum lipids peroxidation** - The extent of lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay (23).

**Human monocyte derived macrophages (HMDM)** - HMDM were separated from the blood (21) and plated at $10^6$/mL in RPMI medium with 10% FCS. After 2h of incubation at 37°C, nonadherent cells were removed, and RPMI with 10% autologous serum (unless specific differently) was added. Macrophages were analyzed 8 days after plating.

**Macrophage cholesterol and lipid peroxides content** - HMDM ($2\times10^5$) lipids were extracted with hexane: isopropanol (3:2, v/v). The hexane phase was dried under nitrogen. The free and esterified cholesterol were separated by thin layer chromatography using the appropriate standards. Then, the spots were scraped from the plate and the lipids extracted. The amount of cholesterol was measured using a commercial kit from Roche, Manheim, Germany. Lipid peroxides content was determined as previously described (2).

**Macrophage PON2 expression** - PON2 mRNA levels and PON2 lactonase activity (towards dihydrocoumarin) were determined as previously described (18).

**Macrophage mediated LDL oxidation** - Human LDL was isolated from plasma of normolipidemic subjects (4). Before oxidation, the LDL (1mg of protein/ml) was dialyzed against PBS. LDL (100µg of protein/mL) was incubated without cells or with HMDM ($2\times10^6$) in the presence of 5µM CuSO$_4$ for up to 20h at 37°C and the extent of cell-mediated oxidation was then determined (4).

**Preparation of oxidized LDL (Ox-LDL), acetylated LDL (Ac-LDL) and aggregated LDL (Agg-LDL)** - Before oxidation LDL (1mg of protein/ml) was dialyzed against PBS
and then incubated with 10µM CuSO₄ at 37°C, under air for 20h. The extent of LDL oxidation was determined by the TBARS assay (23). Ac-LDL was prepared by the method of Basu et al. (24) and Agg-LDL was prepared extensive by vortexing (25). **Statistical analysis-** Statistical analysis was performed using the student paired t-test when comparing the mean of two groups. ANOVA was used when more than 2 groups were compared and results are given as mean ± SEM.
Table I: Serum lipids concentration and biochemical parameters in hypercholesterolemic patients vs. controls: effect of atorvastatin therapy.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>TC mg/dl</th>
<th>HDL-C mg/dl</th>
<th>LDL-C mg/dl</th>
<th>TG mg/dl</th>
<th>Glucose mg/dl</th>
<th>AST U/L</th>
<th>BUN mg/dl</th>
<th>Creatinine mg/dl</th>
<th>CRP mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>175±5</td>
<td>45±3</td>
<td>110±6</td>
<td>98±16</td>
<td>92±3</td>
<td>26±3</td>
<td>13±3</td>
<td>0.89±0.04</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>Patients</td>
<td>348±30*</td>
<td>45±3</td>
<td>271±30*</td>
<td>162±18</td>
<td>92±2</td>
<td>19±4</td>
<td>15±2</td>
<td>0.85±0.04</td>
<td>5.7±1.3*</td>
</tr>
<tr>
<td>Patients-Atorvastatin</td>
<td>237±17#</td>
<td>46±3</td>
<td>162±16#</td>
<td>141±17</td>
<td>91±3</td>
<td>21±3</td>
<td>14±2</td>
<td>0.77±0.04</td>
<td>3.3±0.9#</td>
</tr>
</tbody>
</table>

Serum samples were collected from 10 healthy subjects (Controls), and from 10 hypercholesterolemic patients before and after atorvastatin therapy (20mg/day, for 2 months). Serum total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), triglyceride (TG), glucose, aspartate amino transferase (AST), Blood Urea Nitrogen (BUN), Creatinine, and C-reactive protein (CRP), were measured. Results are given as mean ± SEM (n=10). *p<0.01 vs. Controls, # p<0.01 vs. Patients (before therapy).