Atorvastatin Preferentially Reduces LDL-Associated Platelet-Activating Factor Acetylhydrolase Activity in Dyslipidemias of Type IIA and Type IIB


Abstract—Human plasma platelet-activating factor acetylhydrolase (PAF-AH) is a phospholipase A2 that is primarily associated with low density lipoprotein (LDL). PAF-AH activity has also been found in high density lipoprotein (HDL), although it has recently been indicated that there is no PAF-AH protein in HDL. Plasma paraoxonase 1 (PON1) is an HDL-associated esterase, which also exhibits PAF-AH–like activity. The effect of atorvastatin (20 mg per day for 4 months) on PAF-AH and PON1 activities in patients with dyslipidemia of type IIA (n=55) or type IIB (n=21) was studied. In both patient groups, atorvastatin significantly reduced plasma PAF-AH activity because of the decrease in LDL plasma levels and the preferential decrease in PAF-AH activity on dense LDL subfractions (LDL-4 and LDL-5). Drug therapy did not affect HDL-associated PAF-AH activity or serum PON1 activities toward paraoxon and phenylacetate in either patient group. However, because of the reduction in LDL cholesterol levels, the ratios of HDL-associated PAF-AH and serum PON1 activities to LDL cholesterol levels were significantly increased after drug administration. The reduction of the HDL-associated PAF-AH activity and the elevation in the ratios of HDL-associated PAF-AH and PON1 activities to LDL plasma levels may represent a new dimension in the antiatherogenic effect of atorvastatin. (Arterioscler Thromb Vasc Biol. 2002;22:306-311.)

Key Words: hyperlipidemia • monocytes/macrophages • platelet-activating factor acetylhydrolase • paraoxonase • atorvastatin

Platelet-activating factor (PAF) is a potent lipid mediator involved in inflammatory diseases1 as well as in atherogenesis.2 In plasma, PAF is hydrolyzed and inactivated by PAF-acetylhydrolase (PAF-AH, EC 3.1.1.47), a Ca2+-dependent phospholipase A2.3 PAF-AH has a marked preference for phospholipids with short-chain moieties at the sn-2 position, and with the exception of PAF, PAF-AH can hydrolyze oxidized phospholipids containing a polyunsaturated fatty acyl residue at this position.3 Plasma PAF-AH is complexed to lipoproteins4,5; thus, it is also denoted as lipoprotein-associated phospholipase A2.6 The role of this enzyme in inflammatory and atherosclerotic diseases remains to be established. Indeed, PAF-AH may represent a potent anti-inflammatory and antiatherogenic enzyme because it degrades PAF and proinflammatory oxidized phospholipids, molecules formed during the oxidation of LDL.7 Consistent with the hypothesis that PAF-AH may exert a cardioprotective role are clinical studies showing that loss of plasma PAF-AH activity due to a G994→T mutation in the PAF-AH gene may constitute a genetic determinant of atherosclerotic disease in the Japanese population.8 In contrast to these findings, PAF-AH may exert proinflammatory and proatherogenic actions as a result of the hydrolysis of oxidized phospholipids, because bioactive oxidized free fatty acids9 and lysophosphatidylcholine are generated.9,10 A recent clinical study indicating that the mass of plasma PAF-AH could be a potential risk factor for coronary artery disease is consistent with the latter observations.11

Human plasma paraoxonase 1 (PON1) is an esterase that is present in plasma in association with HDL.12 In vitro, PON1 hydrolyzes paraoxon and phenylacetate; by contrast, the in vivo substrates for this enzyme are considered to be phospholipid hydroperoxides and cholesteryl ester hydroperoxides, molecules that are formed during LDL oxidation.13 Recently, it has been shown that PON1 hydrolyses PAF, thus exhibiting PAF-AH–like activity.14 In this context, it has been further indicated that there is no PAF-AH protein in HDL, suggesting that the HDL-associated PAF-AH activity is due to PON1.14 It has been shown that PON1 is able to retard LDL oxidation15 and to reduce the proinflammatory effects of oxidized LDL.16 Furthermore, PON1 may inhibit HDL oxidation, thereby preserving its antiatherogenic functions.17

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From the Departments of Internal Medicine (V.T., M.E.) and Cardiology (J.A.G.) and the Laboratory of Biological Chemistry (E.B.), Medical School, and the Laboratory of Biochemistry (S.-A.P.K., A.P.T., A.D.T.), Department of Chemistry, University of Ioannina, Ioannina, Greece, and the Institut National de la Santé et de la Recherche (M.J.C.), Unité 551, Hôpital de la Pitié, Paris, France.
Correspondence to Dr Alexandros D. Tselepis, Laboratory of Biochemistry, Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece.
E-mail atselep@cc.uoi.gr
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In the present study, we investigated the effect of atorvastatin therapy on the circulating activities of PAF-AH and PON1 in patients with dyslipidemia of type IIA and type IIB. We also studied the drug effect on PAF-AH production and secretion by the patients’ peripheral blood monocyte-derived macrophages in culture.

Methods

Patients

Seventy-six unrelated hyperlipidemic patients attending the Lipid Clinic of the University Hospital of Ioannina participated in the present study. According to their lipid levels, patients were divided into the primary hypercholesterolemia (dyslipidemia type IIA) group (LDL cholesterol > 160 mg/dL) and the combined hyperlipidemia (dyslipidemia type IIB) group (LDL cholesterol > 160 mg/dL and triglycerides > 200 mg/dL). Among dyslipidemic type I patients, 36 had a diagnosis of heterozygous familial hypercholesterolemia (FH), and 19 had a diagnosis of polygenic hypercholesterolemia (NonFH). Atorvastatin treatment was initiated in all patients at a single dose of 20 mg at bedtime for 4 months; then a second blood analysis was performed after a 14-hour overnight fast. For comparison of PAF-AH and PON1 activities between patient groups and normolipidemic subjects, 45 apparently healthy normolipidemic individuals receiving no medication served as control subjects. For supplementary online material involving patient selection criteria and patient characteristics, please see the text and Table I at http://www.atvb.ahajournals.org.

Subfractionation of Plasma Lipoproteins

Lipoproteins were fractionated by isopycnic density gradient ultracentrifugation into the following subfractions: VLDL+IDL, density (d) < 1.019 g/mL; LDL-1, d = 1.019 to 1.023 g/mL; LDL-2, d = 1.023 to 1.029 g/mL; LDL-3, d = 1.029 to 1.039 g/mL; LDL-4, d = 1.039 to 1.050 g/mL; LDL-5, d = 1.050 to 1.063 g/mL; HDL-2, d = 1.063 to 1.100 g/mL; HDL-3, d = 1.100 to 1.167 g/mL; and VHDL, d = 1.167 to 1.190 g/mL. For more details on lipoprotein subfractionation, please see http://www.atvb.ahajournals.org.

Isolation and Culture of Human Blood Monocytes

Peripheral blood from patients (before the initiation of therapy with atorvastatin) and from healthy volunteers was drawn into EDTA-containing tubes. Blood was centrifuged, and monocytes were isolated from the buffy coats as previously described.22 For more details on cell culture conditions, please see http://www.atvb.ahajournals.org.

RNA Isolation, First-Strand cDNA Synthesis, and Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated with RNA Plus (Q-Biogene) from adherent macrophages before and 48 hours after atorvastatin treatment. Detection and quantification of PAF-AH mRNA was performed by reverse transcription–polymerase chain reaction.23 For more details, please see http://www.atvb.ahajournals.org.

Measurement of PAF-AH and PON1 Activities

PAF-AH activity was measured by the trichloroacetic acid (TCA) precipitation procedure with the use of [3H]PAF (100 nmol/L final concentration) as a substrate. PON1 activities were determined by using paraoxon and phenylacetate as substrates. For more details on enzyme assays, please see http://www.atvb.ahajournals.org.

Analytical Methods and Statistical Analysis

For analytical methods and statistical analysis, please see online supplement at http://www.atvb.ahajournals.org.

Results

Effect of Atorvastatin Therapy on Plasma Lipid Profile

As expected, atorvastatin significantly decreased serum total cholesterol and LDL cholesterol levels as well as serum apoB and apoE levels in both patient groups. A significant reduction in serum triglycerides in both groups was equally observed; the reduction was more profound in dyslipidemic type IIB patients (see online Table II at http://www.atvb.ahajournals.org).

Plasma PAF-AH Activity

As shown in the Table, total plasma PAF-AH activity at baseline was higher in both patient groups compared with the control group (P<0.0001 for both comparisons). Furthermore, baseline enzyme activity in type IIB patients was significantly lower than that in type IIA patients (P<0.01). When the primary hypercholesterolemia group was subdivided into FH and NonFH patients, the enzyme activity in FH patients (95.7±30.5 nmol/mL per minute) was significantly higher than that in NonFH patients (70.2±15.2 nmol/mL per minute, P<0.004). No difference in the HDL-associated PAF-AH activity (HDL-PAF-AH), expressed per milliliter of plasma, was observed between type IIA patients and control subjects, whereas compared with either control subjects or type IIA patients, type IIB patients exhibited lower HDL-PAF-AH (P<0.002 for both comparisons, Table). Furthermore, when type IIA patients were subdivided into FH and NonFH, no differences were observed in the HDL-associated enzyme activity (data not shown). It is important to note that the ratio of HDL-PAF-AH to LDL cholesterol levels (expressed as nanomoles per milligram LDL cholesterol per minute) was significantly lower in both patient groups compared with the control group (P<0.0001 for both comparisons), whereas no difference in this ratio was observed between the patient groups (Table).

Interestingly, atorvastatin treatment led to a reduction in plasma PAF-AH activity in both patient groups (Table). Enzyme activity in type IIB patients was decreased by 42.4% to reach control values (P<0.001). A decrease in enzyme activity (~28.6%, P<0.001) was also observed in type IIA patients; however, in this patient group, enzyme activity remained significantly elevated compared with that in the control group even after atorvastatin therapy (Table). This phenomenon was due to the enzyme activity in FH patients, which remained elevated compared with enzyme activity in the control subjects (69.1±22.7 versus 43.8±13.0 nmol/mL per minute, respectively; P<0.04), whereas the posttreatment values in the NonFH patients (48.4±12.9 nmol/mL per minute) were similar to those in the control group. The reduction of enzyme activity was significantly correlated with the decrease in plasma LDL cholesterol levels (Figure 1).

In contrast to total plasma enzyme activity, atorvastatin therapy did not affect HDL-PAF-AH activity in either patient group (Table). Consequently, the ratio of HDL-PAF-AH to LDL cholesterol levels was significantly increased in both groups (31% in type IIA patients and 91% in type IIB patients, P<0.0002 for both comparisons; Table). Similar
results were obtained when HDL-PAF-AH was expressed per LDL mass or apoB plasma levels (data not shown). Despite the significant increase in the above ratio after atorvastatin therapy, it remained lower in both patient groups compared with the control group (Table).

**Serum PON1 Activity**

Serum PON1 activity toward paraoxon was lower (although not statistically significant) in the type IIA patients than in the control subjects (Table). When this group was subdivided into FH and NonFH patients, the enzyme activity in FH group (67.7±36.1 U/L) was lower than that in the control group (89.9±44.5 U/L) and in the NonFH group (89.2±54.8 U/L, P<0.04 for both comparisons), a finding that is in agreement with previous results. No difference in PON1 activity toward paraoxon was observed between control subjects and type IIB patients. Unlike PON1 activity toward paraoxon, the baseline values of PON1 activity toward phenylacetate were significantly lower in both patient groups compared with the control group (Table). It is important to note that atorvastatin did not affect either of the PON1 activities in the patient groups. However, because of the reduction in LDL cholesterol levels, the ratio of both PON1 activities to LDL cholesterol levels (in units per milligram LDL cholesterol) was significantly increased after drug administration, although it remained lower than the corresponding control values (Table). The same results were obtained when both PON1 activities were expressed per LDL mass or apoB plasma levels (data not shown).

### Effect of Atorvastatin on the Mass Distribution of Plasma Lipoprotein Subspecies

To further investigate the effect of atorvastatin on the PAF-AH activity associated with individual lipoprotein subclasses, we fractionated plasma lipoproteins in each patient before and after atorvastatin therapy. As shown in online Table III (please see http://www.atvb.ahajournals.org), atorvastatin therapy in type IIA patients significantly reduced the ratio of both PON1 activities to LDL cholesterol, as shown in online Table III.

### Table III (please see http://www.atvb.ahajournals.org)

<table>
<thead>
<tr>
<th>Type IIA</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Treatment</td>
<td>After Treatment</td>
</tr>
<tr>
<td>Plasma PAF-AH activity, nmol · mL⁻¹ · min⁻¹</td>
<td>43.8±13.0</td>
</tr>
<tr>
<td>HDL-PAF-AH activity, nmol · mL⁻¹ · min⁻¹</td>
<td>3.5±1.3</td>
</tr>
<tr>
<td>Ratio HDL-PAF-AH/LDL-cholesterol, nmol · mg⁻¹ · min⁻¹</td>
<td>3.20±1.65</td>
</tr>
<tr>
<td>PON-1 activity (paraoxon), U/L</td>
<td>90.0±44.5</td>
</tr>
<tr>
<td>Ratio PON-1 (paraoxon)/LDL cholesterol, U/mg</td>
<td>0.08±0.04</td>
</tr>
<tr>
<td>PON-1 activity (phenylacetate), U/mL</td>
<td>58.7±11.7</td>
</tr>
<tr>
<td>Ratio PON-1 (phenylacetate)/LDL cholesterol, U/mg</td>
<td>51.8±11.6</td>
</tr>
</tbody>
</table>

Values are mean±SD. A paired t test was used for comparisons between baseline and posttreatment values, whereas 1-way ANOVA followed by a least significant difference test was used for comparisons between individual groups. A value of P<0.05 was considered to be significant. *P<0.001 and †P<0.05 vs controls; ††P<0.001 vs baseline values; and †††P<0.001 and ‡‡‡P<0.05 vs type IIA at the same time (baseline or after treatment).
PAF-AH Activity in Plasma Lipoprotein Subspecies

PAF-AH activity was determined in all apoB-containing and apoA-I–containing plasma lipoprotein subspecies. The enzyme activity, expressed per milligram of protein, per milliliter of lipoprotein mass, or per milliliters of plasma, was preferentially associated with the dense LDL-5 subfraction in both patient groups. Figure 2 illustrates the results of PAF-AH activity expressed per milligram of protein. There were no significant differences in enzyme activity in each subfraction between the 2 groups. However, compared with the control group, both patient groups exhibited significantly higher enzyme activity associated with VLDL + IDL, LDL-1, LDL-2, and LDL-5 subfractions (Figure 2). Atorvastatin significantly reduced the total enzyme activity (expressed per milliliter of plasma) associated with the VLDL + IDL subfraction as well as with each LDL subfraction in both patient groups. Remarkably, a significant reduction was also observed in the enzyme activity (expressed either per milligram of protein or per milligram of lipoprotein mass) in the dense LDL subfractions (ie, LDL-4 and LDL-5); this phenomenon was not observed in the other apoB-containing lipoprotein subfractions. Figure 3 illustrates the PAF-AH activity in these subfractions, expressed per milligram of protein. Finally, no changes were observed in the enzyme activity associated with each HDL subfraction after atorvastatin therapy in either patient group.

Effect of Atorvastatin on PAF-AH Secretion From Macrophages

It has been shown that macrophages represent the major source of the plasma- and LDL-associated PAF-AH activity.24 To investigate whether the reduction in plasma- and LDL-associated PAF-AH activity by atorvastatin was due to any drug effect on enzyme secretion, we studied the effect of atorvastatin on PAF-AH production and secretion by peripheral blood monocyte–derived macrophages. Cells isolated from 8 subjects of each group were used in these studies. Incubations were performed for 24 and 48 hours with various concentrations of atorvastatin, ranging from 0.005 to 10 μmol/L. There was a steady increase in total (secreted plus cell-associated) PAF-AH activity in untreated cells (incubated with dimethyl sulfoxide in the absence of atorvastatin) from normolipidemic control subjects, attaining 244±66 nmol/mg DNA per hour at 24 hours of culture and 494±97 nmol/mg DNA per hour at 48 hours of culture. This increase reflected mainly the secreted enzyme activity, which was 146±32 nmol/mg DNA per hour (60% of total) at 24 hours of culture and 395±73 nmol/mg DNA per hour (80% of total) at 48 hours of culture. Similar results were obtained for untreated macrophages from both patient groups (data not shown). Cell treatment with atorvastatin at low doses (0.005 and 0.05 μmol/L) for 48 hours significantly increased the secreted enzyme activity to a similar extent in all studied groups (Figure 4), whereas a slight but not significant increase from 98.8±26 nmol/mg DNA per hour to 110±38 and 117±41 nmol/mg DNA per hour (at 0.005 and 0.05 μmol/L atorvastatin, respectively) in the cell-associated enzyme activity was also observed in all studied groups. Overall, compared with no treatment, atorvastatin treatment significantly increased the total enzyme activity by 33±8% in all studied groups (P<0.01). Surprisingly, this phenomenon was not observed at higher atorvastatin doses (up to 10 μmol/L). It must be noted that in enzyme assays performed in the presence of 0.005 to 10 μmol/L atorvastatin with the use of Figure 4. Effect of atorvastatin on PAF-AH secretion from human macrophages in culture. Cells were incubated in the presence of various atorvastatin concentrations for 48 hours. PAF-AH activity in the cell supernatant was determined by the TCA precipitation method. Values are mean±SD (n=8 for each studied group). *P<0.01 compared with untreated cells (0 μmol/L atorvastatin).
of macrophage supernatant as the source of the enzyme (containing 7.8 nmol/mL per minute of PAF-AH activity), no effect of the drug on PAF-AH activity was observed. To investigate whether the increase in PAF-AH activity induced by low doses of atorvastatin was due to an increase in the enzyme expression, we evaluated the PAF-AH mRNA levels. No differences were observed in the enzyme expression between untreated and atorvastatin-treated cells in all group studied (data not shown), suggesting that the increase in PAF-AH production and secretion induced by atorvastatin is due to a drug effect at the posttranscriptional level.

Discussion

In the present study, we show for the first time that atorvastatin therapy in patients with primary hypercholesterolemia as well as in those with combined hyperlipidemia significantly reduces total plasma PAF-AH activity. This phenomenon is due to not only a reduction in plasma LDL levels but also a decrease in the enzyme activity associated with the dense LDL subspecies. Atorvastatin therapy did not affect HDL-PAF-AH activity, nor did it affect total serum PON1 activities toward paraoxon and phenylacetate in either patient group.

The higher plasma PAF-AH activity observed in type II A patients, compared with control subjects, is in accordance with our published results. In addition, our data reveal that compared with control subjects, type II B patients exhibit significantly higher plasma enzyme activity. Consistent with our previous studies on normolipidemic and hypercholesterolemic subjects, the majority of LDL-associated PAF-AH activity in both patient groups was associated with the dense LDL subfractions.

Atorvastatin therapy significantly reduced plasma PAF-AH activity in both patient groups. A similar effect was observed for lovastatin therapy in patients with non-insulin-dependent diabetes mellitus and in obese individuals as well as in hypercholesterolemic patients. The present study shows for the first time that atorvastatin reduces plasma PAF-AH activity by decreasing the LDL-associated enzyme, which has been recently shown to be a potential risk factor for coronary artery disease. Indeed, plasma levels of PAF-AH activity mainly reflect LDL-associated enzyme activity. The reduction in enzyme activity by atorvastatin could be due to either a decrease in PAF-AH secretion from its main cellular sources or the drug-induced enhancement of LDL clearance from plasma. According to our results, atorvastatin did not reduce PAF-AH secretion from macrophages; thus, the decrease in plasma PAF-AH cannot be attributed to a reduction in enzyme secretion by this drug. Consequently, the enhancement in the rate of LDL removal from the circulation may represent the main mechanism by which atorvastatin reduces plasma PAF-AH activity. This is also supported by the positive correlation observed between the reduction of plasma LDL cholesterol levels and that of plasma PAF-AH activity. However, the decrease in plasma LDL may not represent the sole mechanism by which atorvastatin reduces the plasma PAF-AH activity. Indeed, the decrease of plasma LDL cholesterol levels in type II A patients is due to the reduction in large and intermediate LDL subspecies (LDL-1 to LDL-3), i.e., particles that are poor in PAF-AH activity. By contrast, atorvastatin does not affect the plasma levels of PAF-AH–rich dense LDL particles, whereas it significantly reduces the enzyme activity associated with these particles. Consequently, the decrease of plasma PAF-AH activity in this patient group is due to not only the reduction in the cholesterol-rich LDL particles but also the decrease in the enzyme activity associated with the dense LDL particles. This phenomenon may also contribute to the decrease in total plasma PAF-AH activity (observed in type II B patients) in which the reduction of plasma LDL concerns all LDL subspecies.

PAF-AH activity can be influenced by alterations in the lipid composition of the lipoproteins. We excluded the possibility that the reduction in the enzyme activity on LDL-4 and LDL-5 induced by atorvastatin could be due to alterations in their lipid composition, because no major alteration in the chemical composition of any LDL subfraction was observed after drug therapy (data not shown). It has been reported that apoB conformation is distinct among LDL subspecies and that apoB (and especially its carboxyl terminus) plays a key role in the association of PAF-AH with LDL. Thus, a differential effect of atorvastatin on the interaction of PAF-AH with the apoB moiety of LDL subspecies, which might result in a specific reduction in enzyme activity on LDL-4 and LDL-5, cannot be excluded.

Unlike total plasma- and LDL-associated PAF-AH, there is a paucity of data on the plasma levels of HDL-associated PAF-AH activity in atherosclerotic diseases. According to our results, the ratio of HDL-PAF-AH to LDL cholesterol levels was significantly lower in both patient groups compared with the control group. This observation is consistent with the published data on FH patients as well as with data on patients with unstable angina. Several lines of evidence suggest that HDL protects LDL against oxidation and inhibits the biological activity of oxidized LDL. These effects are at least partially mediated by the HDL-associated PAF-AH activity. The antiatherogenic role of HDL-PAF-AH is further supported by recent studies in apoE-deficient mice. Besides PAF-AH, 2 other HDL-associated enzymes, lecithin-cholesterol acyltransferase and PON1, exhibit PAF-AH–like activity; thus, the HDL-associated PAF-AH activity may represent a pool of similar catalytic activities expressed by 3 different enzymes. In this context, it has recently been shown that there is no PAF-AH protein in HDL, suggesting that the PAF-AH protein may not contribute to the HDL-associated PAF-AH activity. Atorvastatin therapy affected neither total plasma HDL cholesterol levels nor HDL-PAF-AH activity. Consequently, it significantly increased the ratios of HDL-PAF-AH to LDL cholesterol levels because of a decrease in the LDL cholesterol levels. Similarly, atorvastatin affected neither PON1 activity toward paraoxon nor PON1 activity toward phenyl acetate. However, because of the decrease in plasma LDL cholesterol levels, a significant increase in the ratios of both PON1 activities to LDL cholesterol levels was observed. Thus, atorvastatin may exert a beneficial antioxidant and antiatherogenic effect by improving the HDL potency against LDL oxidation and against the atherogenic biological effects of oxidized LDL.

In conclusion, atorvastatin therapy in dyslipidemic type II A and type II B patients significantly decreases LDL-associated PAF-AH activity. This effect along with the improvement in the ratios of HDL-associated PAF-AH and
PON1 activities to LDL plasma levels may represent a new and important overall antiatherogenic effect of this potent hypolipidemic agent.

Acknowledgments

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Methods

Patients

Seventy-six unrelated hyperlipidemic patients participated in the study. Secondary causes of dyslipidemia (diabetes, liver or renal diseases, abnormalities of thyroid function, alcoholism, etc) were excluded by personal history, physical examination and appropriate laboratory analyses. None of the study participants was obese (BMI>30 Kg/m²), hypertensive (blood pressure>140/90 mmHg on repeated measurements) or was taking lipid-lowering drugs or other medications known to interfere with lipid metabolism, including hormonal replacement therapy. Finally, no patient had any clinical evidence of cardiovascular disease. After the initial screening, patients were advised to follow the National Cholesterol Education Program (NCEP) step 1 diet (which limits dietary intake of cholesterol to 300mg/day, that of saturated fats to 10% of total energy intake and total fats to 30% of total energy intake) for three months. At the end of this period, a complete laboratory baseline analysis was performed. Atorvastatin treatment was initiated in all patients at a single dose of 20mg at bedtime for 4 months; then a second blood analysis was performed after a 14-hour overnight fast. The main criterion used to assess compliance to atorvastatin treatment was the percentage of pills missed during the study period according to the patients’ interview validated by tablet count. The compliance was greater than 98% and no patient missed more than two tablets during the study period. During the treatment period a food record rating score was determined three times in order to assess compliance to the diet. There was no significant change in patients’ body weight or smoking habits during the study. Additionally, food record rating scores remained stable throughout the trial (the mean cholesterol intake was less than 260 mg per day in all three times determined, whereas the mean consumption
of saturated and total fats was constantly below 10% and 30%, respectively). The study was approved by the Ethics Committee of the University Hospital of Ioannina and all study patients gave written informed consent for participation in the study.

Subfractionation of plasma lipoproteins

Lipoproteins were fractionated by isopycnic density gradient ultracentrifugation as described (1). After ultracentrifugation, 24 fractions of 0.4 mL each were collected and analyzed for their protein content. Subsequently, equal volumes of certain gradient fractions were pooled to constitute the lipoprotein subfractions, as follows: fractions 1 and 2 (VLDL+IDL; d<1.019 g/mL), 3 and 4 (LDL-1; d=1.019-1.023 g/mL), 5 and 6 (LDL-2; d=1.023-1.029 g/mL), 7 and 8 (LDL-3; d=1.029-1.039 g/mL), 9 and 10 (LDL-4; d =1.039-1.050 g/mL), 11 and 12 (LDL-5; d =1.050-1.063 g/mL), 13 to 16 (HDL-2; d=1.063-1.100 g/mL), 17 to 22 (HDL-3; d=1.100-1.167 g/mL), 23 and 24 (VHDL; d=1.167-1.190 g/mL). All subfractions were extensively dialyzed in 10 mmol/L PBS containing 2 mmol/L EDTA, pH 7.4 at 4°C, then filter-sterilized and stored at 4°C for up to 2 weeks.

Isolation and culture of human blood monocytes

Peripheral blood monocytes were cultured and grown in 24-well plastic tissue culture dishes (1 x 10⁶/well) (for PAF-AH activity) or in 6-well plastic tissue culture dishes (3 x 10⁶/well) (for mRNA purification) with RPMI medium, containing L-glutamine, 40 µg/mL gentamycin and 10% human serum (heat inactivated). After 3 days, the medium was replaced and at 6 days of culture it was removed, and the cells were washed twice with PBS. Treatments of these cells with atorvastatin (provided as a powder by Parke-Davis and dissolved in DMSO) were performed for 24 and 48 hours in RPMI medium containing 10%
human serum in which the endogenous PAF-AH was completely and irreversibly inactivated by incubation with 1mmol/L Pefabloc as previously described (2). After treatment, supernatants were recovered and the cell layers were washed twice with PBS and then detached and lysed by the addition of 0.2 mL of a lysis solution containing 1% EDTA and 0.1% Triton X-100. Both supernatants and cell lysates were centrifuged (500 x g for 10 min at 4ºC), stored at 4ºC and analyzed for PAF-AH activity and lactate dehydrogenase activity (kit from Boehringer Mannheim GmbH, Germany) within 24 hours from the collection. Cell lysates were further analyzed for their protein content, determined with the bicinchoninic acid (BCA) method (Pierce), and DNA content, determined as previously described (3). Viability under all culture conditions was determined by trypan blue dye exclusion and the absence of lactate dehydrogenase release; viability was > 95%.

**RNA isolation, first strand cDNA synthesis and RT-PCR**

Total RNA was isolated with RNA Plus from adherent macrophages. First strand cDNA synthesis was performed with 5µg of total RNA in the presence of oligo dT (2µg) and 500µU of SuperScript M-MLV reverse transcriptase (4). Detection and quantification of PAF-AH mRNA was performed by RT-PCR in the presence of two specific oligonucleotides, AHPAF2 (TCTTGGAACA CACTGGCTTA TGGGC) and AHPAF6R (GGACTGAACCC TCGATTGTA A) and compared to the amplification of actin as previously described (4,5).

**Assay of PAF-acetylhydrolase activity**

PAF-AH activity was measured by the trichloroacetic acid (TCA) precipitation procedure using [3H]-PAF (100 mmol/L final concentration) as a substrate. Four µg of protein from each lipoprotein subfraction or 50 µL of either plasma diluted 1:50, v/v with HEPES buffer,
pH 7.4, or the HDL-containing supernatant after treatment of plasma with magnesium chloride/dextran sulphate (diluted 1:3, v/v with HEPES) were mixed with HEPES in a final volume of 90 µL and used as the source of the enzyme. Incubations were performed for 10 min and PAF-AH activity was expressed as nmol PAF degraded per min per mg of protein or mL of plasma. PAF-AH activity in macrophage supernatants or cell lysates was determined by the same method using 50 µL from each sample as the source of the enzyme. Incubations were performed for 1 hour and PAF-AH activity was expressed as nmol PAF degraded per min per mg of cell DNA.

**Measurement of PON1 activity**

PON1 activity was determined using paraoxon and phenyl acetate as substrates. The rate of paraoxon hydrolysis was measured by monitoring the increase in absorbance at 412 nm, 25°C for 90 sec in a microelisa spectrophotometer (SpectraMax 190, Molecular Devices). The assays were performed in a final volume of 250 µL containing 25 µL of serum, 5.5 mmol/L paraoxon, 2 mmol/L CaCl2 and 100 mmol/L Tris-HCl, pH 8.0. The amount of p-nitrophenol generated was calculated from the molar absorptivity at pH 8.0, which is 17,000 L mol⁻¹ cm⁻¹ (6). PON1 activity towards paraoxon was expressed in U/L serum with 1U/L defined as 1 µmol of p-nitrophenol formed per min. The assays for PON1 activity towards phenyl acetate were also performed in a final volume of 250 µL containing 50 µL of serum (diluted 1/100 v/v), 1mmol/L phenyl acetate, 2 mmol/L CaCl₂ and 20 mmol/L Tris-HCl, pH 8.0. (6). The rate of phenyl acetate hydrolysis was recorded at 270 nm at 25°C for 90 sec. Enzymatic activity was calculated from the molar extinction coefficient 1310 L mol⁻¹ cm⁻¹. PON1 activity towards phenyl acetate was expressed in U/mL with 1 U/mL defined as 1 µmol of phenyl acetate hydrolyzed per min.
Analytical methods

Serum total cholesterol, triglycerides, and HDL-cholesterol were determined with an automatic analyzer (Olympus AU560, Hamburg, Germany). Serum LDL-cholesterol was calculated using the Friedewald formula (provided that triglycerides’ levels were lower than 350mg/dL. In three Type IIB patients with higher triglycerides’ values LDL-cholesterol was not determined). Serum apolipoproteins B (apo B), AI (apo AI) and E (apo E) were measured by immunonephelometry (Behring Diagnostics GmbH, Liederbach, Germany). Serum Lp(a) levels were measured by an enzyme immunoassay method (Macra Lp(a), Terumo Medical Corporation Diagnostic Division, Elkton, MD, USA) (7). The total cholesterol, free cholesterol, triglyceride and phospholipid content in each lipoprotein subfraction were measured enzymatically, whereas cholesteryl ester mass was calculated as (total-free cholesterol mass) x 1.67. The protein content of the lipoprotein subfractions was measured by the BCA method. The lipoprotein mass of each subfraction was calculated as the sum of the mass of the individual lipid and protein components. Total LDL or HDL mass was calculated as the sum of the mass of the corresponding subfractions.

Statistical analysis

Data were expressed as mean ± SD, except for Lp(a) which was expressed as the median and range. Statistical analyses were performed using paired t-test for comparisons between baseline and post treatment values, while one-way analysis of variance (ANOVA) followed by LSD test was used for comparisons between individual groups. Because of the skewed distribution of Lp(a), the nonparametric Wilcoxon matched-pairs test was used for comparisons between baseline and post treatment values. Correlations between PAF-AH
activity and lipid parameters were estimated using linear regression analysis, while Yates corrected chi-square test was used for differences in proportions.

References


<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Type IIA</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>55</td>
<td>21</td>
</tr>
<tr>
<td>Men / Women</td>
<td>22 / 33</td>
<td>10 / 11</td>
</tr>
<tr>
<td>Mean Age (range), years</td>
<td>49 (10-68)</td>
<td>52 (31-69)</td>
</tr>
<tr>
<td>BMI, Kg/m²</td>
<td>24.1±3.6</td>
<td>26.6±4.3</td>
</tr>
<tr>
<td>Smokers / Non Smokers</td>
<td>29 / 26</td>
<td>12 / 9</td>
</tr>
</tbody>
</table>
### TABLE II. Effect of atorvastatin on plasma lipid and lipoprotein levels in patients with dyslipidemias Type IIA and IIB.

<table>
<thead>
<tr>
<th></th>
<th>Type IIA</th>
<th></th>
<th></th>
<th>Type IIB</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>%Change</td>
<td>P</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>347.5±73.2</td>
<td>260.7±54.6</td>
<td>-25.0</td>
<td>0.000</td>
<td>318.4±45.4</td>
<td>220.1±43.2</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>121.9±45.6</td>
<td>111.9±36.6</td>
<td>-8.20</td>
<td>0.048</td>
<td>287.9±83.2</td>
<td>182.8±73.3</td>
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<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>50.8±13.1</td>
<td>49.8±11.4</td>
<td>-2.0</td>
<td>NS</td>
<td>44.1±10.1</td>
<td>47.7±15.8</td>
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<tr>
<td>LDL-cholesterol, mg/dL</td>
<td>272.2±71.7</td>
<td>189.7±55.4</td>
<td>-30.3</td>
<td>0.000</td>
<td>211.6±33.1</td>
<td>132.4±36.9</td>
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<tr>
<td>ApoAI, mg/dL</td>
<td>146.1±27.1</td>
<td>145.4±22.4</td>
<td>-0.5</td>
<td>NS</td>
<td>154.2±27.6</td>
<td>158.3±37.1</td>
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<tr>
<td>ApoB, mg/dL</td>
<td>179.8±39.7</td>
<td>134.5±34.1</td>
<td>-25.2</td>
<td>0.000</td>
<td>170.4±31.6</td>
<td>113.1±24.5</td>
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<tr>
<td>ApoE, mg/dL</td>
<td>49.9±11.1</td>
<td>41.7±8.3</td>
<td>-16.4</td>
<td>0.000</td>
<td>62.8±15.3</td>
<td>43.2±10.4</td>
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<tr>
<td>Lp(a), mg/dL</td>
<td>12.0 (0.8-50.0)</td>
<td>15.5 (1.3-67.0)</td>
<td>+29.2</td>
<td>NS</td>
<td>13.2 (0.8-67.0)</td>
<td>16.1 (1.2-41.2)</td>
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</table>

Values represent the mean ± SD. Paired t-test was used for comparisons between baseline and post treatment values except for Lp(a) levels where Wilcoxon matched-pairs test was used. A P value < 0.05 was considered to be significant.
TABLE III. Effect of atorvastatin on lipoprotein mass in patients with dyslipidemias Type IIA and IIB.

<table>
<thead>
<tr>
<th></th>
<th>Type IIA</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
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<tr>
<td>VLDL+IDL</td>
<td>42.3±21.7</td>
<td>25.7±8.5</td>
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<tr>
<td>Total LDL</td>
<td>461.2±83.2</td>
<td>340.1±80.1</td>
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<tr>
<td>LDL-1</td>
<td>31.0±13.9</td>
<td>14.1±4.6</td>
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<tr>
<td>LDL-2</td>
<td>72.7±20.4</td>
<td>29.8±7.3</td>
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<tr>
<td>LDL-3</td>
<td>197.9±27.6</td>
<td>140.3±35.1</td>
</tr>
<tr>
<td>LDL-4</td>
<td>123.8±29.2</td>
<td>112.6±54.5</td>
</tr>
<tr>
<td>LDL-5</td>
<td>35.7±8.5</td>
<td>43.1±5.5</td>
</tr>
<tr>
<td>Total HDL</td>
<td>234.1±25.3</td>
<td>228.4±25.7</td>
</tr>
<tr>
<td>HDL-2</td>
<td>68.1±12.3</td>
<td>60.3±12.2</td>
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<tr>
<td>HDL-3</td>
<td>138.7±13.1</td>
<td>139.2±17.5</td>
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<tr>
<td>VHDL</td>
<td>27.2±5.8</td>
<td>28.9±8.3</td>
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</tbody>
</table>

Values represent mean ± SD. Paired t-test was used for comparisons between baseline and post treatment values and a P value <0.05 was considered to be significant.