Differential Effect of Hypolipidemic Drugs on Lipoprotein-Associated Phospholipase A₂

Vasilios G. Saougos, Afroditi P. Tambaki, Mihalis Kalogirou, Michael Kostapanos, Irene F. Gazi, Robert L. Wolfert, Moses Elisaf, Alexandros D. Tselepis

Objective—Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is a predictor for incident atherosclerotic disease. We investigated the effect of 3 hypolipidemic drugs that exert their action through different mechanisms on plasma and lipoprotein-associated Lp-PLA₂ activity and mass.

Methods and Results—In 50 patients with Type IIA dyslipidemia were administered rosuvastatin (10 mg daily), whereas in 50 Type IIA dyslipidemic patients exhibiting intolerance to previous statin therapy were administered ezetimibe as monotherapy (10 mg daily). Fifty patients with Type IV dyslipidemia were given micronised fenofibrate (200 mg daily). Low- and high-density lipoprotein (LDL and HDL, respectively) subclass analysis was performed electrophoretically, whereas lipoprotein subfractions were isolated by ultracentrifugation. Ezetimibe reduced plasma Lp-PLA₂ activity and mass attributable to the reduction in plasma levels of all LDL subfractions. Rosuvastatin reduced enzyme activity and mass because of the decrease in plasma levels of all LDL subfractions and especially the Lp-PLA₂ on dense LDL subfraction (LDL-5). Fenofibrate preferentially reduced the Lp-PLA₂ activity and mass associated with the VLDL+IDL and LDL-5 subfractions. Among studied drugs only fenofibrate increased HDL-associated Lp-PLA₂ (HDL-Lp-PLA₂) activity and mass attributable to a preferential increase in Lp-PLA₂ associated with the HDL-3c subfraction.

Conclusion—Ezetimibe, rosuvastatin, and fenofibrate reduce Lp-PLA₂ activity and mass associated with the atherogenic apoB-lipoproteins. Furthermore, fenofibrate improves the enzyme specific activity on apoB-lipoproteins and induces the HDL-Lp-PLA₂. The clinical implications of these effects remain to be established. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: hyperlipidemia ■ lipoproteins ■ PAF-acetylhydrolase ■ Lp-PLA₂ ■ ezetimibe ■ fenofibrate ■ rosuvastatin

Platelet-activating factor (PAF) acetylhydrolase exhibits a Ca²⁺-independent phospholipase A₂ activity and degrades PAF and oxidized phospholipids by catalyzing the hydrolysis of the ester bond at the sn-2 position. PAF-acetylhydrolase in plasma is complexed to lipoproteins; thus it is also referred as lipoprotein-associated phospholipase A₂ (Lp-PLA₂). Lp-PLA₂ is associated mainly with apolipoprotein B (apoB)-containing lipoproteins and primarily with low-density lipoprotein (LDL), whereas a small proportion of circulating enzyme activity is also associated with high-density lipoprotein (HDL). The majority of the LDL-associated Lp-PLA₂ activity is bound to the atherogenic small-dense LDL (sdLDL) particles, and we recently showed that the enzyme activity is a marker of sdLDL particles in plasma. Lp-PLA₂ is principally produced by hematopoietic cells including monocytes-macrophages. Lp-PLA₂ has been identified in atherosclerotic plaques; however, its role in atherosclerosis is still under investigation. In this regard, it is suggested that this enzyme might have an antiatherogenic role because it degrades and inactivates proinflammatory PAF and oxidized phospholipids; other studies showed that Lp-PLA₂ may have a proinflammatory and proatherogenic role, because it generates lysophosphatidylycholine (lysoPC) and bioactive oxidized fatty residues. Data from large White population studies demonstrated an independent association between plasma Lp-PLA₂ and cardiovascular disease (CVD) risk. In contrast to total plasma enzyme, which mainly represents the LDL-associated Lp-PLA₂, several lines of evidence suggest that HDL-associated Lp-PLA₂ activity, although at low levels, contributes to the atherogenic effects of this lipoprotein. However, the clinical value of HDL-associated Lp-PLA₂ as a potent inhibitor of the atherosclerotic process remains to be established.

The clinical implications of these effects remain to be established.
influence plasma Lp-PLA₂.₁⁵,₁⁶ Thus several statins (atorvastatin, lovastatin, simvastatin, and fluvastatin) reduce the enzyme activity in plasma in parallel to a reduction in LDL-cholesterol levels.¹⁷–²¹ In contrast, pravastatin increased plasma Lp-PLA₂ activity,²² whereas other investigators suggested that pravastatin reduced plasma Lp-PLA₂ mass.²³ Fibrates reduce plasma Lp-PLA₂ activity but significantly increase the HDL-associated Lp-PLA₂ activity.¹⁶,²⁴ These data suggest that there are significant differences on the effect of various hypolipidemic drugs on Lp-PLA₂ activity or mass in total plasma and in lipoprotein subfractions. To provide more insights into the effect of hypolipidemic drugs on plasma Lp-PLA₂, we investigated the effect of 3 agents that exert their action through different mechanisms (rosuvastatin, ezetimibe, and fenofibrate) on Lp-PLA₂ activity and mass in total plasma and in lipoprotein subfractions in hyperlipidemic patients.

Materials and Methods
For a detailed Materials and Methods section, please see the data supplement, available online at http://atvb.ahajournals.org.

Results
Effect of Hypolipidemic Therapy on Serum Lipid Profile
As shown in supplemental Table I (available online at http://atvb.ahajournals.org), ezetimibe significantly decreased serum total cholesterol, LDL-cholesterol, non-HDL-cholesterol, and apoB levels. Furthermore, ezetimibe induced a slight but significant reduction in serum HDL-cholesterol levels whereas it did not affect apoA-I levels. Rosuvastatin significantly decreased serum triglycerides, total cholesterol, LDL-cholesterol, non–HDL-cholesterol, and apoB levels. The reduction in these serum lipid parameters was more profound compared with the ezetimibe group. Fenofibrate significantly decreased serum levels of triglycerides, total cholesterol, LDL-cholesterol, non–HDL-cholesterol, and apoB. Furthermore, fenofibrate induced a significant increase in the serum levels of HDL-cholesterol and apoA-I, a phenomenon not observed in the other 2 groups. Finally, no alterations in the baseline Lp(a) levels were induced by any lipid-lowering therapy (supplemental Table I).

Table 1. Effect of Hypolipidemic Drugs on ApoB- and apoA-I-Containing Lipoprotein Subclasses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ezetimibe (n=50)</th>
<th>Rosuvastatin (n=50)</th>
<th>Fenofibrate (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL-cholesterol, mmol/L</td>
<td>1.3±0.2</td>
<td>1.3±0.2</td>
<td>1.6±0.3*</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>1.6±0.3</td>
<td>1.4±0.3*</td>
<td>1.2±0.3*</td>
</tr>
<tr>
<td>Buoyant LDL-cholesterol, mmol/L</td>
<td>3.5±0.8</td>
<td>2.9±0.6*</td>
<td>2.6±1.0</td>
</tr>
<tr>
<td>sdLDL-cholesterol, mmol/L</td>
<td>0.20±0.08</td>
<td>0.13±0.08*</td>
<td>0.52±0.30</td>
</tr>
<tr>
<td>sdLDL proportion, %</td>
<td>5±2</td>
<td>4±2</td>
<td>12±4</td>
</tr>
<tr>
<td>Mean LDL size, Å</td>
<td>269±4</td>
<td>269±3</td>
<td>258±6</td>
</tr>
<tr>
<td>HDL-2 cholesterol, mmol/L</td>
<td>0.57±0.23</td>
<td>0.65±0.23</td>
<td>0.28±0.16</td>
</tr>
<tr>
<td>HDL-3 cholesterol, mmol/L</td>
<td>1.2±0.3</td>
<td>1.1±0.2*</td>
<td>1.0±0.25</td>
</tr>
</tbody>
</table>

Data represent the mean± SD values. *P<0.05, †P<0.01, and ‡P<0.001 compared with baseline values. §P<0.01 and ¶P<0.001 compared with the baseline values of either ezetimibe or rosuvastatin group.

Effect of Hypolipidemic Therapy on Lipoprotein Subclasses
Type IV dyslipidemic patients had higher plasma levels of VLDL-cholesterol and lower levels of IDL-cholesterol and buoyant LDL-cholesterol at baseline compared with type IIA dyslipidemic patients of either the ezetimibe or the rosuvastatin group. Type IV dyslipidemic patients had significantly higher baseline levels of sdLDL-cholesterol and a higher proportion of sdLDL whereas the mean LDL size was lower compared with type IIA patients. Treatment with either ezetimibe or rosuvastatin significantly reduced the mass of all apoB-containing lipoprotein subclasses, with the exception of VLDL-cholesterol, which was not reduced by ezetimibe. However, neither drug affected sdLDL proportion and mean LDL size. Fenofibrate significantly reduced VLDL-cholesterol levels but it did not affect IDL-cholesterol or buoyant LDL-cholesterol levels. Finally, fenofibrate reduced sdLDL-cholesterol levels (and therefore the proportion of sdLDL) and increased mean LDL size (Table 1).

Type IV dyslipidemic patients had lower levels of HDL-2 and HDL-3 subclasses at baseline compared with type IIA patients of either ezetimibe or rosuvastatin group. Ezetimibe significantly reduced the concentration of small HDL-3 subclass without affecting the concentrations of the large HDL-2 subclass. In contrast, rosuvastatin did not affect the mass of either HDL subclass. Finally, fenofibrate significantly increased the mass of both HDL subclasses.

Lipoprotein Profile
Ezetimibe significantly decreased total plasma and non-HDL-Lp-PLA₂ activity and mass; however, it did not affect the enzyme specific activity or the Lp-PLA₂ activity to apoB ratio. Fenofibrate significantly reduced Lp-PLA₂ activity or the ratios of Lp-PLA₂ activity or mass to apoB. Furthermore, fenofibrate induced a significant increase in the enzyme specific activity or the Lp-PLA₂ activity to apoB ratio (in nmol/mg/min, 0.58±0.13 before versus 0.60±0.15 posttreatment) and the Lp-PLA₂ mass to apoB ratio (in ng/mg, 3.75±0.51 before versus 3.83±0.98 posttreatment; Table 2). Rosuvastatin significantly reduced total plasma and non-HDL Lp-PLA₂ activity and mass; these reductions were more pronounced compared with those induced by ezetimibe. Like ezetimibe, rosuvastatin did not alter the enzyme specific activity or the ratios of Lp-PLA₂ activity or mass to apoB. Fenofibrate significantly reduced total plasma and non-HDL Lp-PLA₂ activity. Importantly, fenofibrate induced a signifi-
TABLE 2  Effect of Hypolipidaemic Drugs on Plasma and Lipoprotein-Associated Lp-PLA₂ Activity, Mass, and Specific Activity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ezetimibe (n=50)</th>
<th>Rosuvastatin (n=50)</th>
<th>Fenofibrate (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Lp-PLA₂ activity, nmol/mL/min</td>
<td>59.7 ± 10.5</td>
<td>66.0 ± 15.4</td>
<td>59 ± 13.8</td>
</tr>
<tr>
<td>Plasma Lp-PLA₂ mass, ng/mL</td>
<td>397 ± 72</td>
<td>449 ± 59</td>
<td>456 ± 73</td>
</tr>
<tr>
<td>Plasma Lp-PLA₂ specific activity, nmol/ng/min</td>
<td>0.15 ± 0.04</td>
<td>0.15 ± 0.03</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>Non-HDL-Lp-PLA₂ activity, nmol/mL/min</td>
<td>56.7 ± 10.3</td>
<td>63.5 ± 21.4</td>
<td>55.7 ± 14.0</td>
</tr>
<tr>
<td>Non-HDL-Lp-PLA₂ mass, ng/mL</td>
<td>324 ± 71</td>
<td>386 ± 77</td>
<td>411 ± 65</td>
</tr>
<tr>
<td>HDL-Lp-PLA₂ activity, nmol/mL/min</td>
<td>2.95 ± 0.82</td>
<td>2.41 ± 0.72</td>
<td>2.10 ± 0.80</td>
</tr>
<tr>
<td>HDL Lp-PLA₂ mass, ng/mL</td>
<td>72.7 ± 21.5</td>
<td>63.0 ± 24.3</td>
<td>49.5 ± 16.5</td>
</tr>
<tr>
<td>HDL Lp-PLA₂ specific activity, nmol/ng/min</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.02</td>
</tr>
</tbody>
</table>

Data represent the mean±SD. *P<0.05, †P<0.01, and ‡P<0.001 compared with baseline values.

Ezetimibe induced a slight but significant reduction in HDL-Lp-PLA₂ activity and mass but it did not affect the enzyme specific activity of the apoB-containing lipoprotein subfractions, as shown in Figure 1.

Ezetimibe significantly reduced the Lp-PLA₂ activity and mass in all patient groups at baseline (Figure 2A and 2B), a finding that is in accordance with previously published results. Consequently, ezetimibe significantly reduced the enzyme activity and mass when it was expressed per mg of protein (data not shown).

Ranolazine reduced the enzyme activity and mass (expressed per mL of plasma) associated with the apoA-1-containing lipoprotein subfractions (Figure 2A and 2B), whereas it did not affect the enzyme activity or mass when it was expressed per mg of protein (data not shown).

Table 2 shows the effect of hypolipidaemic drugs on plasma and lipoprotein-associated Lp-PLA₂ activity, mass, and specific activity.

**Lp-PLA₂ Activity and Mass on Lipoprotein Subfractions**

We further investigated the effect of hypolipidaemic drugs on the Lp-PLA₂ activity and mass associated with LDL subfractions (1 to 5). Remarkably, it significantly reduced the enzyme activity and mass (expressed per mg of protein) in the dense LDL-5 subfraction (Figure 2A and 2B), whereas it did not affect the enzyme activity or mass when it was expressed per mL of plasma (data not shown).

Ranolazine reduced the enzyme activity and mass (expressed per mL of plasma) associated with the apoB-containing lipoprotein subfractions (Figure 2A and 2B). Remarkably, it significantly reduced the enzyme activity and mass expressed per mg of protein in the dense LDL-5 subfraction (Figure 2A and 2B), whereas it did not affect the enzyme activity or mass when it was expressed per mL of plasma (data not shown).

**Importantly, fenofibrate increased HDL-Lp-PLA₂ activity and mass (expressed per mg of protein) in the dense LDL-5 subfraction in all patient groups (Figure 2A and 2B), whereas it did not affect the enzyme-specific activity of Lp-PLA₂ associated with this subfraction (Figure 2C).**

**Fenofibrate significantly reduced the Lp-PLA₂ activity and mass associated with the VLDL+IDL subfraction (Figure 3A and 3B), whereas it did not affect the enzyme-specific activity of this subfraction (Figure 3C). Fenofibrate did not affect the enzyme activity, mass, or specific activity of the enzyme associated with large and intermediate LDL particles (LDL-1 to LDL-4); however, it significantly reduced the enzyme activity and mass associated with LDL-5 (Figure 3A and 3B). Furthermore, fenofibrate induced a significant increase in the specific activity of Lp-PLA₂ associated with this subfraction (Figure 3C).**

**Finally, it should be noted that no detectable amounts of Lp(a) were found in any lipoprotein subfraction at baseline or after treatment with any hypolipidaemic drug. Thus it is unlikely that the alterations in the Lp-PLA₂ associated with LDL subfractions induced by hypolipidaemic therapy are influenced by changes in the Lp(a) levels and in the Lp(a)-associated Lp-PLA₂.**
Among the HDL subfractions, Lp-PLA$_2$ activity and mass in all patient groups at baseline were preferentially associated with HDL-3c, a finding which is in accordance with our previously published results.$^6,^{17,24}$ Ezetimibe slightly, albeit significantly, reduced Lp-PLA$_2$ activity and mass associated with the HDL-3c subfraction, a phenomenon not observed after rosuvastatin administration. By contrast, fenofibrate significantly increased Lp-PLA$_2$ activity and mass associated with HDL-3c (Figure 3A and 3B). Neither drug influenced the activity or mass of Lp-PLA$_2$ associated with the other HDL subfractions. Finally, neither drug affected the enzyme specific activity on any HDL subfraction (in nmol/ng/min, 0.32±0.12 for HDL-2b, 0.24±0.10 for HDL-2a, 0.12±0.04 for HDL-3a, 0.07±0.02 for HDL-3b, and 0.04±0.01 for HDL-3c).

It should be emphasized that the mean value of enzyme specific activity in total HDL formed by mixing of equal volumes of all HDL subfractions (0.15 nmol/ng/min), is much lower compared with that of total LDL formed by mixing of equal volumes of the subfractions LDL-1 to LDL-5 (1.34 nmol/ng/min). Importantly, when both lipoproteins were dissociated by treatment with 0.1% Triton X-100, the Lp-PLA$_2$ specific activity on LDL was significantly reduced to 0.93 nmol/ng/min ($P<0.03$) because of the increase by 36% in the enzyme mass (from $23.8±4.2$ to $32.4±5.1$ ng/mg of total protein, $P<0.03$). Neither the enzyme activity in both lipoproteins nor the enzyme mass in HDL was significantly influenced by this treatment. These results show that the method used for the determination of Lp-PLA$_2$ mass may not detect all active enzyme in LDL, a phenomenon not observed for HDL.
Discussion

The present study compares for the first time the effect of 3 hypolipidemic drugs (ezetimibe, rosuvastatin, fenofibrate), which exert their action through different mechanisms, on plasma Lp-PLA₂ activity and mass. All drugs reduce Lp-PLA₂ activity and mass associated with the atherogenic apoB-containing lipoproteins. Furthermore, fenofibrate increases the specific activity of the enzyme associated with these lipoproteins and specifically that of the most atherogenic dense LDL-5 subfraction.

Fenofibrate reduces Lp-PLA₂ activity and mass associated with apoB-containing lipoproteins, an effect that could be mainly attributed to the preferential reduction of the enzyme associated with LDL-5 particles,²⁴ i.e., those particles carrying the majority of LDL-associated enzyme.²⁶ In accordance with our previously published results,²⁴ the present study shows that the above reduction is attributed to the fenofibrate action to decrease sdLDL and to increase large buoyant LDL particles, which have a higher clearance rate than sdLDL.²⁶ A contributory role to the reduction of Lp-PLA₂ by fenofibrate plays also the decrease in enzyme associated with the triglyceride-rich VLDL + IDL subfraction (attributable to the drug-induced reduction in the plasma concentration of this subfraction).
An important observation of the present study is that the non-HDL-Lp-PLA2 specific activity is significantly increased by fenofibrate. According to our previous results, the specific activity of Lp-PLA2 associated with large buoyant LDL is higher than that of either LDL-5 or VLDLIDL subfraction. In this regard, the results of the present study show that the method used for the determination of Lp-PLA2 mass may not detect all active enzyme in LDL, a phenomenon not observed for HDL, suggesting that structural differences among lipoprotein species may significantly influence the determination of enzyme mass, a hypothesis that needs further investigation. Based on the above observations, we may suggest that the increase in non-HDL-Lp-PLA2 specific activity by fenofibrate is attributed to the drug-induced preferential reduction in the enzyme associated with LDL-5 and VLDL+IDL subfractions. A contributory role in the above phenomenon may also play the fenofibrate-induced increase in the specific activity of Lp-PLA2 associated with LDL-5.

In accordance with our previously published results, fenofibrate treatment increases the HDL-Lp-PLA2 activity. It also increases the HDL-Lp-PLA2 mass, thus it does not affect the enzyme specific activity. This effect is attributable to the drug-induced increase in plasma levels of both HDL-2 and HDL-3 subspecies as well as to the preferential enrichment of the HDL-3c in Lp-PLA2. We had previously suggested that the latter phenomenon is attributed to enzyme transfer from triglyceride-rich apoB-containing lipoproteins to HDL during

![Figure 3. Bar graphs illustrating the effect of fenofibrate therapy on the Lp-PLA2 activity (A), Lp-PLA2 mass (B), and Lp-PLA2 specific activity (C) associated with VLDL+IDL and dense LDL-5 subspecies in patients with Type IV dyslipidemia. Lipoprotein subspecies were fractionated by isopycnic density gradient ultracentrifugation of plasma. Enzymatic activity was determined by the trichloroacetic acid (TCA) precipitation procedure and enzyme mass by use of a dual monoclonal antibody immunoassay. Lp-PLA2 specific activity was expressed as a ratio of the enzyme activity to the enzyme mass. Values represent the mean±SD. *P<0.001 and **P<0.01 compared with respective baseline values.](image-url)
their enhanced lipolysis by lipoprotein lipase induced by fenofibrate. Although the role of the HDL-LpPLA2 in humans has not been established yet, data from in vitro experiments as well as in vivo studies in animal models suggest that this enzyme may significantly contribute to the antiatherogenic effects of HDL (reviewed in ). Consequently, the increase of HDL-Lp-PLA2 induced by fenofibrate may represent an important antiatherogenic effect of this drug, a hypothesis that needs further investigation.

The present study further demonstrates that the administration of rosuvastatin in type IIA dyslipidemic patients significantly reduces Lp-PLA2 activity and mass associated with apoB-containing lipoproteins. This reduction is the highest observed among all statins used in previous studies, and it could be primarily attributed to the drug-induced reduction in the plasma concentration of all LDL subfractions and to the preferential reduction of Lp-PLA2 induced by fenofibrate may represent an important antiatherogenic effect of this drug, a hypothesis that needs further investigation.

The present study further demonstrates that the administration of rosuvastatin in type IIA dyslipidemic patients significantly reduces Lp-PLA2 activity and mass associated with apoB-containing lipoproteins. This reduction is the highest observed among all statins used in previous studies, and it could be primarily attributed to the drug-induced reduction in the plasma concentration of all LDL subfractions and to the preferential reduction of Lp-PLA2 induced by fenofibrate. This mechanism may explain our results on the preferential reduction in Lp-PLA2 associated with LDL-5 (expressed per mg of protein) induced by rosuvastatin.

Ezetimibe is a drug that acts by inhibiting the absorption of cholesterol at the brush border of the intestinal wall. The present study shows for the first time that it reduces the plasma levels of Lp-PLA2 mass and activity (although to a lesser extent compared with rosuvastatin and fenofibrate), by reducing the plasma concentration of all apoB-containing lipoprotein subfractions. Furthermore, ezetimibe induces a slight but significant decrease in the HDL-Lp-PLA2 activity and mass. This follows the lowering effect of ezetimibe on plasma HDL-cholesterol levels a finding, which is not consistent in all studies and may reflect the relatively high pretreatment levels of HDL-cholesterol in our population resulting in a regression to the mean effect. Because ezetimibe does not influence the ratio of enzyme activity or mass to apoA-I levels, we suggest that the reduction in HDL-3 plasma concentration may account for the ezetimibe-induced decrease in the HDL-Lp-PLA2. This is further supported by the finding that ezetimibe decreases the enzyme associated only with HDL-3c subfraction. It should be noted that all patients treated with ezetimibe were statin-intolerant,
Therefore, the above results may not be representative of other groups given ezetimibe.

In addition to LDL and HDL, another carrier of Lp-PLA2 in plasma is Lp(a). Interestingly, we and others have demonstrated that Lp(a) is enriched in Lp-PLA2 compared with LDL. However, Lp(a) can influence the distribution of Lp-PLA2 between LDL and HDL in plasma only when its plasma levels exceed 8 mg/dL. Thus, it is unlikely that the Lp(a)-associated Lp-PLA2 could influence the enzyme changes induced by hypolipidemic drugs, because the present results showed that no alterations in Lp(a) levels were induced by any lipid-lowering therapy and no patient exhibited a baseline or posttreatment Lp(a) levels above 8 mg/dL. Finally, no detectable amounts of Lp(a) were found in any lipoprotein subclass at baseline or after treatment with any hypolipidemic drug.

Clinical studies have shown an independent association between plasma levels of Lp-PLA2 mass or activity and CVD. The present study further shows that the determination of Lp-PLA2 mass, activity, and specific activity on individual lipoprotein subfractions may be pathophysiologically and clinically important. In this regard, we have previously shown that the preferential enzyme distribution on sSDL particles compared with large buoyant apoB-containing has as a consequence an increased production of lysoPC, the main metabolite of Lp-PLA2, during oxidation of this subfraction. Several studies have supported the important role of lysoPC in atherogenesis, and more recently it was shown that local coronary production of lysoPC is associated with endothelial dysfunction and early atherosclerosis. Furthermore, the preferential association of Lp-PLA2 with HDL-3 subfraction as compared with other apoA-I-containing lipoproteins may contribute to the antiinflammatory and antioxidant effects of these particles. Finally the Lp(a)-associated Lp-PLA2 may play an important role in the metabolism of oxidized phospholipids in humans, in view of emerging data showing that oxidized phospholipids in plasma are preferentially sequestered on Lp(a). Taking into account our previous results showing that the type of dyslipidemia and the underlying metabolic defect significantly influence the enzyme distribution among lipoprotein subtypes, we suggest that the determination of enzyme parameters on specific lipoprotein subtypes may provide useful information on both pathophysiological and clinical basis, in addition to the valuable information provided from the measurement of Lp-PLA2 mass and activity in total plasma.

A limitation of the present study could be the selection of patients who were allocated to different therapeutic agents according to the NCEP ATP III goals that resulted in populations with different types of dyslipidemias. Thus it must be acknowledged that the different lipid abnormalities observed between Type IIA and Type IV patient groups may have contributed to the differential effect on the lipoprotein-associated Lp-PLA2 levels of fenofibrate (administered in Type IV patients) compared with rosuvastatin or ezetimibe (given to Type IIA individuals).

In conclusion the present study demonstrates for the first time that fenofibrate, rosuvastatin, and ezetimibe (acting through different mechanisms) reduce Lp-PLA2 activity and mass associated with the atherogenic apoB-containing lipoproteins, the rosuvastatin exhibiting the most potent effect. Additionally, fenofibrate increases the specific activity of the non-HDL–Lp-PLA2 as well as the HDL–Lp-PLA2 mass and activity. The clinical implications of these effects remain to be established.

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Disclosures

None.

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Methods

Patients

One hundred and fifty hyperlipidaemic patients attending the Outpatient Lipid Clinic of the University Hospital of Ioannina participated in the present study. Exclusion criteria were: prior atherosclerotic disease (myocardial infarction, unstable angina, ischemic stroke, peripheral arterial disease, percutaneous transluminal coronary angioplasty and coronary artery bypass graft), diagnosed diabetes mellitus (fasting glucose >6.9 mmol/L), liver disease (serum aminotranferase activity greater than 3-fold, e.g. >120 IU/L, normal range 5-40 IU/L), renal disease (serum creatinine levels greater than 132 mmol/L, normal range 53-106 mmol/L) and hypothyroidism (TSH greater than 5 μIU/ml, normal range 0.5-4.8 μIU/L). Moreover, patients receiving drugs that could affect lipid metabolism as well as renal or hepatic function were also excluded from the present study. Finally, all patients with serum levels of lipoprotein (a) [Lp(a)] over the cut-off point of 8 mg/dL were excluded from the study since according to our previously published results, higher concentrations of Lp(a) significantly affect the distribution of Lp-PLA2 among plasma lipoprotein subfractions separated by gradient ultracentrifugation. After the initial screening, patients were advised to follow the National Cholesterol Education Program (NCEP) step 1 diet for three months. At the end of this period, a complete laboratory baseline analysis was performed. According to their lipid levels, patients were divided into the following groups: 1. Primary hypercholesterolaemia [Type IIA dyslipidaemia, i.e. serum levels of low-density lipoprotein cholesterol (LDL-cholesterol) >160 mg/dL (4.1 mmol/L) and serum triglyceride levels <200 mg/dL (2.3mmol/L), n= 50, who were administered rosuvastatin (10 mg daily)]. 2. Type IIA dyslipidaemia [n= 50, intolerant in previous statin therapy [expressed as elevated creatine kinase (CK) levels]
3. Primary hypertriglyceridaemia [type IV dyslipidaemia, i.e. serum levels of LDL-cholesterol <160 mg/dL (4.1 mmol/L) and serum triglyceride levels ≥200 mg/dL (2.3 mmol/L), n= 50, who were given micronised fenofibrate (200 mg daily)] in order to achieve the NCEP Adult Treatment Panel III goals for the LDL-cholesterol levels² (Table I). After 2 months of active treatment, a second blood analysis was performed. The Ethics Committee of the University Hospital of Ioannina gave its approval for the conduction of the study and every participant gave written consent.

**Biochemical parameters**

All lipid and lipoprotein determinations were carried out after an overnight fast. Serum levels of total cholesterol, HDL-cholesterol and triglycerides were determined using an Olympus AU 600 analyzer (Olympus Diagnostica, Hamburg, Germany). LDL-cholesterol was calculated using the Friedewald formula. Apolipoproteins (apo) B and A-I were measured with a Behring Nephelometer BN100 (Liederbach, Germany). The protein content of the lipoprotein subfractions was measured by the bicinechonic acid method (Pierce)³. Lipoprotein [a] (Lp[a]) levels in serum and in lipoprotein subfractions were measured by an enzyme immunoassay method (Macra Lp[a], Terumo Medical Corporation Diagnostic Division, Elkton, MD, USA) as we have previously described¹.

**Electrophoretic analysis of apoB and apoA-I lipoprotein subclasses**

Lipoprotein subclass analysis, was performed electrophoretically by use of high-resolution 3% polyacrylamide gel tubes and the Lipoprint LDL System (Quantimetrix, Redondo Beach, CA), following the instructions provided by the
manufacturer. Analysis of the apoB-containing lipoproteins was performed as we recently described. After electrophoresis, very low-density lipoprotein (VLDL) remained in the origin [retention factor (Rf) = 0.0], whereas HDL migrated at the front (Rf = 1.0). In between, several bands can be detected: MID bands C, B, and A, which correspond mainly to intermediate-density lipoprotein (IDL), as well as up to 7 LDL bands. The LDL1 and LDL2 bands correspond to large, buoyant LDL particles, whereas bands LDL3 to LDL7 correspond to sdLDL particles. We determined the cholesterol mass of each apoB-lipoprotein subfraction, the mean LDL particle size (in Å), and the proportion (%) of the cholesterol mass of sdLDL particles over the total LDL cholesterol mass.

Analysis of the apoA-I-containing lipoproteins was performed using the same electrophoretic procedure with some modifications. Briefly, 25 µL of sample was mixed with 300 µL of Lipoprint Loading Gel and placed upon the upper part of the high resolution 3% polyacrylamide gel. After 30 minutes of photopolymerization in room temperature, electrophoresis was performed for 50 minutes with 3 mA for each gel tube. After electrophoresis, VLDL and LDL remained in the origin (Rf = 0.0) whereas albumin migrated at the front (Rf = 1.0). In between, up to 9 bands of HDL can be detected whose Rf's are 0.05, 0.10, 0.15, 0.20, 0.25, 0.29, 0.38, 0.48 and 0.53. Bands 1 to 3 (Rf = 0.05 to 0.15) correspond to large HDL-2 subfraction, whereas bands 4 to 9 (Rf = 0.20 to 0.53) comprised the small HDL-3 subfraction. The cholesterol mass of each HDL subfraction was determined as previously described.

**Subfractionation of plasma lipoproteins by ultracentrifugation**

Lipoproteins were fractionated by isopycnic density gradient ultracentrifugation as previously described. To study the effect of hypolipidemic drugs on Lp-PLA₂
activity and mass associated with apoB-containing lipoprotein subspecies, total plasma was subjected to isopycnic density gradient ultracentrifugation as previously described. According to previously published data, a proportion of LDL-bound Lp-PLA₂ dissociates and is redistributed to apoA-I-containing lipoprotein subspecies, during ultracentrifugation of total plasma. Thus, to avoid any contamination of the HDL-associated Lp-PLA₂ with the LDL-associated enzyme during ultracentrifugation, we studied the effect of hypolipidemic drugs on Lp-PLA₂ associated with the HDL subspecies, using plasma depleted of apoB-lipoproteins, i.e., the HDL-containing supernatant, after treatment of plasma with magnesium chloride–dextran sulfate (to precipitate all apoB-containing lipoproteins). When plasma was used, the following apoB-containing subfractions were prepared: VLDL - IDL (d = 1.019 g/mL), LDL-1 (d = 1.019–1.023 g/mL), LDL-2 (d = 1.023–1.029 g/mL), LDL-3 (d = 1.029 –1.039 g/mL), LDL-4 (d = 1.039–1.050 g/mL), and LDL-5 (d = 1.050–1.063 g/mL). When the HDL-containing supernatant was used, the following apoA-I-containing subfractions were prepared: HDL-2b (d = 1.063–1.091 g/mL), HDL-2a (d = 1.091–1.100 g/mL), HDL-3a (d = 1.100–1.133 g/mL), HDL-3b (d = 1.133–1.156 g/mL), and HDL-3c (d = 1.156–1.179 g/mL). All subfractions were dialyzed extensively at 4°C in 10 mmol/L phosphate-buffered saline, pH 7.4 (PBS) containing 2 mmol/L EDTA, filter-sterilized, and maintained at 4°C under nitrogen until analysis. Under these storage conditions, no oxidation has been reported to occur.

Measurement of Lp-PLA₂ activity

Lp-PLA₂ activity in total plasma, in apoB-depleted plasma, after the sedimentation of all apo B-containing lipoproteins with dextran sulfate-magnesium chloride (HDL-Lp-PLA₂ activity), as well as in lipoprotein subfractions, was
determined by the trichloroacetic acid precipitation procedure using [³H]-PAF (100 μmol/L final concentration) as a substrate. Lp-PLA₂ activity was expressed as nmol PAF degraded per min per mL of plasma or mg of LDL subfraction protein. The non-HDL Lp-PLA₂ activity was calculated by subtracting the HDL-Lp-PLA₂ activity from the total plasma enzyme activity. In separate experiments, Lp-PLA₂ activity was determined in LDL subfractions LDL-3 and LDL-5 of type IIA dyslipidaemic patients at baseline (using 8 μg of protein) in the presence of either human serum albumin (at concentrations ranging from 4 to 8 g/dL) or the total plasma proteins (at concentrations ranging from 4 to 8 g/dL) prepared by mixing of equal volumes from the gradient fractions 25-30 (d = 1.190-1.210 g/mL) isolated by ultracentrifugation of the HDL-containing plasma supernatant (as described above). According to our previously published results these subfractions do not contain any Lp-PLA₂ activity. In other experiments equal volumes of the five LDL subfractions (LDL-1 to LDL-5) or the 5 HDL subfractions HDL-2b to HDL-3c from type IIA dyslipidaemic patients at baseline, were mixed to form the total LDL or total HDL. Subsequently, each lipoprotein was treated with 0.1% Triton X-100 to dissociate the particle. Lp-PLA₂ activity was determined before and after Triton treatment using 8 μg of protein as the source of the enzyme. The final Triton concentration in the enzyme assay was 0.01%. It should be noted that according to our previously published results the above Triton concentration does not affect the enzyme activity.

To study the recovery of Lp-PLA₂ activity after ultracentrifugation in the presence and absence of either albumin or Triton X-100, we mixed equal volumes from the gradient fractions 1-24 containing the total enzyme activity. Lp-PLA₂ activity was determined after treatment of the reconstituted gradient fractions with either albumin 6 g/dL or 0.1% Triton X-100. Enzyme activities were determined as above. In the
absence of any agent, the enzyme recovery (compared to enzyme activity in total plasma) was 150±15%. In the presence of albumin or Triton X-100 the enzyme recovery was 90±5% or 146±12% respectively.

**Measurement of Lp-PLA₂ mass**

Lp-PLA₂ mass in total plasma, in apoB-depleted plasma (HDL Lp-PLA₂ mass), as well as in lipoprotein subfractions, was determined by a dual monoclonal antibody immunoassay standardized to recombinant Lp-PLA₂ (PLAC test, diaDexus, Inc.), following the manufacturer instructions, as we recently described⁵. The non-HDL-Lp-PLA₂ mass was calculated by subtracting the HDL-Lp-PLA₂ mass from the total plasma enzyme mass⁵. In separate experiments, Lp-PLA₂ mass was determined in LDL subfractions LDL-3 and LDL-5 in the presence of either human serum albumin (at concentrations ranging from 4 to 8 g/dL) or the total plasma proteins (at concentrations ranging from 4 to 8 g/dL) prepared as described above. In other experiments Lp-PLA₂ mass was determined in the total LDL or total HDL prepared as described above, before or after treatment with 0.1% Triton X-100. It should be noted that the presence of the Triton did not affect the standard calibrator curves of enzyme mass assay. Lp-PLA₂ specific activity was expressed as a ratio of the enzyme activity to the enzyme mass (nmol/ng/min)⁵. To study the recovery of Lp-PLA₂ mass after ultracentrifugation in the presence and absence of either albumin or Triton X-100, we mixed equal volumes from the gradient fractions 1-24 containing the total enzyme mass. Lp-PLA₂ mass was determined after treatment of the reconstituted gradient fractions with either albumin 6 g/dL or 0.1% Triton X-100. In the absence of any agent the enzyme recovery (compared to enzyme mass in total
plasma) was 94±4%. In the presence of albumin or Triton X-100 the enzyme recovery was 91±6% or 119±16%, respectively.

**Statistical analysis**

Data are presented as mean ± SD, except for Lp(a) which was expressed as the median and range. Statistical analysis was performed using paired Student’s t-test for comparisons between baseline and posttreatment 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. Yates’s corrected chi-square test was used for differences in proportions. A p value of less than 0.05 was considered to be significant. All analyses were carried out using SPSS 13.0 softpack.
Table I. Effect of hypolipidaemic drugs on serum lipid and lipoprotein levels in Type IIA, and Type IV dyslipidaemic patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ezetimibe (n=50)</th>
<th>Rosuvastatin (n=50)</th>
<th>Fenofibrate (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (Males/Females)</td>
<td>23/27</td>
<td>19/31</td>
<td>20/30</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.1±19.5</td>
<td>54.6±14.6</td>
<td>55.9±11</td>
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<tr>
<td>BMI (Kg/m²)</td>
<td>24.5±7.9</td>
<td>25.8±4.2</td>
<td>34.3±7</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.8±0.9</td>
<td>5.7±0.9</td>
<td>6.1±0.9</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.6±0.5</td>
<td>1.5±0.6</td>
<td>1.4±0.6</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.6±0.4</td>
<td>1.5±0.4</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>4.4±0.8</td>
<td>3.6±0.6</td>
<td>3.8±0.9</td>
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<tr>
<td>Non-HDL cholesterol, mmol/L</td>
<td>5.2±0.7</td>
<td>4.3±0.6</td>
<td>4.0±0.9</td>
</tr>
<tr>
<td>ApoAI, g/L</td>
<td>1.4±0.3</td>
<td>1.4±0.2</td>
<td>1.2±0.3</td>
</tr>
<tr>
<td>ApoB, g/L</td>
<td>1.2±0.2</td>
<td>1.0±0.2</td>
<td>0.8±0.3</td>
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<tr>
<td>Lp(a), mg/dL</td>
<td>4.2 (2.0-6.1)</td>
<td>4.0 (2.0-5.8)</td>
<td>3.9 (2.0-7.5)</td>
</tr>
</tbody>
</table>

Data represent the mean±SD, except for Lp(a) values which are expressed as median (range). *p<0.05, †p<0.01 and ‡p<0.001 compared with the corresponding baseline values.
References


