

The elevation of apoB in hypercholesterolemic patients is primarily attributed to the relative increase of apoB/Lp-PLA₂

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Abstract Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is a risk factor of cardiovascular disease. Plasma Lp-PLA₂ is mainly associated with apolipoprotein (apo)B-containing lipoproteins, primarily with low density lipoproteins (LDLs). Importantly, only a proportion of circulating lipoproteins contain Lp-PLA₂. We determined the plasma levels of Lp-PLA₂-bound apoB (apoB/Lp-PLA₂) in patients with primary hypercholesterolemia. The effect of simvastatin therapy was also addressed. The plasma apoB/Lp-PLA₂ concentration in 50 normolipidemic controls and 53 patients with primary hypercholesterolemia at baseline and at 3 months posttreatment with simvastatin (40 mg/day) was determined by an enzyme-linked immunosorbent assay. The concentration of the apoB-containing lipoproteins that do not bind Lp-PLA₂ [apoB/Lp-PLA₂(-)] was calculated by subtracting the apoB/Lp-PLA₂ from total apoB. The apoB/Lp-PLA₂ levels were 3.6-fold higher, while apoB/Lp-PLA₂(-) were 1.3-fold higher in patients compared with controls. After 3 months of simvastatin treatment apoB/Lp-PLA₂ and apoB/Lp-PLA₂(-) levels were reduced by 52% and 33%, respectively. The elevation in apoB-containing lipoproteins in hypercholesterolemic patients is mainly attributed to the relative increase in the proatherogenic apoB/Lp-PLA₂, while simvastatin reduces these particles to a higher extent compared with apoB/Lp-PLA₂(-). Considering that Lp-PLA₂ is proatherogenic, the predominance of apoB/Lp-PLA₂ particles in hypercholesterolemic patients may contribute to their higher atherogenicity and incidence of cardiovascular disease.—Tellis, C. C., E. Moutzouri, M. Elisaf, R. L. Wolfert, and A. D. Tselepis. The elevation of apoB in hypercholesterolemic patients is primarily attributed to the relative increase of apoB/Lp-PLA₂. *J. Lipid Res.* 2013. 54: 3394–3402.

Supplementary key words lipoprotein-associated phospholipase A₂-bound apolipoprotein B • hypercholesterolemia • low density lipoprotein • simvastatin

Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) [platelet-activating factor (PAF)-acetylhydrolase] exhibits

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a Ca²⁺-independent PLA₂ activity and degrades PAF as well as oxidized phospholipids (oxPLs). Such phospholipids are accumulated in the artery wall and may play key roles in vascular inflammation and atherosclerosis. Lp-PLA₂ is secreted by macrophages and other inflammatory cells in the arterial wall, and circulates in plasma associated with lipoproteins (1). The majority of plasma Lp-PLA₂ is bound on apolipoprotein B (apoB)-containing lipoproteins, primarily on low density lipoprotein (LDL) particles. A small proportion of the circulating enzyme is also associated with high density lipoproteins (HDLs). The majority of the LDL-associated Lp-PLA₂ is bound to atherogenic small dense LDL (sdLDL) particles (2–4). Through hydrolysis of oxPLs, Lp-PLA₂ generates pro-inflammatory oxidized nonesterified fatty acids and lysophosphatidylcholine (LPC), which are involved in various stages of atherosclerotic plaque development and may also play an important role in plaque vulnerability. Thus Lp-PLA₂ is considered to be a proatherogenic enzyme (5, 6).

Several epidemiologic studies suggest that plasma Lp-PLA₂ is an independent predictor of cardiovascular events in primary and secondary prevention (7–14). A meta-analysis, which included 79,036 participants with or without coronary artery disease (CAD) from 32 prospective studies, showed that Lp-PLA₂ activity and mass each had a continuous association with the risk of CAD (9).

In all epidemiologic studies, the total Lp-PLA₂ plasma enzyme has been determined; this mainly represents the LDL-associated Lp-PLA₂. Thus plasma Lp-PLA₂ is considered as a promising therapeutic target, and two ongoing

Abbreviations: apoB/Lp-PLA₂, lipoprotein-associated phospholipase A₂-bound apoB; apoB/Lp-PLA₂(-), apoB-containing lipoproteins that do not bind Lp-PLA₂; CAD, coronary artery disease; hsCRP, high sensitivity C-reactive protein; IDL, intermediate density lipoprotein; Lp(a), lipoprotein (a); LPC, lysophosphatidylcholine; Lp-PLA₂, lipoprotein-associated phospholipase A₂; oxLDL, oxidized LDL; oxPL, oxidized phospholipid; PAF, platelet-activating factor; sdLDL, small dense LDL; TG, triglyceride.

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phase 3 trials on a selective enzyme inhibitor, darapladib, are currently in progress (15). Furthermore, previous studies by our group (16, 17) and others (18–20) have demonstrated that among existing cardiovascular drugs, statins effectively reduce plasma levels of Lp-PLA₂ activity and mass in parallel with the significant reduction of LDL-cholesterol levels, suggesting that the rate of LDL removal from the circulation may represent an important mechanism for the reduction of plasma Lp-PLA₂ levels (16, 17).

Importantly, only a proportion of circulating apoB-containing lipoproteins contain Lp-PLA₂ (3). Thus it remains to be established whether these particles differ from those that do not carry Lp-PLA₂ in terms of their pathophysiological role in atherosclerosis and their clinical significance, as well as their behavior to pharmacological intervention.

The aim of the present study was to establish a new method in order to directly determine the plasma concentration of Lp-PLA₂-bound apoB (apoB/Lp-PLA₂) in normolipidemic healthy volunteers, as well as in patients with primary hypercholesterolemia. The effect of simvastatin therapy was also addressed.

MATERIALS AND METHODS

Populational studies

The study included 53 hypercholesterolemic subjects (30 women and 23 men) and 50 controls (27 women and 23 men). Consecutive patients with primary hypercholesterolemia aged 20 to 70 years attending the Outpatient Lipid and Obesity Clinic of the University Hospital of Ioannina, Greece participated in the present study. Patients were eligible if their LDL-cholesterol levels were above those recommended by the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) based on each patient's risk factors, following a 3 month period of lifestyle changes (21).

Exclusion criteria were known CAD, symptomatic carotid artery disease, peripheral arterial disease, abdominal aortic aneurysm, diabetes mellitus, renal disease (serum creatinine levels >1.6 mg/dl), hypothyroidism (thyroid stimulating hormone >5 IU/ml), liver disease (alanine aminotransferase and/or aspartate aminotransferase levels >3-fold upper limit of normal in two consecutive measurements), and neoplasia, as well as clinical and laboratory evidence of an inflammatory or infectious conditions within 6 months preceding the study. Patients with hypertension were included in the study if they were on stable medication for at least 3 months and their blood pressure was adequately controlled (no change in their treatment was allowed during the study). Patients taking anti-inflammatory drugs were excluded. Patients currently taking lipid-lowering drugs or having stopped them less than 12 weeks before study entry as well as patients with a history of adverse reactions to statins were excluded. All patients received simvastatin, 40 mg daily, for 3 months. Compliance with treatment was assessed by questionnaire and tablet count. Dietary composition, level of physical activity, smoking habits, and all concurrent medications were maintained unchanged throughout the study period. The control group included 50 age-, sex-, and weight-matched subjects with normal lipid levels [LDL-cholesterol <130 mg/dl, HDL-cholesterol >40 mg/dl, and triglycerides (TGs)

<150 mg/dl]. The Ethics Committee of the University Hospital of Ioannina gave approval for the study and all participants gave written consent prior to their enrollment in the study.

Biochemical parameters

Venous blood samples were obtained in the morning after 12 h fasting. Concentrations of serum total cholesterol, TGs, and HDL-cholesterol were determined enzymatically on the Olympus AU 600 clinical chemistry analyzer (Olympus Diagnostica, Hamburg, Germany). HDL-cholesterol was determined by a direct assay (Olympus Diagnostica) (22). LDL-cholesterol was calculated with the Friedewald formula. apoA-I, apoB, and apoE as well as lipoprotein a [Lp(a)] were measured with a Behring BN-100 nephelometer and with reagents (antibodies and calibrators) from Dade Behring Holding GmbH (Liederbach, Germany) (22). Serum concentrations of high sensitivity C-reactive proteins (hsCRPs) were measured with a high sensitivity immunonephelometric assay (Beckman Instruments, Fullerton, CA). The reference range of this assay is 1.0 to 80 mg/l. The detection limit is 1.0 mg/l. Serum creatinine, liver, and muscle enzymes as well as thyroid function tests were performed by conventional methods, as we have previously described (22).

Electrophoretic analysis of apoB-containing lipoprotein subclasses

Analysis of the apoB-containing lipoprotein subclasses was performed electrophoretically by use of high-resolution 3% polyacrylamide gel tubes and the Lipoprint LDL system (Quantimetrix, Redondo Beach, CA), as we have previously described (3). After electrophoresis, very low density lipoprotein (VLDL) remained in the origin [retention factor (R_f) = 0.0], whereas HDL migrated at the front (R_f = 1.0). In between, several bands were detected: midbands C, B, and A, which correspond mainly to intermediate density lipoprotein (IDL), as well as up to seven 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-containing 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 (3).

Measurement of plasma Lp-PLA₂ activity and mass

The Lp-PLA₂ activity in total plasma was determined by the trichloroacetic acid precipitation procedure using [³H]PAF (100 μmol/l final concentration) as a substrate (4). The reaction was performed for 10 min at 37°C. Lp-PLA₂ activity was expressed as nanomoles of PAF degraded per minute per milliliter of plasma. The Lp-PLA₂ mass in total plasma was determined by a dual monoclonal antibody immunoassay standardized to recombinant Lp-PLA₂ (PLAC test kits kindly provided by diaDexus Inc., San Francisco, CA), following the manufacturer's instructions, as we previously described (23).

Measurement of oxidized LDL

Plasma levels of oxidized LDL (oxLDL) were measured by a competitive enzyme-linked immunosorbent assay using a specific murine monoclonal antibody 4E6 and following the instructions provided by the manufacturer (Mercodia, Uppsala, Sweden) as we have previously described (24). Intra- and interassay coefficients of variation of the assay were 6.0% and 7.0%, respectively.

Determination of apoB/Lp-PLA₂

To quantify the apoB/Lp-PLA₂ plasma levels, we established an enzyme-linked immunosorbent assay (ELISA) as follows.

Microtiter 96-well plates (Costar, Corning Inc., NY) were coated overnight at 4°C with 100 µl/well of the anti-Lp-PLA₂ monoclonal antibody 2C10 (kindly provided by diaDexus Inc.) in 0.2 mol/l sodium phosphate buffer, pH 6.5 (10 µg protein/ml). Wells were then washed four times with a 50 mmol/l Tris-HCl buffer saline solution, pH 7.4 (TBS) containing 0.1% Tween-20 to remove any excess antibody, and the noncoated surface was then blocked by an overnight incubation at room temperature with 300 µl/well of a blocking solution containing 1% free fatty acid-bovine serum albumin (FFA-BSA) and 2.5% sucrose solution in 10 mmol/l potassium phosphate buffer, pH 7.4. Subsequently, the blocking solution was completely removed, and the coated plates were air-dried at room temperature, sealed with desiccant, and stored at 4°C. The apoB/Lp-PLA₂ levels were determined using 40 µl plasma samples that were added to each well, and the volume was completed to 200 µl with assay buffer that consisted of 0.2% FFA-BSA, 0.2% mouse serum, 0.2% calf serum, and 0.1% Proclin-150 in TBS (pH 7.4). A calibration curve was simultaneously prepared using LDL at a range from 1 to 60 µg apoB. Wells containing either plasma samples or LDL calibrators were incubated for 90 min at room temperature on a plate shaker at 600 rpm and then washed four times with TBS. Two hundred microliters of an anti-apoB horseradish peroxidase (HRP)-conjugated polyclonal antibody (1 mg/ml; Acris Antibodies GmbH, Herford, Germany), diluted 1:10,000 v/v with assay buffer (final concentration 0.1 µg protein/ml), were added to each well and incubated for 90 min at room temperature on a plate shaker at 600 rpm. Subsequently, the plate was washed four times with a 0.025% Tween-20 solution in TBS and then 100 µl of a HRP substrate (TMB, 3,3', 5,5"-tetramethylbenzidine; Cell Signaling Inc., Danvers, MA) were added to each well. The plate was incubated at room temperature for 20 min in the dark. The reaction was stopped with 100 µl/well 1N HCl. The optical density of plasma samples and LDL calibrators was measured versus blank (100 µl of a HRP substrate plus 100 µl 1N HCl) at 450 nm using a microwell plate reader. Negative controls were also prepared by following the above procedure in wells which were not covered with the anti-Lp-PLA₂ monoclonal antibody 2C10.

Preparation of LDL calibrators. The LDL used for the calibration curve was isolated from pooled fresh plasma from healthy normolipidemic volunteers by sequential ultracentrifugation at $d = 1.019\text{--}1.063$ g/ml (25). LDL protein was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) (26). The Lp-PLA₂ mass content of LDL was determined by the PLAC[®] test (diaDexus Inc.) using 10 µg of LDL protein, whereas the LDL apoB content was determined with a Behring Holding GmbH BN-100 nephelometer (Liederbach Inc., Germany) (27). In some experiments oxLDL instead of native LDL was used as a calibrator. LDL was oxidized in the presence of copper sulfate (5 µM final concentration). The kinetics of oxidation was determined by continuously monitoring the increase in absorbance at 234 nm for 6 h, as we have previously described (2, 28). The purity and the oxidative modification of the LDL preparations were evaluated by agarose gel electrophoresis (Hydragel Lipo and Lp(a) kit, Sebia Inc., France) (28). The LDL calibrators as well as the oxLDL preparations were stored in the dark in sealed tubes overlaid with N₂ at 4°C and used within 2 weeks. The total protein content of various LDL preparations ranged from 2.5 to 3.5 mg/ml, the Lp-PLA₂ mass ranged from 5 to 10 ng/mg protein, and the apoB levels ranged from 2 to 3 mg/ml. In selected experiments HDL ($d = 1.063\text{--}1.210$ g/ml, prepared by sequential ultracentrifugation as we have previously described (29) was used instead of LDL, at a range from 1 to 60 µg apoA-I.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) and median (range) for parametric and nonparametric data, respectively. The differences of study parameters between controls and hypercholesterolemic participants, as well as baseline and post-treatment values for patients, were evaluated by paired samples *t*-tests (or Wilcoxon's rank test for non-Gaussian variables). Significance was defined at $P < 0.05$ (two-tailed). Analyses were performed using the Statistical Package for the Social Sciences (SPSS) 16.0 (SPSS Inc., Chicago, IL).

RESULTS

Clinical and biochemical characteristics of the study population

Fifty-three hypercholesterolemic subjects (30 women and 23 men, aged 57 ± 13 years) and 50 controls (27 women and 23 men, aged 54 ± 11 years) participated in the study. The clinical and biochemical characteristics of the study population are shown in **Table 1**. Hypercholesterolemic patients exhibited significantly higher body mass index values as well higher serum levels of total cholesterol, LDL-cholesterol, oxLDLs, and hsCRPs compared with controls. Hypercholesterolemic patients also exhibited higher levels of buoyant LDL-cholesterol and sdLDL-cholesterol compared with controls, whereas no difference in the TGs, Lp(a), sdLDL proportion, and mean LDL size was observed between the two study groups (Table 1). apoB as well as Lp-PLA₂ activity and mass levels were also significantly higher in hypercholesterolemic patients compared with controls, whereas no difference in the ratio of Lp-PLA₂ mass/apoB was observed between hypercholesterolemic patients and controls (Table 1).

Effect of simvastatin therapy on lipid parameters and Lp-PLA₂

As expected, simvastatin therapy significantly reduced serum levels of total cholesterol, LDL-cholesterol, and oxLDLs (Table 1). Furthermore, simvastatin significantly reduced buoyant LDL-cholesterol and sdLDL-cholesterol levels, but it did not affect sdLDL proportion and mean LDL size (Table 1). Finally, simvastatin significantly decreased hsCRP and apoB levels as well as Lp-PLA₂ activity and mass; however, it did not affect the ratio of Lp-PLA₂ mass/apoB (Table 1).

Calibration curve for apoB/Lp-PLA₂ determination

For the determination of plasma apoB/Lp-PLA₂ levels, we established an ELISA method in which microtiter 96-well plates were coated with the anti-Lp-PLA₂ monoclonal antibody 2C10 to capture the plasma Lp-PLA₂-containing lipoprotein particles. An anti-apoB HRP-conjugated polyclonal antibody was subsequently used to specifically quantify the plasma apoB-containing lipoproteins that were captured by the anti-Lp-PLA₂ monoclonal antibody. Different concentrations of LDL, isolated by sequential ultracentrifugation from pooled

TABLE 1. Clinical and biochemical characteristics of control subjects and hypercholesterolemic patients at baseline and 3 months posttreatment with simvastatin

Parameter	Controls (n = 50)	Simvastatin (n = 53)	
		Baseline	Posttreatment
Age (years)	54 ± 11	57 ± 13	—
Sex (male/female)	23/27	23/30	—
BMI (kg/m ²)	25 ± 3	29 ± 5*	28 ± 3*
Current smoking (n) ^a	15	18	—
Total cholesterol (mg/dl)	165 ± 30	257 ± 40*	175 ± 30**
TGs (mg/dl)	101 ± 26	112 ± 57	97 ± 32
HDL-cholesterol (mg/dl)	58 ± 7	57 ± 13	58 ± 11
LDL-cholesterol (mg/dl)	107 ± 26	176 ± 34*	97 ± 26**
Buoyant LDL-cholesterol (mg/dl)	68 ± 16	88 ± 20*	51 ± 17**
sdLDL-cholesterol (mg/dl)	5 (0–18)	9 (4–22)*	5 (0–16)**
sdLDL proportion (%)	4.9 ± 1.9	5.1 ± 2.3	5.2 ± 2.0
Mean LDL size, nm	26.9 ± 0.2	26.6 ± 0.3	26.7 ± 0.4
apoB (mg/dl)	76 ± 17	113 ± 24*	72 ± 19**
apoA-I (mg/dl)	151 ± 19	154 ± 26	158 ± 24
apoE (mg/dl)	40 ± 8	44 ± 10	41 ± 8
oxLDL (U/l)	45 ± 12	89 ± 18*	55 ± 20**
Lp(a) (mg/dl)	6.8 (2.3–19.1)	7.5 (2.3–21.3)	7.3 (2.4–25.4)
hsCRP (mg/l)	2.2 (1.0–6.4)	3.0 (1.2–12.9)*	2.5 (1.0–9.7)**
Lp-PLA ₂ activity (nmol/ml/min)	48 ± 9	64 ± 16*	44 ± 16**
Lp-PLA ₂ mass (ng/ml)	261 ± 41	397 ± 77**	251 ± 71**
Lp-PLA ₂ mass/apoB ratio (ng/mg)	344 ± 26	351 ± 35	349 ± 39

Values are expressed as mean ± SD except for Lp(a) and hsCRP which are expressed as median (range).

P* < 0.005 compared with controls; *P* < 0.001 compared with baseline values. BMI, body mass index.

^aCurrent smoking was defined as smoking at least one cigarette daily.

fresh plasma of healthy normolipidemic volunteers, were used as calibrators. A standard point-to-point calibration curve was constructed by plotting the absorbance obtained for each LDL calibrator on the vertical axis versus the mass of LDL calibrator expressed as apoB mass in micrograms on the horizontal axis (Fig. 1). No absorbance against blank was observed in wells used as negative controls or in wells where HDL was placed instead of LDL (data not shown). To evaluate whether LDL oxidation influences the apoB/Lp-PLA₂ assay, we used oxLDL instead of native LDL as a calibrator. As is shown in Fig. 1, oxidation of LDL does not affect the calibration curve for apoB/Lp-PLA₂ determination. The minimum detection limit of this method is 0.9 µg/ml, whereas the intra- and inter-assay coefficients of variation are 7.6 and 9.1%, respectively.

apoB/Lp-PLA₂ levels in the study population and effect of simvastatin therapy

Using the standard calibration curve, we determined the concentration of apoB/Lp-PLA₂ in our plasma samples (expressed as apoB levels in milligrams per deciliter). These apoB levels correspond to the concentration of the Lp-PLA₂-bound apoB. The concentration of the apoB lipoproteins that do not carry Lp-PLA₂ [apoB/Lp-PLA₂(–)] was calculated by subtracting the apoB/Lp-PLA₂ from total apoB levels. The present study shows for the first time that apoB/Lp-PLA₂ are significantly higher (3.6-fold) in hypercholesterolemic patients compared with controls (Fig. 2A). apoB/Lp-PLA₂ in hypercholesterolemic patients represents the 22.1% of total apoB levels while in normolipidemic controls it represents the 9.2% of total apoB. The apoB/Lp-PLA₂(–) are modestly, albeit significantly,

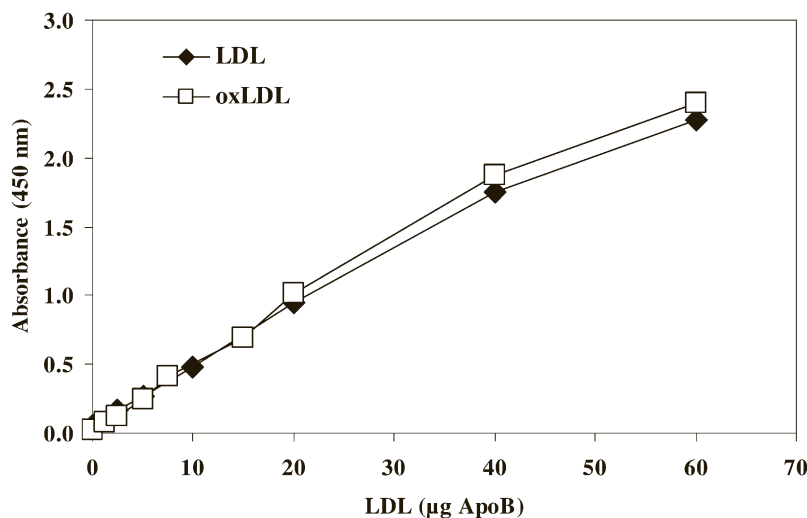


Fig. 1. A standard calibration curve for the determination of apoB/Lp-PLA₂ levels. This curve was constructed by plotting the absorbance obtained for each calibrator (LDL or oxLDL) on the y axis versus the amount of LDL or oxLDL, respectively, expressed in micrograms of apoB.

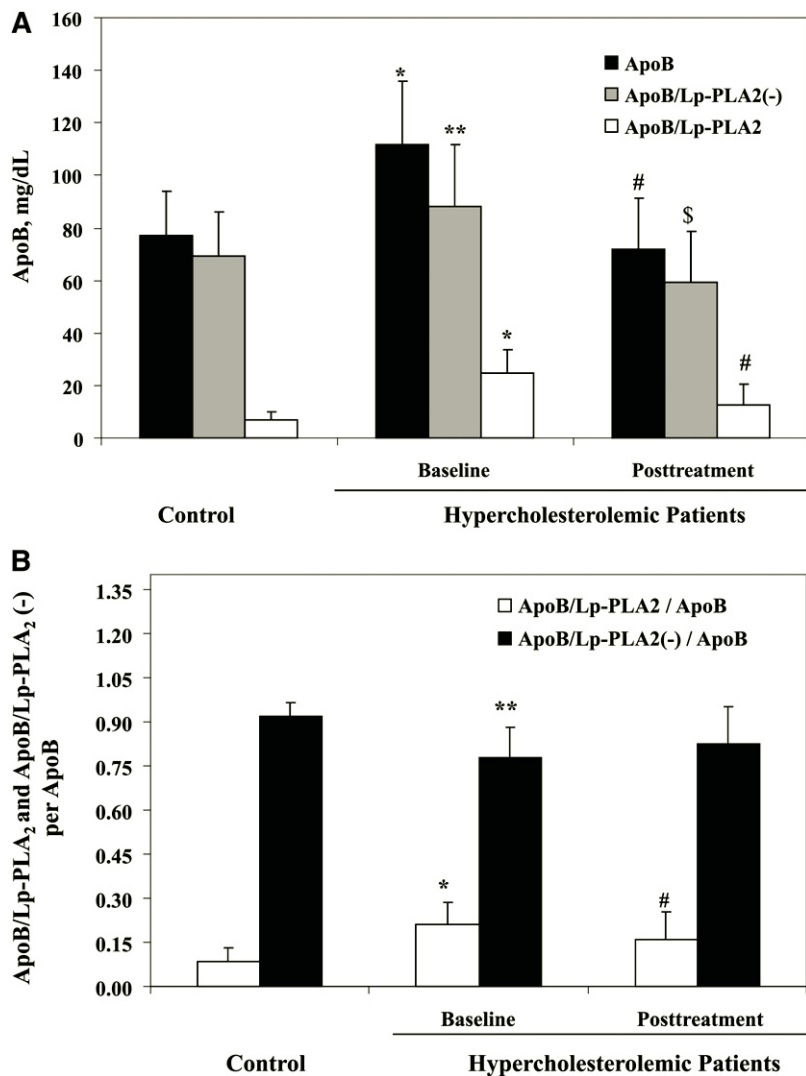


Fig. 2. A: Bar graph showing the total apoB, apoB/Lp-PLA₂(-) and apoB/Lp-PLA₂ levels in normolipidemic controls as well as in hypercholesterolemic patients at baseline, as well as after 3 months of treatment with 40 mg/day of simvastatin. Data are mean ± SD. **P* < 0.005 and ***P* < 0.001 compared with the control group. #*P* < 0.001 and \$*P* < 0.005 compared with baseline values. B: Bar graph showing the ratios of apoB/Lp-PLA₂/apoB, and apoB/Lp-PLA₂(-)/apoB in normolipidemic controls as well as in hypercholesterolemic patients at baseline and after 3 months of treatment with 40 mg/day of simvastatin. Data are mean ± SD. **P* < 0.001 and ***P* < 0.05 compared with the control group; #*P* < 0.001 compared with baseline values.

higher (1.3-fold) compared with controls (Fig. 2A), and this increase is similar to that of total apoB (1.5-fold increase). Furthermore, the ratio of apoB/Lp-PLA₂/apoB in hypercholesterolemic patients is significantly higher compared with normolipidemic controls (Fig. 2B), whereas the ratio of apoB/Lp-PLA₂(-)/apoB was modestly, albeit significantly, lower in hypercholesterolemic patients than in controls, primarily due to the relatively higher elevation of apoB compared with apoB/Lp-PLA₂(-) in hypercholesterolemic patients (Fig. 2B). These results suggest that among apoB-containing lipoproteins, the particles that are mostly elevated in hypercholesterolemic patients at baseline are apoB/Lp-PLA₂ particles.

After 3 months of treatment with simvastatin, the plasma apoB/Lp-PLA₂ levels were significantly reduced by 52% compared with baseline, whereas the apoB/Lp-PLA₂(-) levels were reduced by 33% (Fig. 2A). It should be mentioned that simvastatin reduced the total apoB levels by 36% (Table 1). Consequently, the apoB/Lp-PLA₂/apoB ratio was significantly reduced by simvastatin, whereas the ratio of apoB/Lp-PLA₂(-)/apoB was not significantly altered (Fig. 2B).

Correlations

apoB/Lp-PLA₂ and apoB/Lp-PLA₂(-) levels in controls as well as in hypercholesterolemic patients at baseline were positively correlated with total cholesterol, TGs, LDL-cholesterol, apoB, Lp(a), sdLDL-cholesterol, buoyant LDL-cholesterol, oxLDL levels, and sdLDL proportion (Table 2). apoB/Lp-PLA₂, but not apoB/Lp-PLA₂(-), levels were positively correlated with hsCRP levels as well as Lp-PLA₂ mass and activity (Table 2). The changes in plasma apoB/Lp-PLA₂ levels in response to simvastatin therapy in hypercholesterolemic patients were positively correlated with the changes in total cholesterol, LDL-cholesterol, apoB, sdLDL-cholesterol, buoyant LDL-cholesterol, and oxLDL, as well as with hsCRP, Lp-PLA₂ mass, and activity (Table 3).

DISCUSSION

In the present study, we propose an ELISA method to quantify the Lp-PLA₂-bound apoB in human plasma. This method is specific for apoB-containing lipoproteins, primarily LDLs, because apoA-I-containing HDL particles do

TABLE 2. Correlation between plasma apoB/Lp-PLA₂ and apoB/Lp-PLA₂(-) levels and lipid inflammatory parameters in the total population

Parameters	apoB/Lp-PLA ₂		apoB/Lp-PLA ₂ (-)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Total cholesterol	0.692	<0.001	0.801	<0.001
TGs	0.389	<0.01	0.286	<0.03
HDL-cholesterol	-0.178	NS	-0.003	NS
LDL-cholesterol	0.732	<0.001	0.830	<0.001
apoA-1	-0.104	NS	0.097	NS
apoB	0.679	<0.001	0.897	<0.001
apoE	0.190	NS	0.300	<0.03
Lp(a)	0.262	<0.01	0.241	<0.01
sdLDL-cholesterol	0.248	<0.03	0.278	<0.02
sdLDL proportion	0.464	<0.001	0.473	<0.001
Buoyant LDL-cholesterol	0.498	<0.001	0.480	<0.001
oxLDL	0.452	<0.001	0.571	<0.001
hsCRP	0.264	<0.02	0.081	NS
LpPLA ₂ mass	0.786	<0.001	0.081	NS
LpPLA ₂ activity	0.703	<0.001	0.066	NS

not have any reactivity. According to the present results, apoB/Lp-PLA₂ represents 9.2% of total apoB levels in controls. In a previous study (3), we had reported that only a small proportion of circulating apoB-containing lipoproteins carry Lp-PLA₂. According to our calculations, one molecule of Lp-PLA₂ corresponds to approximately 100 sdLDL (LDL5) and to 4,000 buoyant LDL (LDL2 and LDL3) particles (3). In this regard, we should point out that only one molecule of Lp-PLA₂ can bind (mainly through residues Tyr-205, Trp-115, and Leu-116) to one molecule of apoB (at residues 4119 to 4279 of the carboxyl terminus) and thus to one LDL particle (30). The underestimation of the amount of Lp-PLA₂ associated with apoB-containing lipoproteins in our previous study, compared with the present study, is most likely attributed to the fact that in the previous study we used density gradient ultracentrifugation to subfractionate plasma lipoproteins. During ultracentrifugation, a proportion of enzyme dissociates from apoB-containing lipoprotein particles and is recovered in the very dense portion of the gradient with proteins (31).

The present study further shows that LDL oxidation does not affect the assay of apoB/Lp-PLA₂ because the standard plots are similar when we use either native LDLs or oxLDLs as calibrators. This is in line with the significant

positive correlations obtained between apoB/Lp-PLA₂ and LDL-cholesterol or oxLDL levels. We and others have previously demonstrated that Lp-PLA₂ is also associated with Lp(a) in human plasma (32–34). Lp(a) contains 1.5- to 2-fold higher enzyme mass (32, 33) and several-fold greater Lp-PLA₂ activity compared with LDL when assayed at equimolar protein concentrations (34). In the present study apoB/Lp-PLA₂ is positively correlated with Lp(a), a finding consistent with the suggestion that the present method also determines Lp(a) particles containing Lp-PLA₂. However, this correlation is relatively weak, possibly due to the low plasma Lp(a) levels in our population. Indeed, we have previously demonstrated that the distribution of Lp-PLA₂ between LDLs and HDLs can be influenced by the presence of Lp(a) only when plasma levels of this lipoprotein exceed 30 mg/dl (32). Thus detectable amounts of Lp(a)-associated Lp-PLA₂ in plasma can be obtained with at least three times higher Lp(a) concentration than that observed in the present study.

Lp-PLA₂ is also associated with the TG-rich VLDLs and IDLs representing only 3.3 ± 1.6% in human plasma (4). The significant correlation observed between apoB/Lp-PLA₂ and TG levels is consistent with the suggestion that the present method also determines VLDL+IDL particles containing Lp-PLA₂. By contrast, apoB/Lp-PLA₂ was not correlated with either apoA-I or HDL-cholesterol levels, which accords with the lack of any reactivity of apoA-I-containing HDL particles in the calibration curve. Overall, the present method specifically determines the apoB-containing lipoproteins carrying Lp-PLA₂ in human plasma, primarily LDLs. Because our population exhibits low Lp(a) and TG levels, further studies are required in a population with high Lp(a) and TG levels to further support the suggestion that this method also determines Lp(a)-associated Lp-PLA₂ and VLDL+IDL-associated Lp-PLA₂.

An important observation of the present study is that the increase in the apoB/Lp-PLA₂ in hypercholesterolemic patients at baseline is relatively higher than that of apoB/Lp-PLA₂(-) (3.6-fold vs. 1.3-fold, respectively, compared with controls). Thus we may suggest that the defective metabolism of apoB-containing lipoproteins observed in

TABLE 3. Correlation between percent changes of plasma apoB/Lp-PLA₂ levels and lipid as well as inflammatory parameters after simvastatin treatment in hypercholesterolemic patients

Parameters	apoB/Lp-PLA ₂ (% Change)	
	<i>r</i>	<i>P</i>
Total cholesterol	0.314	<0.03
TGs	0.058	NS
LDL-cholesterol	0.490	<0.001
apoB	0.377	<0.005
Lp(a)	0.049	NS
sdLDL-cholesterol	0.247	<0.03
Buoyant LDL-cholesterol	0.463	<0.004
oxLDL	0.513	<0.003
hsCRP	0.321	<0.01
Lp-PLA ₂ mass	0.554	<0.001
Lp-PLA ₂ activity	0.496	<0.001

primary hypercholesterolemia mainly reflects the apoB/Lp-PLA₂ particles and could be primarily attributed to the elevation of LDL and oxLDL particles, because neither TG (VLDL+IDL) nor Lp(a) levels are increased in hypercholesterolemic patients. This is further supported by the positive correlation between the reduction of apoB/Lp-PLA₂ and LDL-cholesterol or oxLDL levels induced by simvastatin. A feature characteristic of patients with primary hypercholesterolemia is the reduction in the rate of LDL removal from the circulation (35). The present study suggests that this metabolic LDL abnormality, which leads to the increase in plasma LDL-cholesterol levels, predominantly reflects the LDL particles carrying Lp-PLA₂. Our study does not provide the biochemical basis for this phenomenon. However, we may suggest that Lp-PLA₂ could be involved in the LDL receptor-dependent and -independent pathways of LDL catabolism. In line with this hypothesis are the results of a recent study showing that cholesteryl ester transfer between HDLs and apoB-containing lipoproteins in plasma may be positively influenced by Lp-PLA₂ (36), suggesting a new proatherogenic function of this enzyme. The above hypothesis needs to be further elucidated. apoB/Lp-PLA₂ is positively correlated with sdLDL-cholesterol levels, and previous studies have shown that sdLDL particles undergo decreased recognition by the LDL receptor, thus resulting in lower clearance rates from the circulation and in their increased plasma half-life (37–40). Because sdLDL particles are enriched in Lp-PLA₂ (2, 4), we suggest that this enzyme may play a role in the decreased clearance rates of sdLDLs.

apoB/Lp-PLA₂ at baseline is positively correlated with plasma Lp-PLA₂ activity and mass which are significantly reduced by simvastatin. This finding is in accordance with previously published results by our group (16, 17) and others (18–20), demonstrating that statins effectively reduce plasma levels of Lp-PLA₂. The present study further shows that simvastatin significantly reduces apoB/Lp-PLA₂ levels, this reduction being positively correlated with that of plasma Lp-PLA₂ activity and mass. The above correlations prompted us to suggest that the increase in apoB/Lp-PLA₂ observed in hypercholesterolemic patients may also be attributed to the increased plasma Lp-PLA₂ levels.

Circulating Lp-PLA₂ derives primarily from cells of the hematopoietic lineage, mainly monocyte-derived macrophages (41, 42), i.e., cells that do not produce apoB-containing lipoproteins. Thus, the release of Lp-PLA₂ occurs independently of lipoprotein secretion; the enzyme subsequently associates with these particles (1). Lp-PLA₂ is actively produced and secreted by monocyte-derived macrophages in response to inflammatory cytokines and microbial lipopolysaccharides (43–47). Hypercholesterolemic patients exhibit a low grade inflammation, as is indicated by the higher hsCRP levels compared with controls, a finding that is in accordance with our previously published results (48). hsCRP levels are positively correlated with apoB/Lp-PLA₂ levels while the percent reduction of hsCRP induced by simvastatin is positively correlated with that of apoB/Lp-PLA₂. In this regard it has been demonstrated that simvastatin effectively reduces Lp-PLA₂ expression and secreted

activity in macrophages primarily during inflammatory stimulation with lipopolysaccharides, through inhibition of the mevalonate-geranylgeranyl pyrophosphate-RhoA-p38 mitogen-activated protein kinase pathway (49). It should be noted that this reduction by statins is not observed in the absence of inflammatory stimuli, a finding that accords with previously published data by our group and others (16, 50). Thus it is possible that the low grade inflammation occurring in our hypercholesterolemic patients may lead to increased secretion of Lp-PLA₂ and subsequently to increased levels of apoB/Lp-PLA₂ which are less well cleared from the circulation, while simvastatin under these inflammatory conditions reduces the secretion of Lp-PLA₂ leading to the reduced formation of apoB/Lp-PLA₂.

The higher apoB/Lp-PLA₂ levels may confer hypercholesterolemic patients with higher atherogenicity, because apoB/Lp-PLA₂ may be more atherogenic compared with apoB/Lp-PLA₂(-) particles. According to previously published data, the existence of Lp-PLA₂ on LDL may influence its atherogenic potency by degrading the oxPLs formed during LDL oxidation and generating LPC (51), which is a central actor in the inflammatory reactions occurring during atherosclerotic plaque development and rupture. Indeed, LPC may contribute to the development of atherosclerotic plaques as well as to plaque vulnerability and rupture. This hypothesis is also supported by studies showing that the Lp-PLA₂ and LPC content of human carotid plaques predict future cardiovascular events, and that the plaque content of Lp-PLA₂ and LPC is increased in symptomatic human carotid plaques (52, 53).

In conclusion, by establishing an ELISA method to specifically determine the Lp-PLA₂-bound apoB, we show for the first time that in patients with primary hypercholesterolemia the increase in apoB/Lp-PLA₂ is relatively higher compared with apoB/Lp-PLA₂(-). Furthermore, simvastatin therapy reduces these particles to a higher extent compared with apoB/Lp-PLA₂(-), suggesting a role of Lp-PLA₂ in the metabolism of apoB-containing lipoproteins. Because Lp-PLA₂ plays an important proatherogenic role by degrading oxPLs formed during LDL oxidation and generating LPC, the predominance of apoB/Lp-PLA₂ particles in the plasma of patients with primary hypercholesterolemia may be an important factor contributing to their higher atherogenicity and incidence of cardiovascular disease. ■■

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