Platelet-Activating Factor Acetylhydrolase Is Mainly Associated With Electronegative Low-Density Lipoprotein Subfraction

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Background—Electronegative LDL [LDL(−)], a modified subfraction of LDL present in plasma, induces the release of interleukin-8 and monocyte chemotactic protein-1 from cultured endothelial cells.

Methods and Results—We demonstrate that platelet-activating factor acetylhydrolase (PAF-AH) is mainly associated with LDL(−). LDL(−) had 5-fold higher PAF-AH activity than the nonelectronegative LDL subfraction [LDL(+)] in both normolipemic and familial hypercholesterolemic subjects. Western blot analysis after SDS-PAGE confirmed these results, because a single band of 44 kDa corresponding to PAF-AH appeared in LDL(−) but not in LDL(+). Nondenaturing polyacrylamide gradient gel electrophoresis demonstrated that PAF-AH was bound to LDL(−) regardless of LDL size. In accordance with the above findings, nonesterified fatty acids, a cleavage product of PAF-AH, were increased in LDL(−) compared with LDL(+).

Conclusions—The high PAF-AH activity observed in LDL(−) could be related to the proinflammatory activity of these lipoproteins toward cultured endothelial cells. (Circulation. 2003;108:92-96.)

Key Words: lipoproteins inflammation fatty acids

Platelet-activating factor acetylhydrolase (PAF-AH) is a serine lipase that hydrolyzes the sn-2 ester bond of PAF and attenuates its high biological activity.1 PAF-AH circulates mainly bound to LDL and, owing to its phospholipase A2 (PLA2) activity, is also known as lipoprotein-associated PLA2.2 Growing evidence suggests that PAF-AH is involved in atherosclerosis, although controversy exists regarding its proinflammatory or antiatherogenic properties. Its capacity to hydrolyze PAF and other PAF-like lipids suggests an antiatherogenic role.1 However, hydrolysis of PAF-like molecules produces lysophosphatidylcholine (LPC) and oxidized or fractionated nonesterified fatty acids (NEFA), which are also well-known inflammation mediators.3 Furthermore, plasma levels of lipoprotein-associated PLA2 appeared to be an independent risk factor for coronary artery disease in normolipemic and hypercholesterolemic subjects.4,5 The observation that PAF-AH inhibition in rabbits slows atherosclerosis progression provided further evidence of the relationship between PAF-AH and atherogenesis.6 Electronegative LDL [LDL(−)] is a subfraction of LDL present in plasma that induces the release of interleukin-8 and monocyte chemotactic protein-1.7,8 and is cytotoxic in cultured human endothelial cells.9,10 The mechanism by which LDL(−) induces chemokine release remains unknown, although it could be related to some compositional differences compared with native LDL, with the most remarkable difference being an increased NEFA content in LDL(−) compared with native LDL.7,8 The aim of the present work was to study whether the origin of increased NEFA in LDL(−) could be related to LDL-associated PLA2 activity.

Methods

Lipoprotein Isolation

Plasma samples were obtained from normolipemic (NL) or familial hypercholesterolemic (FH) subjects, as described previously.8 The study was approved by the Ethics Committee of the hospital, and all subjects gave informed consent. Total LDL (1.019 to 1.050 g/mL) was isolated by sequential ultracentrifugation and fractionated into electrophoretic [LDL(+); elution at 0.22 mol/L NaCl] and LDL(−) (elution at 0.50 mol/L NaCl) by anion-exchange chromatography.7 This density range was chosen to avoid contamination with lipoprotein(a) [Lp(a)], which is known to have a high amount of PAF-AH.11 Composition of LDL fractions, including total and free cholesterol, triglyceride, phospholipid, apolipoprotein (apo) B, and NEFA, was determined as described previously.7 Because ultracentrifugation was reported to promote the displacement of PAF-AH from LDL to HDL or lipoprotein-deficient serum,12 an alternative method to ultracentrifugation was used. Total LDL from human NL plasma was isolated by gel filtration chromatography, as described previously.13 Briefly, 10 mL of plasma was subjected to chromatography consecutively (1 mL in each chromatograph) in a Superose 6 column (Amersham Biosciences) and collected in 1-mL fractions, and cholesterol, triglyceride, apoB, and...
apoA-I were measured. Fraction 11, corresponding to the purest LDL fraction (apoB = 0.20 g/L, apoA-I = 0.00 g/L, cholesterol = 0.68 mmol/L, triglyceride = 0.07 mmol/L, total volume 10 mL), was subfractionated in LDL(+)+ and LDL(−) by anion-exchange chromatography as described above. LDL(+) and LDL(−) subfractions were concentrated with an Amicon ultrafiltration concentrator.

**PAF-AH Activity**

A commercial colorimetric assay (Cayman Chemical) was used. This method uses 2-thio-PAF as a substrate and purified human PAF-AH as a positive control. LDL fractions (10 μL of LDL at 0.25 g/L apoB, in Tris 20 mmol/L, EDTA 1 mmol/L, pH 7.4) were incubated with 2-thio-PAF and 5,5'-dithio-bis(2-nitrobenzoic acid) as indicated by the manufacturer. Absorbance was measured at 414 nm at increased times in a microtiter plate, and the slope was used to calculate PAF-AH activity (expressed as micromoles per minute per milliliter).

**Lecithin:Cholesterol Acyltransferase Activity**

Lecithin:cholesterol acyltransferase activity in both LDL fractions was determined by measuring the esterification of labeled free cholesterol with liposomes formed by 3H-free cholesterol/phosphatidylcholine/cholate, as described previously.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis**

Commercial 3% to 8% polyacrylamide gels (Novex) were used. Gels were prerun for 30 minutes with Tris-glycine buffer (Tris 50 mmol/L, glycine 384 mmol/L, pH 8.3) containing 0.1% (v/v) SDS. LDL subfractions (5 μg of apoB) diluted vol/vol in Laemmli sample buffer without 2-mercaptoethanol (BioRad) were electrophoresed for 10 minutes at 25 V, 10 minutes at 50 V, and 1 hour at 100 V. Rainbow marker (Amersham) was used as the molecular weight standard.

**Nondenaturing Polyacrylamide Gradient Gel Electrophoresis**

Nondenaturing polyacrylamide gradient gel electrophoresis (GGE) gels (2% to 16% polyacrylamide) were made as described previously, and electrophoresis developed for 30 minutes at 30 V, 30 minutes at 70 V, and 12 hours at 100 V. Five micrograms of apoB of each LDL subfraction were applied in each lane. A mixture of plasma containing 4 LDL bands of known size was used as standard.

**Western Blot Analysis**

Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immun-blot PVDF, BioRad) in a Mini Trans-blot electrophoretic transfer cell (BioRad) for 1 hour at 30 V with Tris 25 mmol/L, glycine 192 mmol/L, methanol 20%, and SDS 0.1% buffer. PVDF membranes were blocked overnight with Tris-HCl 10 mmol/L, NaCl 150 mmol/L, pH 7.4 buffer (TBS) containing 5% nonfat dry milk, incubated for 1.5 hours with PAF-AH or secretory PL(A2, sPLA2) polyclonal antiserum (Cayman Chemical) diluted 1/1000 with TBS containing 2.5% nonfat dry milk, washed 4 times with TBS containing 0.1% Tween 20 (TTBS), and then incubated for 1.5 hours with the secondary antibody labeled with peroxidase (dilution 1/5000, goat anti-rabbit IgG, Sigma). After 4 washes with TTBS, membranes were developed with a chemoluminescent substrate (Supersignal CL-HRP substrate system, Pierce).

**Statistical Analysis**

Results are expressed as mean ± SD. Differences between groups were tested with Wilcoxon test (for paired data) and Mann-Whitney U test (for unpaired data). A value of P < 0.05 was considered significant.

**Results**

The LDL(−) proportion was 8.0 ± 2.3% in NL samples (n = 8) and 15.6 ± 2.9% in FH samples (n = 8; P < 0.05). Composition of LDL subfractions (Table 1) confirmed features observed previously, including increased NEFA content in LDL(−) compared with LDL(+). PAF-AH activity was approximately 5-fold higher in LDL(−) than in LDL(+) from both NL and FH subjects (Table 1).

Western blot analysis confirmed that PAF-AH was preferably associated with LDL(−) (Figure 1). Western blot of SDS-PAGE gels showed an intense band of 44 kDa in LDL(−) from NL and FH, corresponding to PAF-AH. This band was absent or very faint in LDL(+) fractions (Figure 1B). There is an apparent discrepancy between the 5-fold enrichment of PAF-AH in LDL(−) measured by enzymatic activity and Western blot that suggests greater enrichment. However, these differences could be more apparent than real. First, Western blot is not an appropriate method for quantification; thus, the absence of PAF-AH in LDL(+) observed in some Western blots is probably due to lack of sensitivity. The absolute amount of PAF-AH carried in LDL fractions is unknown, but the molar ratio apoB/PAF-AH is clearly higher than 1, even in LDL(−). Packard et al reported that normolipemic subjects have ~2 mg/L PAF-AH in plasma, in contrast to ~1 g/L apoB. This indicates that the amount of PAF-AH electrophoresed in each lane could be in the order of nanograms.

**Table 1. Composition and PAF-AH Activity of LDL Subfractions (n = 8) Isolated From NL and FH Subjects**

<table>
<thead>
<tr>
<th></th>
<th>NL Subjects</th>
<th>FH Subjects</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>LDL(+)</td>
<td>LDL(−)</td>
</tr>
<tr>
<td>Total cholesterol, % of LDL mass</td>
<td>40.5 ± 1.0</td>
<td>40.7 ± 0.5</td>
</tr>
<tr>
<td>Free cholesterol, % of LDL mass</td>
<td>11.7 ± 0.9</td>
<td>12.2 ± 1.0</td>
</tr>
<tr>
<td>Triglycerides, % of LDL mass</td>
<td>7.3 ± 1.5</td>
<td>8.7 ± 2.1*</td>
</tr>
<tr>
<td>Phospholipids, % of LDL mass</td>
<td>25.9 ± 2.6</td>
<td>25.9 ± 2.7</td>
</tr>
<tr>
<td>ApoB-100, % of LDL mass</td>
<td>26.3 ± 1.6</td>
<td>24.6 ± 1.9*</td>
</tr>
<tr>
<td>NEFA, mol/mol apoB</td>
<td>8.9 ± 1.9</td>
<td>16.8 ± 6.2*</td>
</tr>
<tr>
<td>PAF-AH activity, μmol·min⁻¹·mL⁻¹</td>
<td>1.17 ± 0.55</td>
<td>6.71 ± 1.29*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs LDL (+).
it is clear that a difference in PAF-AH from 5- to 6-fold between LDL(+) and LDL(−) would result in the absence [or the observation of very faint bands, as in LDL(+) or the presence of a well-defined band corresponding to PAF-AH [as in LDL(−)].

Western blot of GGE demonstrated that PAF-AH was bound to LDL(−), because PAF-AH bands resembled the bands of LDL(−) observed in gels stained with Coomassie Brilliant Blue (Figure 3). LDL(−) from FH subjects presented an intense band of greater than normal-sized LDL bands. This band could be due to the presence of small aggregates or to contamination with Lp(a). Evidence indicates that this band was not Lp(a). First, the density range was chosen to avoid contamination with Lp(a) in LDL. Second, Lp(a) content in both LDL fractions, measured by immunoturbidimetry (Roche), was lower than 0.9% in LDL(+) and 2.1% in LDL(−) in all LDL preparations, in accordance with previous reports. Finally, the intensity of this band by protein staining (Figure 3A) was much higher than when revealed by Western blot (Figure 3B); this observation is inconsistent with the finding that Lp(a) contains a 7-fold higher amount of PAF-AH than LDL. Hence, we concluded that this band of greater than normal-sized LDL was due to small aggregates.

The possibility that PAF-AH was coisolated with LDL(−) because of artifacts produced during ultracentrifugation was tested by isolating total LDL from plasma by gel filtration chromatography. Western blot in GGE (Figure 4) and PAF-AH activity of LDL subfractions separated from total LDL isolated by gel filtration showed a patent increase in PAF-AH associated with both LDL fractions compared with samples isolated by ultracentrifugation [PAF-AH activity (μmol · min⁻¹ · mL⁻¹) of total LDL isolated by chromatography, LDL(+) 0.55, LDL(−) 0.75; total LDL isolated by ultracentrifugation, LDL(+) 0.35, LDL(−) 4.50], in accordance with previous reports. However, this decrease does not modify the basic observation that most PAF-AH is bound to LDL(−).

The presence in LDL fractions of other enzymes with PLA₂ activity, such as sPLA₂ and lecithin:cholesterol acyltransferase, was studied. sPLA₂ was not observed in either LDL(+) or LDL(−) by Western blot (data not shown). Moreover, the presence of EDTA 1 mmol/L in LDL samples during the activity assay also ruled out the possibility of 2-thio-PAF degradation being due to sPLA₂, because this enzyme needs calcium for its enzymatic activity. Lecithin:cholesterol acyltransferase activity was very low and equal in both LDL fractions (0.03 ± 0.01 nmol · h⁻¹ · mL⁻¹). Preincubation of both LDL fractions with Pefabloc 500 μmol/L (Sigma), a known specific inhibitor of PAF-AH, abolished the hydrolyzing activity of PAF-AH by up to 90% (Table 2), which indicates that hydrolysis of thio-PAF was specific for PAF-AH.

**Discussion**

PAF-AH has been proposed as a potential risk factor for coronary artery disease, and its plasma concentration and activity have been shown to be directly correlated with plasma and LDL cholesterol. The present work demonstrates that LDL-borne PAF-AH is preferentially bound to LDL(−). Increased PAF-AH activity associated with LDL(−) in NL subjects concurs with reports of preferential distribution of PAF-AH in dense LDL subfractions, because LDL(−) from NL subjects has been reported to be more abundant in dense subfractions. However, LDL(−) from FH patients is abundant in light subfractions; hence, the
The observation that PAF-AH was also preferentially carried in LDL(−) from FH suggests that its association depends on lipoprotein characteristics other than density or size.

Some concerns could be raised based on the possibility that the predominant association of PAF-AH with LDL(−) is an artifactual feature due to the methods used to isolate LDL(−). It was described that ultracentrifugation results in the loss of PAF-AH in LDL and a parallel enrichment of PAF-AH in HDL and lipoprotein-deficient serum,12 but a redistribution of PAF-AH from LDL(+) to LDL(−) is highly improbable because both fractions are of very similar density. Nevertheless, we tested this possibility. Ultracentrifugation did not modify the fact that most PAF-AH was bound to LDL(−), as demonstrated by the experiment using gel filtration chromatography to isolate total LDL. Concerning the use of anion-exchange chromatography to separate LDL(−) from LDL(+), no alternative methods based on the electric charge of molecules exist, because electrophoresis is an excellent analytical technique but is not suitable as a preparative method. However, several arguments indicate that it is very improbable that anion-exchange chromatography promoted a preferential association of PAF-AH with LDL(−). According to previous reports by Stafforini and colleagues,19 it is unlikely that PAF-AH leaves LDL as a consequence of a relatively small change in ionic strength. Those authors reported that the interaction between PAF-AH and LDL involves protein-protein interactions between specific residues of PAF-AH and apoB.19 They also suggested that these interactions were tight because PAF-AH was copurified with a carboxyl-terminal fragment of apoB.20 In earlier works,21,22 these authors failed to isolate PAF-AH from LDL using changes in the ionic composition and strength of the buffer and had to use detergents (Tween 20) to separate LDL from PAF-AH. In the same report, it was stated that PAF-AH activity elutes in a DEAE-Sepharose column (an anion-exchange resin similar to Q-Sepharose) at a lower ionic strength than most of the LDL/VLDL precipitate injected, which indicates that PAF-AH has a lower electronegativity than most LDL. In a more recent work,23 in which CHAPS was used instead of Tween 20 to isolate PAF-AH, it was reported that PAF-AH elutes in a MonoQ column (which contains the same quaternary amine as Q-Sepharose as active group) at an ionic strength of 0.15 to 0.20 mol/L NaCl; hence, PAF-AH alone would elute in the first step (0.22 mol/L NaCl) of the stepwise gradient that we used to separate both LDL fractions, i.e., in the step containing LDL(−). These observations indicate that it is very unlikely that either ultracentrifugation or anion-exchange chromatography could induce an artifactual binding of PAF-AH to LDL(−).

The association between LDL(−) and PAF-AH suggests several implications concerning the atherogenicity of these lipoproteins. The mechanisms by which LDL(−) induces chemokine release are poorly understood. Although some authors reported that LDL(−) contained increased oxidation products9,18,24 other investigators found no evidence of oxidative modification.7,8,10 The high activity of PAF-AH in LDL(−) points to a possible role of this enzyme in the inflammatory effect of LDL(−). PAF-AH activity generates LPC and short-chain or oxidized NEFA. In accordance with this, increased NEFA7,8 and LPC24 content was observed in LDL(−). Both LPC and NEFA are known inflammation mediators. LPC is chemotactic for monocytes,1,25 induces apoptosis in endothelial and smooth muscle cells,26 and involves protein-protein interactions between specific residues of PAF-AH and apoB.19 They also suggested that these interactions were tight because PAF-AH was copurified with a carboxyl-terminal fragment of apoB.20 In earlier works,21,22 these authors failed to isolate PAF-AH from LDL using changes in the ionic composition and strength of the buffer and had to use detergents (Tween 20) to separate LDL from PAF-AH. In the same report, it was stated that PAF-AH activity elutes in a DEAE-Sepharose column (an anion-exchange resin similar to Q-Sepharose) at a lower ionic strength than most of the LDL/VLDL precipitate injected, which indicates that PAF-AH has a lower electronegativity than most LDL. In a more recent work,23 in which CHAPS was used instead of Tween 20 to isolate PAF-AH, it was reported that PAF-AH elutes in a MonoQ column (which contains the same quaternary amine as Q-Sepharose as active group) at an ionic strength of 0.15 to 0.20 mol/L NaCl; hence, PAF-AH alone would elute in the first step (0.22 mol/L NaCl) of the stepwise gradient that we used to separate both LDL fractions, i.e., in the step containing LDL(−). These observations indicate that it is very unlikely that either ultracentrifugation or anion-exchange chromatography could induce an artifactual binding of PAF-AH to LDL(−).

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| TABLE 2. Effect of LDL Subfraction Preincubation With the PAF-AH–Specific Inhibitor Pefabloc (500 μmol/L) on 2-thio-PAF Degradation |
|---|---|---|
| **PAF-AH Activity** (μmol·min⁻¹·mL⁻¹) | Control | Pefabloc (500 μmol/L) | PMSF (4 mmol/L) |
| NL-LDL(+) | 0.50 ± 0.14 | 0.18 ± 0.11 | 0.30 ± 0.14 |
| NL-LDL(−) | 5.82 ± 0.95 | 0.20 ± 0.14 | 0.08 ± 0.04 |
| FH-LDL(+) | 0.80 ± 0.28 | 0.28 ± 0.11 | 0.20 ± 0.07 |
| FH-LDL(−) | 4.48 ± 0.67 | 0.10 ± 0.07 | 0.10 ± 0.07 |

The effect of PMSF (4 mmol/L), a general inhibitor of serine lipases, is also shown. Results are mean of duplicates from 2 independent experiments.
increases monocyte chemotactic protein-1 expression in endothelial cells.\textsuperscript{27} Oxidized NEFA is also a chemoattractant to monocytes.\textsuperscript{3} The present findings suggest that chemokine release induced by LDL(−) could be a consequence of the high PAF-AH activity in LDL(−) through the production of LPC, NEFA, or both.

This observation is consistent with a proinflammatory role for PAF-AH, in accordance with its positive correlation with plasma cholesterol and coronary artery disease risk.\textsuperscript{4,5} The use of specific inhibitors also supports this possibility, because PAF-AH inhibition decreased monocyte chemotactic protein-1 expression in endothelial cells.\textsuperscript{27} Oxidized NEFA is also a chemoattractant to monocytes.

In summary, we report that most PAF-AH activity is bound to LDL(−) and suggest that this enzyme could be related to the chemokine release induced by LDL(−) in endothelial cells. Further studies are required to define the role of LDL(−)-bound PAF-AH.

Acknowledgments

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References

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