Susceptibility of phospholipids of oxidizing LDL to enzymatic hydrolysis modulates uptake by P388D$_1$ macrophage-like cells

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Abstract Addition of the phospholipids 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PLE) and 1-*O*-hexadecyl-2-desoxy-2-amino-arachidonoyl-*sn*-glycero-3-phosphocholine (PLA) to [^{125}I]LDL and subsequent Cu^{2+}-induced oxidation result in significant differences in protein modification and uptake by P388D$_1$ macrophage-like cells. PLE-treated LDL is ingested at a 1.27-fold rate compared to PLE-treated LDL and displays enhanced electrophilic mobility. Similar results (1.43-fold enhanced uptake of LDL preloaded with PLE) are obtained when the uptake of phospholipid-enriched oxLDL particles are examined. The preference for ingestion as well as protein modification of both preparations is, however, reversed under experimental conditions allowing diffusion and inactivation of a fraction of the peroxidation products. These findings suggest that LDL-associated PAF-acetylhydrolase can exert a dual role and, to be protective to LDL, require an appropriate microenvironment, capable of binding certain species of oxidatively fragmented lipids.

Key words: Low density lipoprotein; PAF-acetylhydrolase; Lipid oxidation; Macrophage-uptake; Phospholipid

1. Introduction

Oxidation of LDL, an event generally thought to be critical in the development of atherosclerosis [1–6], is accompanied by the generation of lysophospholipids [7]. One source of phospholipase activity, a PAF-acetylhydrolase [8], has been identified, an enzyme which is associated with low and high density lipoproteins (LDL and HDL) in human plasma [9,10]. It is Ca^{2+}-independent and exhibits an apparent molecular weight of 43.000 kDa [11,12]. The enzyme is secreted by human macrophages [13] and exerts a marked substrate specifity for PAF and phospholipids carrying short and polar *sn*-2 residues. It has been demonstrated recently that phospholipids carrying unsaturated fatty acids like arachidonic acid at the *sn*-2 position are fragmented by peroxidative processes to afford biologically active PAF-like compounds. These products are also substrates of PAF-acetylhydrolase [14–16], suggesting the possibility that this enzyme affects the concentration and half-life of oxidatively degraded phospholipids in vivo.

Lipid peroxide decomposition products cause derivatization of the LDL apolipoprotein B100 (apo B100) [17]; scavenger-mediated uptake of LDL by macrophages apparently involves the modification of the ε-amino groups of apo B100 lysines [18–20]. Therefore, factors enhancing LDL modification may be critical in the development and progression of atherosclerotic lesions. Reaction of LDL with lipid peroxidation products and aldehydes results in increased uptake of such modified particles by macrophages, leading to an accumulation of lipids and subsequently to a conversion of macrophages to foam cells [21]. Since PAF-acetylhydrolase catalyzes the hydrolysis of fragments derived from polyunsaturated phospholipids [14], the enzyme may affect the composition of oxidation products and thereby their potential to act as LDL-modifying agents. If this hypothesis is correct, PAF-AH should modulate the degree and type of LDL modification which determines the degree of interaction with the scavenger receptor. Thus, the action of PAF-acetylhydrolase on oxidatively fragmented phospholipids could be critical in atherogenesis.

In this study we provide evidence that modification and cellular uptake of oxidizing LDL is modulated by the susceptibility of phospholipids to enzymatic hydrolysis, and that the PAF-acetylhydrolase, as well as other phospholipases, could play a dual role in LDL modification, depending on their microenvironments.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Aldrich Chemical Co. (Steinheim, Germany), Fluka (Neu-Ulm, Germany) and E. Merck (Darmstadt, Germany). Biochemicals were obtained from Boehringer Mannheim GmbH (Mannheim, Germany), Sigma (Munich, Germany) or Serva (Heidelberg, Germany). Material for cell culture was obtained from Nunc (Wiesbaden, Germany). [^{3}H]Arachidonic acid, [^{3}H]PAF and [^{14}C]PAF were products of DuPont de Nemours (NEN, Bad Homburg, Germany).

[^{125}I]LDL (7.70 TBq/mmol), prepared according to the method of Sinn et al. [22], was purchased from Immuno Diagnostik GmbH (Bensheim, Germany) and diluted with unlabeled LDL to attain a specific activity of 100 kBq/mg protein.

Protein concentration was determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA) and BSA as the standard. 1-*O*-Hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine was purchased from Biomol (Hamburg, Germany). Chloroform and methanol were distilled from P_2O_5 and from Mg prior to use. Silica gel (grade 60, 70–230 mesh) for column chromatography was from Machery-Nagel (Düren, Germany); TLC plates for analytical and preparative use (0.25 and 1 mm, F 254) were from E. Merck (Darmstadt, Germany) and were pre-eluted with methanol. Phospholipids were spotted with Phospray (Supelco, Bad Homburg, Germany) on TLC. Proton nuclear magnetic resonance spectra were obtained on a Bruker WM 300 spectrometer

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Abbreviations: LDL, low density lipoprotein; LPS, lipopolysaccharides; HBSS, Hank's balanced salt solution; PAF, platelet activating factor; PAH-AH, platelet activating factor-acetylhydrolase; PLA, 1-*O*-hexadecyl-2-desoxy-2-amino-arachidonyl-*sn*-glycero-3-phosphocholine; PLE, 1-*O*-hexadecyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; [^{125}I]E-LDL, ^{125}I-labeled LDL preloaded with PLE; [^{125}I]A-LDL, ^{125}I-labeled LDL preloaded with PLA; PLA$_2$-PLE/PLA-LDL, LDL modified with oxidized phospholipase-treated phospholipids (PLA/PLE); TNBS, 2,4,6-trinitrobenzene sulfonic acid; BHT, butylated hydroxytoluene; DCC, dicyclohexylcarbodiimide; DMAP, dimethylaminopyridine; REM, relative electrophoretic mobility; PLA-LDL, LDL oxidized in the presence of PLA; PLE-LDL, LDL oxidized in the presence of PLE.

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(Bruker Physik AG, Karlsruhe, Germany) and proton chemical shifts are relative to tetramethylsilane (TMS), the internal reference. The spectra were run in $CDCl_3$ and multiplicities are reported as singlet (s), doublet (d), triplet (t) or multiplet (m). FAB mass spectra were recorded on a MAT 311 A (Varian, Bremen, Germany) using a FAB gun from Ion Tech (Teddington, UK) and glycerol as matrix (Xe, 6 kV, ion current = 1 mA). LSC counting was performed on an Isocap 300 (Searle, Chicago, USA). ^{125}I radioactivity was quantified with a LKB-Wallac RiaGamma 1274 (Turku, Finland).

2.2. LDL-preparation and (co)oxidation, preparation of phospholipid-enriched LDL

LDL was isolated from pooled human plasma containing EDTA (1 mg/ml) by sequential flotation in an ultracentrifuge at preselected densities (between 1.019 and 1.063 g/ml) as described [23] and dialyzed extensively against Tris-buffer (20 mM, pH 7.8, 150 mM NaCl, 0.2 mM EDTA); LDL was kept sterile under nitrogen in Tris-buffer at 4°C and used within 2 weeks. For oxidation, LDL was dialyzed against the same buffer (except EDTA) prior to use. Oxidation of ^{125}I-labeled LDL (100 kBq/mg protein, 1 mg of protein/ml) was carried out in Tris-buffer by addition of $CuSO_4$(20 μM) and incubation for up to 24 h at 37°C. Reactions were stopped by addition of EDTA (0.2 mM) and BHT (20 μM). For co-oxidation with phospholipids, phospholipids were added in Tris-buffer (20 mM; pH 7.8) to attain a final concentration of 300 μM.

[^{125}I]LDL enriched with PLA or PLE was obtained according to a published protocol [18]. Briefly, PLE and PLA phospholipids were dissolved in ethanol and dried under N_2 in a silanized glass tube. Then phosphate buffered saline and 2.5 mg of protein of a density >1.21 g/ml plasma fraction (as a source of plasma transfer proteins) were added, and the mixture (PLE 300 μM or PLA 300 μM) was vortexed vigorously for 1 min. To an aliquot of these mixtures [^{125}I]LDL (1.7 MBq/mg, 1 mg/ml, EDTA 0.2 mM, BHT 20 μM) was then added and the samples were incubated for 1 h at 37°C. LDL was re-isolated by density gradient ultracentrifugation as described, dialyzed, and oxidation was performed as outlined above. To modify native LDL with oxidized phospholipids, PLE and PLA were oxidized ($CuSO_4$ 20 μM, 24 h, 37°C) followed by a 1 h incubation with native [^{125}I]LDL at 37°C (PLE 300 μM or PLA 300 μM) and subsequent dialysis against Tris-buffer (20 mM; pH 7.8, EDTA 0.2 mM, BHT 20 μM) to obtain phospholipid-modified [^{125}I]LDL (100 kBq/mg). In some experiments immobilized PLA_2 attached to beaded agarose (bee venom 4200 units/g; Sigma, Deisenhofen, Germany) was added to the oxidized lipids (10 units/300 μl, phospholipids 300 μM) and the samples were incubated at 37°C (0.5 h). Prior to addition to the LDLs, the PLA_2 was separated by centrifugation (5 min, 1500 × *g*).

2.3. Synthesis of phospholipids

PLA was prepared by a route via 1-*O*-hexadecyl-2-(*S*)-aminopropanol as published by this laboratory previously [24]. The reaction of 1-O-hexadecyl-2-desoxy-2-amino-*sn*-glycero-3-phosphocholine (50 mg, 0.104 mmol) with arachidonic acid (300.4 mg, 1 mmol) was performed in chloroform in the presence of DCC (35.4 mg, 0.3 mmol) and DMAP (183.3 mg, 1.5 mmol) at room temperature. Stirring for 18 h under an argon atmosphere yielded crude 1-*O*-hexadecyl-2-desoxy-2-amino-arachidonoyl-*sn*-glycero-3-phosphocholine purified by column chromatography eluting first with a mixture of chloroform/methanol (5:1, v/v), then with chloroform/methanol/water (65:45:8, v/v). Subsequent preparative TLC (elution with chloroform/methanol/water 65:45:8, v/v, under argon) and extraction with chloroform/methanol (5:1, v/v) afforded 30.7 mg (0.04 mmol, 40%) of pure compound. 1-*O*-Hexadecyl-2-desoxy-2-amino-5,6,8,9,11,12,14,15-[^3H]arachidonoyl-*sn*-glycero-3-phosphocholine (4.2 mg, 0.0055 mmol, 45 MBq/mM) was synthesized and purified in an analogous way using [^3H]arachidonic acid (21 mg, 0.07 mmol, 45 MBq/mmol) in the presence of DCC (2.4 mg, 0.02 mmol) and DMAP (12.3 mg, 0.1 mmol). The concentration of phospholipid-containing samples was estimated by phosphate determination according to Ames and Dubin [25].

MS (FAB, glycerol, pos. mode): m/z = 767 $[M + H]^+$. ^1H NMR ($CDCl_3$) δ ppm: 0.85 (6H, m, -CH_3), 1.25 (34H, m, -$(CH_2)_{14}$-CH_3; -$(CH_2)_3$-CH_3), 1.5 (2H, m, -O-CH_2-$(CH)_{14}$-CH_3), 1.65 (2H, m, -NH-CO-CH_2-CH_2-), 2.0–2.1 (4H, m, -CH_2-CH = CH-), 2.3 (2H, t, -CO-CH_2-), 2.8 (6H, m, -CH = CH-CH_2-CH = CH-), 3.32 (9H, s, -$N^+(CH_3)_3$), 3.55 (2H, m, -CH_2-$N^+(CH_3)_3$), 3.7 (1H, m, -CH-NH-), 3.75 (2H, m, -CH-CH_2-O-CH_2), 3.9 (2H, m, -CH-CH_2-O-P-), 4.3 (2H, m, -P-O-CH_2-CH_2), 5.35 (8H, m, -CH = CH-). Tritiated phospholipids were characterized by FAB-MS.

2.4. Cell Culture, uptake of lipoproteins

The $P388D_1$ macrophage cell line (clone 3124, provided by American Type Culture Collection, MD, USA) was cultered at 37°C in RPMI 1640 supplemented with glutamine (2 mM), 10% heat inactivated fetal calf serum (FCS), penicillin (50 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere (5% CO_2:95% air).

Uptake studies were carried out using cultured macrophages (3×10^6) in 50 mm-dishes (Greiner, Frickenhausen, Germany). 2 h after seeding, the macrophages were treated with LPS (*Escherichia coli*, serotype 026:B6, 100 ng/ml) for 1 h at 37°C, washed three times with 2 ml of HBSS and incubated with another 2 ml of HBSS for 20 min at 37°C. After the preincubation, HBSS was removed and 1.5 ml HBSS containing 0.1% of BSA and ^{125}I-labeled lipoproteins (80 μg protein/dish) were added for 30 min at 37°C. The cells were washed three times with 1.5 ml of ice-cold HBSS and then solubilized with 1.0 ml sodium hydroxide (0.3 M) and Triton X-100 (0.1%) for 1 h at 37°C. Aliquots were taken for determination of radioactivity (500 μl) and protein content (5 μl).

2.5. Electrophoresis

The electrophoretic mobility (30 mA, 50 V, 1.5 h) of the lipoproteins was determined in an agarose gel (1.0%) in Tris-CH_3COOH (pH 8.3, 8 mM) supplemented with EDTA (0.2 mM). Protein staining was carried out with Coomassie blue R250. The electrophoretic mobility was determined relative to bromophenol blue.

2.6. Determination of reactive lysines

The number of lysine residues was measured by titration with TNBS as described [26] using valine as standard. LDL (5 μg) was mixed with 100 μl $NaHCO_3$ (4%, w/v) and 5 μl of TNBS (0.1%, w/v). After incubation for 1 h at 37°C, 10 μl of HCl (1 mol) and 10 μl of SDS (10%) were added.

2.7. PAF-acetylhydrolase assay

An aliquot of the respective lipoprotein (30 μg) was transfered to a substrate solution ([^3H]PAF, 200 μM) and incubated for 1 h at 37°C. The reaction was stopped by the addition of glacial acetic acid (20 μl), and *N*-methyl-[^{14}C]PAF (5 kBq/sample) was added as internal standard. The lipids were extracted according to the method of Bligh and Dyer [27], and the lyso-PAF concentration was determined by TLC separation and scraping of spots visualized with Phospray by liquid scintillation counting.

3. Results

The aim of this study was to analyze the effect of LDL-associated phospholipase activity on the oxidative modification of LDL by supplementation with two ether phospholipids, PLE and PLA. Phospholipids carrying an arachidonic acid ester at the *sn*-2 position are natural components of the lipoprotein [28] and are degraded in the course of lipid peroxidation [29]. The two compounds used in this study differ only at the *sn*-2 position: whereas PLE carries an ester oxygen, the other compound (PLA) is substituted with an amide nitrogen. This difference creates significant differences in hydrolytic stability as shown for the analogue 2-amino-2-desoxy-acetyl-PAF [30]. Also, the replacement of the ester moiety by an amide function abolishes the hydrolytic activity of the enzyme; therefore, selective hydrolysis of short-chain oxidation products from endogenous and exogenous ester phospholipids of LDL by PAF-AH could result in significant differences in terms of apoB modification when compared to alterations caused by unhydrolyzable fragments of an amide analogue like PLA. To test this hypothesis, LDL samples were oxidized in the presence of equal amounts of the two exogenous phospholipids PLE and PLA

(300 μM) and the rates of the receptor-mediated endocytosis were monitored. When LDL was subjected to Cu^{2+}-initiated oxidation (24 h) in the presence of PLE, the uptake of the modified lipoprotein (PLE-LDL) by P388D_1 macrophage-like cells was enhanced by an additional 17% compared to oxLDL which was not subjected to any additions except copper (Table 1). Radioiodinated LDL oxidized together with PLA (PLA-LDL) was ingested at somewhat reduced rates: approximatively 9% less radioactivity was recovered from the cells than from oxLDL. Relative to PLA-LDL, PLE-LDL was taken up at a 1.33-fold rate. After 12 h of oxidation this difference was less pronounced (1.13-fold increased rate). The uptake of LDL modified with preoxidized PLE differed from native LDL-uptake by an additional 10%. As for treatment of LDL with the preoxidized amide analog PLA, the uptake was even reduced by 13% (relative to native LDL). Treatment of oxidized phospholipids with immobilized bee venom phospholipase prior to its addition to native LDL, however, markedly enhanced the difference found for the ingestion of both particles (Table 1). Thirty-five percent more PLA_2-PLE-LDL than PLA_2-PLA-LDL was ingested. The results obtained from quantification by endocytosis did not correspond to the degree of modification estimated by TNBS titration of reactive amino groups (Table 1). No significant difference in the number of free lysine groups of oxLDL, PLE- LDL and PLA-LDL could be obtained, while a total reduction of about 40% compared to native LDL was found.

To ensure that differences in uptake were not partly due to interactions of oxidizing LDLs with exogenous phospholipid vesicles, experiments were partly repeated using LDLs enriched with one of the two compounds. Pretreatment of radioiodinated LDL with PLA or PLE in the presence of a phospholipid transfer protein-containing fraction yielded lipoproteins preloaded with unsaturated phospholipids [^{125}I]E-LDL (using PLE) and [^{125}I]A-LDL (using PLA). To estimate the PLA or PLE content of these LDLs, phospholipids carrying [^{3}H]arachidonic acid were synthesized and used as markers. Typically, 10.5% of the radioactivity of both compounds was incorporated, corresponding to an enrichment of about 27 additional phospholipid molecules and an additional 30% of arachidonic acid/particle [29]. Radioiodinated particles, [^{125}I]E-LDL and [^{125}I]A-LDL, preloaded with non-labeled phospholipids were then oxidized as described above. Relative differences in uptake of [^{125}I]A-LDL and [^{125}I]E-LDL were similar to those of the previous experiment; the latter LDL was ingested at an increased rate of 1.43-fold compared to [^{125}I]A-LDL (Table 1). The absolute values of the pre-loaded oxidized lipoproteins ingested, TNBS-sensitive modification and lipoprotein bound tritium were somewhat smaller than found in the first set of experiments. During oxidation, the LDL-associated acetylhydrolase activity decreased progressively (Fig. 1).

After 24 h, 14.0% of the starting activity was left in [^{125}I]E-LDL and 18.5% in the corresponding amide-loaded [^{125}I]A-LDL. Similarly the activity of PLE- and PLA-LDL decreased.

The results described so far apparently imply that PAF-acetylhydrolase or any other phospholipase A_2 activity in oxidizing LDL enhances lipid accumulation in macrophages as a consequence of an increased degree of protein modification. However, due to experimental conditions, all lipid peroxidation products were trapped with LDL in a closed vial. In an attempt to mimic physiological conditions more closely, copper-induced oxidation of phospholipid-enriched [^{125}I]E-LDL and [^{125}I]A-LDL was carried out in dialysis bags. Albumin (15 μM) was placed outside the membrane thus generating a concentration gradient for compounds released from oxidizing lipoproteins to an environment capable of functioning as an acceptor for aldehydes and fatty acids. Under these conditions, modification and uptake of the LDLs were found to be reduced when the data were compared to values obtained from the albumin-free experiments. As shown in Table 1, samples oxidized in dialysis bags in the presence of albumin were characterized by a corresponding reduction in the degree of modification of apoB lysines and by changes of REM in agarose electrophoresis.

Most importantly, both the order of the REM values and the preference in cellular uptake of the respective phospholipid-

Table 1
Effect of phospholipid addition and phospholipid enrichment of LDL on oxidative protein modification and uptake by P388D_1 cells

LDL sample	% uptake[a]	Electrophoretic mobility	Reactive amino groups (mol NH_2/mol apoB)[b]	[^{3}H]-LDL-bound radioactivity (%)[c]
nLDL	3.73 ± 1.47	0.31	325 ± 18	1.9 ± 1.3
oxLDL (24 h)	6.73 ± 1.06	0.73	181 ± 17	8.5 ± 5.2
PLE-LDL (12 h)	5.90 ± 1.07	0.59	200 ± 13	n.d.
PLE-LDL (24 h)	7.84 ± 0.13[d]	0.70	191 ± 18	9.7 ± 6.1
[^{125}]E-LDL	8.46 ± 0.72[e]	0.76	184 ± 24	10.5 ± 5.9
oxPLE + nLDL	4.11 ± 0.13	0.45	256 ± 12	n.d.
PLA_2-PLE-LDL (24 h)	9.11 ± 1.26	0.82	174 ± 34	n.d.
PLE-LDL + BSA (24 h)	5.45 ± 0.65	0.57	247 ± 17	n.d.
PLA-LDL (12 h)	5.22 ± 0.28	0.55	238 ± 19	n.d.
PLA-LDL (24 h)	6.18 ± 0.50[d]	0.67	205 ± 22	9.4 ± 6.0
[^{125}]A-LDL	5.92 ± 0.32[e]	0.62	211 ± 18	10.5 ± 4.8
oxPLA + nLDL	3.30 ± 0.13	0.48	250 ± 28	n.d.
PLA_2-PLA-LDL (24 h)	6.75 ± 0.87	0.64	222 ± 29	n.d.
PLA-LDL + BSA (24 h)	7.68 ± 0.35	0.70	212 ± 18	n.d.

n.d. not determined.
[a] Uptake of [^{125}]-labelled lipoproteins relative to native LDL (100%). Mean ± S.D.
[b] Estimated as TNBS reactivity. Mean ± S.D.
[c] Mean ± S.D.
[d] Significant difference as evaluated by single tailed t-test ($P < 0.01$, $n = 6$).
[e] Significant difference as evaluated by single tailed t-test ($P < 0.01$, $n = 6$).

enriched particles ([^{125}I]E-LDL and [^{125}I]A-LDL) were reversed. Relative to [^{125}I]A-LDL, 43% more radioactivity was measured in cells treated with [^{125}I]E-LDL (Table 1). While REM and uptake of radioactivity in macrophages showed good correlation (r^2 = 0.831, including all data displayed in Table 1), correlation of uptake and lysine modification was poor (r^2 = 0.655).

4. Discussion

Our results indicate that the addition of the two exogenous phospholipids PLA and PLE to oxidizing LDL causes distinct effects in terms of modification and uptake by P388D$_1$ cells. While PLE enhances electrophoretic mobility, protein modification and ingestion by P388D$_1$ macrophage-like cells in the first set of experiments, the opposite is observed for the amide analog PLA.

A similar result is obtained when native LDL is modified with peroxidized phospholipids. The degree of modification is, however, found to be somewhat reduced, which suggests that in situ scavenging of oxidation products by apolipoprotein B may be more efficient. The short interaction time of oxidized phospholipids with LDL (2 h) may contribute to this effect. Addition of exogenous PLA_2 to oxidized phospholipids prior to incubation with LDL, however, markedly increases the differences between the two LDLs in terms of protein modification and macrophage uptake. Therefore, variations in the hydrolytic stability of the *sn*-2-substituents are apparently accompanied by changes in the peroxidative modification of apoB 100 and thus in receptor recognition. Since both exogenously added phospholipids differ only marginally in their physicochemical properties, the finding of distinct rates of cellular uptake is most likely a consequence of a discriminating action of a LDL-associated phospholipase A_2, most likely identical with PAF-acetylhydrolase.

During LDL oxidation a concomittant loss of enzyme activity has been observed. Our data are in good agreement with previous reports dealing with the inactivation of this enzyme in the course of oxidation [31]. The authors suggested the generation of non-competitively inhibiting oxidation products as 4-hydroxynonenal rather than substrate dilution by oxidized phospholipids. In line with that, the inhibitory effect was not found to be altered dramatically by non-hydrolyzable short-chain substrate analogs potentially generated by oxidative fragmentation of PLA. On the contrary, a somewhat larger hydrolase activity remained when [^{125}I]A-LDL was oxidized. Despite a progressive loss of enzyme activity, data from both studies are not in conflict with the hypothesis that acetylhydrolase affects the composition of peroxidation products since significant enzymatic activity (appr. 15–25%) is left after 24 h of oxidation.

Insertion of the phospholipids in the LDL layer with transfer proteins slightly diminished the differences between the LDL samples, probably due to an overall increased ratio of endogenously available to exogenously added unsaturated components, diminishing the differences present in both preparations. A detailed analysis of parameters, like concentration and packing of phospholipids in LDL, however, was not within the scope of this study, although other results suggest that experiments involving other phospholipids may provide further insight into factors determining LDL oxidation and modification [32]. While it is reasonable to assign differences in uptake to the LDL-associated acetylhydrolase, one critical point in this context is to precisely quantify the PAF-AH-mediated effect. Since endogenous *sn*-2-ester phospholipids still represent the main part in samples treated with PLA, as determined by means of tritiated compounds, differences of the LDLs in terms of modification and uptake may represent only a part of a PAF-AH-mediated effect possible.

Our findings can be interpreted as depicted in Scheme 1.

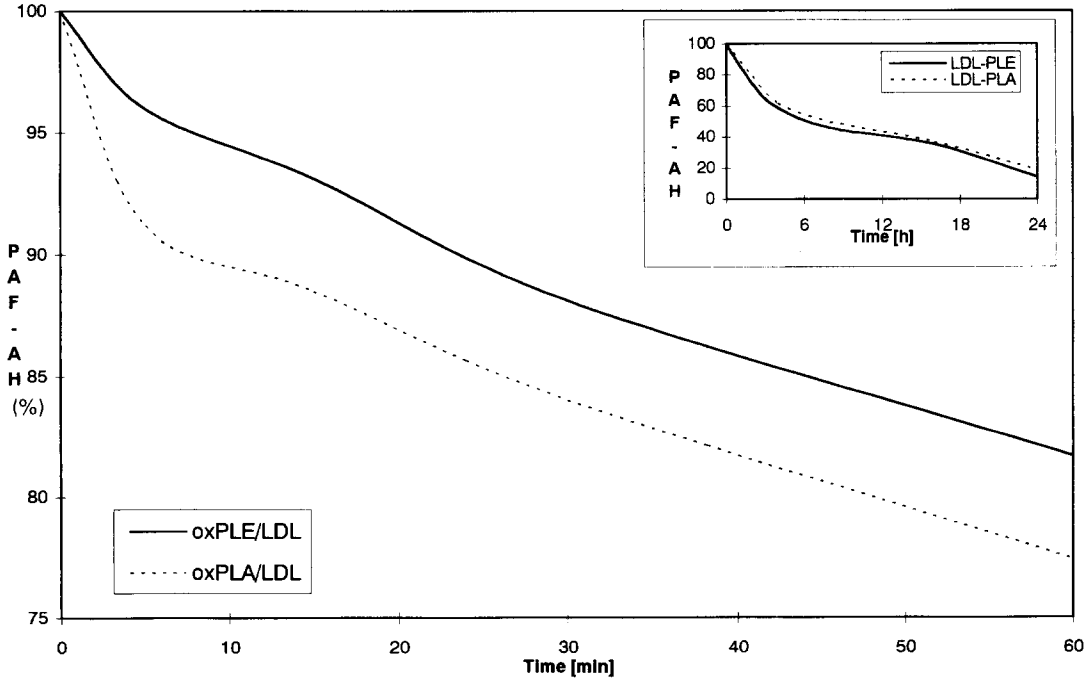
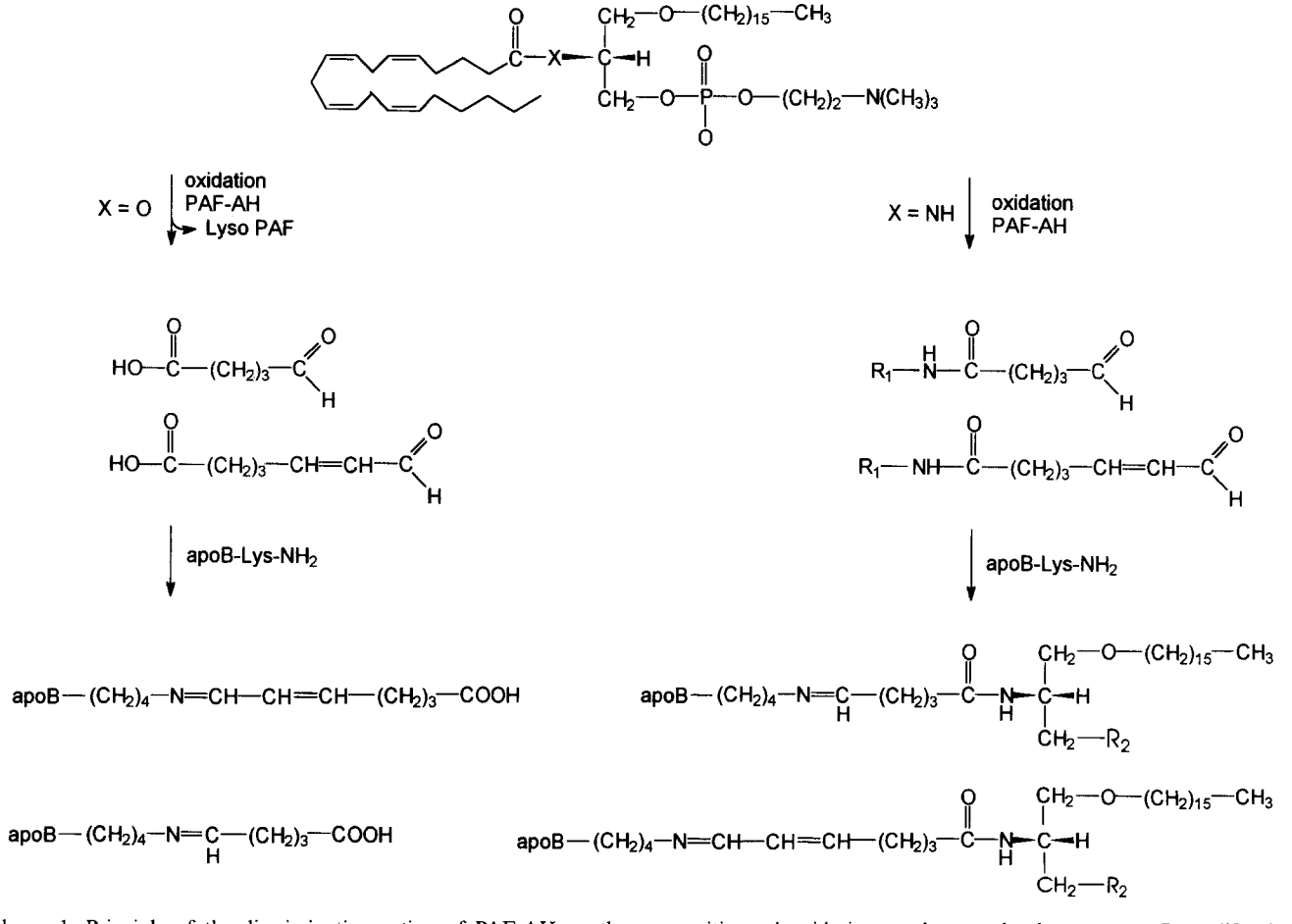


Fig. 1. Change in PAF-AH activity (%) during 24-h co-oxidation of [^{125}I]LDL with PLE and PLA or by modification of 24-h preoxidized PLE or PLA with native [^{125}I]LDL for 1 h (means of three samples).

Oxidative degradation of PLA gives phospholipids with fragmented *sn*-2 residues. The action of PAF-AH on peroxidatively fragmented short-chain ester phospholipids from PLE yields lysophospholipids and, among other products, bifunctional aldehydic carboxylic acids. A very simplistic stoichiometric rationalization of potentially PAF-AH-dependent processes suggests that one molecule of phospholipid PLA initially gives two compounds, one aldehydic phospholipid and one aldehyde. Provided that there is complete hydrolysis, oxidation of PLE in the presence of an active hydrolase affords at least one molecule of lysophospholipid, one aldehyde and one acidic aldehyde. PAF-acetylhydrolase-catalyzed hydrolysis in concert with lipid peroxidation thus may provide more reactive hydrophilic products and consistently, a phospholipid carrying a 5-oxovalerate at the *sn*-2 position has been identified in oxidized phosphatidylcholine [14]. On the other hand, consecutive products of PLA-LDL probably comprise compounds which are more lipophilic and less reactive due to steric hindrance created by the carbon chain length. Hydrolytic activity consequently may cause enhanced protein modification, scavenger receptor binding and subsequent cellular uptake of PLE-LDL. Reactions of lipid fragments with proteins, besides of chemical reactivity, therefore may be affected by water solubility and partitioning between an aqueous and a lipophilic environment. Whether PAF-AH-dependent generation of aldehydic acids plays a significant role in modification of apoprotein B cannot be assessed at the present time. A formation of such species has been previously postulated by Esterbauer [33,34], however, experimental evidence of such species has been hampered so far by the lack of an appropriate method for quantitative determination.

It must be taken into account that the experimental design in the first set of experiments precludes free diffusion of reactive hydrophilic products, an issue which most certainly results in an overall enhanced degree of apoB modification due to an increased reaction rate of LDL-unbound fragments. Despite the fact that the site of LDL oxidation is unknown, it is reasonable to assume deviating conditions for LDL oxidation in vivo. Factors affecting LDL modification in vivo may include the presence of scavenging proteins capable of competing with apoB 100 for reaction with aldehydic agents; in short this means that the composition of the microenvironment may be critical. Therefore modification and uptake was investigated in a second set of experiments. As reported by this laboratory previously [35], albumin may be capable of protecting LDL from degradation via scavenger-dependent pathways by properties distinct to its funtion as antioxidant. Moreover albumin, besides its ability to bind lysophospholipids [36–38] and its antioxidant properties [39], has multiple binding sites for fatty acids [40,41]. This may further favor the scavenging of acidic peroxidation products and macrophages, capable of incorporating albumin-bound fatty acids [42], may contribute to neutralizing reactive



Scheme 1. Principle of the discriminating action of PAF-AH on the composition od oxidation products and subsequent apoB modification. R_1 = 1-*O*-hexadecyl-2-desoxy-*sn*-glycero-3-phosphocholine; R_2 = phosphocholine.

acidic compounds. In addition, the release of PAF-acetylhydrolase activity markedly increases with the differentiation of monocytes to macrophages [43].

In a recently published study, LDL protection by PAF-AH has been suggested and LDL-modification has been analyzed by an alternative approach [44]. Supplementation of purified PAF-acetylhydrolase from erythrocytes as well as inhibitor pretreatment caused a reduced electrophoretic mobility of oxidized LDL. However, despite an apparent correlation between cellular uptake and electrophoretic mobility, this does not necessarily imply causal linkage and changes in conformation and/or apolipoprotein oxidation may be critical as well [45, 46].

Taken together, the results of the current study are in accord with a LDL protective role of PAF-acetylhydrolase activities in terms of oxidative modification and subsequent uptake by macrophages. Our experimental design ensured that sample groups to be compared were supplemented with an equal amount of exogenous phospholipid thus excluding any parameters except the varying susceptibility of oxidatively generated phospholipid derivatives to enzymatic hydrolysis with high probability. The most likely interpretation of the differences observed in the LDL preparations is that the presence of a hydrolytic activity in LDL favors the release of oxidation products which tend to escape from reaction with LDL due to diffusion in an aqueous environment. Accordingly, diffusion and/or attachment to additional binding sites appear to be a prerequesite for a beneficial effect of phospholipases in this context. However, since hydrolytic activity is likely to affect the whole pattern of oxidation products, more experiments are needed to analyze variations in biological activity, like cellular toxicity and mutagenicity, in order to establish a protective physiological role of PAF-AH during LDL-oxidation.

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