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Effect of platelet activating factor on the kinetics of LDL oxidation in vitro

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Oxidatively modified low-density-lipoprotein (LDL) might contribute to the atherosclerotic process. This study was performed to examine an effect of platelet-activating factor (PAF) and of synthetic PAF analogs on Cu(II) induced oxidation of LDL in vitro: The D- and L-isomers of PAF and analogs with short-chain *sn*-2-substituents, 1-*O*-alkyl-2-butyryl-*sn*-glycero-3-phosphocholine and 1-*O*-alkyl-2-heptanoyl-*sn*-glycero-3-phosphocholine, were found to be the most effective inhibitors of LDL oxidation. Oxidation was inhibited completely at PAF concentrations above 100 μM. Lyso-PAF and analogs carrying longer chains at the *sn*-2 position were less effective. These results thus provide evidence for the involvement of other parameters in LDL oxidation beyond the content of natural antioxidants like vitamin E and β-carotene.

Platelet-activating factor; Low-density lipoprotein; Oxidation; Phospholipid

1. INTRODUCTION

Evidence is now accumulating for a critical role of oxidized lipoproteins in the pathophysiology of atherosclerosis [1–3]. Several studies have found a correlation between the lipid peroxides in the circulating plaque and arteriosclerosis [4]; oxidized low-density lipoprotein (ox-LDL) has been demonstrated in atherosclerotic plaques from human and the Watanabe rabbit [5–7]. Ox-LDL potentially has several modes of action in atherogenesis. First, in particular macrophagocytic foam cells of the atheromatous lesion very intensively exhibit immunocytochemical staining for ox-LDL epitopes [5]. Macrophages avidly take up ox-LDL isolated from the atherosclerotic lesion by scavenger receptors and form cytoplasmic cholesteryl ester-rich lipid droplets in the cytoplasma, but do not metabolize native plasma LDL at increased rates. Uptake of ox-LDL by macrophages induces their conversion to cholesteryl ester-rich 'foam cells' [8]. Second, ox-LDL might perpetuate plaque growth by enhancing monocyte recruitment and macrophage retention in the atherosclerotic lesions: it exhibits chemoattractive activity for monocytes and migration inhibitory activity for macrophages [9–11]. Third, ox-LDL is cytotoxic at low concentrations and is antigenic; these potentials of ox-LDL could cause endothelial cell death, formation of thrombi, and further enhance the rate of progression from the early lesions to the fibrous plaque stage and the formation of thrombi [12].

At this point, the mechanisms underlying the LDL oxidation in vivo are not well understood. Several in vitro model systems for studying oxidation of LDL were established. LDL can be oxidized by cultured monocytes/macrophages [13–15], endothelial cells [16,17], smooth muscle cells [18], by treatment with lipoxygenase and phospholipase A_2 [19–21], and incubation with redox metals [17,22]. It is believed that the oxidation of the polyunsaturated fatty acids of the LDL particles in these systems generate aldehydic thiobarbituric acid-reactive substances which might modify the positively charged amino-acid residues of apolipoprotein B. Lipophilic and hydrophilic antioxidants such as vitamin E [23], vitamin C, uric acid [22] and redox metal chelators protect LDL in these in vitro systems and indicate a causative role of reactive oxygen species in the LDL 'modification process. Preventive antioxidant treatment with probucol was shown to reduce the rate of LDL degradation in the arterial wall in Watanabe rabbits and to dramatically prevent the formation and progression of atheromatous lesions in their aortas [24–27].

The extent of modification of LDL in vitro by reactive oxygen species could be diminished to some extent when HDL were added to the in vitro LDL oxidation assay [28]. The protective effect of HDL is potentially very interesting, since an inverse correlation of HDL and premature atherosclerosis was observed. However,

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Abbreviations: PAF, platelet-activating factor; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ox-LDL, oxidized low-density lipoprotein; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid reactive substances; TLC, thin-layer chromatography.

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the mechanism by which HDL protects LDL is puzzling and could not be clarified in detail. It was found that only the rate of ox-LDL degradation by macrophages but not the formation of TBARS in the LDL oxidation assay was altered by HDL. Several possibilities were discussed. It was proposed that the protection of LDL from oxidative modification by HDL might be related to a rapid exchange of oxidized phospholipids from LDL with unoxidized HDL phospholipids, or a competition between LDL and HDL phospholipids for oxidation, or trapping of reactive lipophilic aldehydes in the lipid phase of HDL [28]. In addition, a phospholipase A_2 in the apolipoprotein B of the LDL [20] and/or the platelet-activating factor acetylhydrolase associated with LDL and HDL [29–31] could further modulate the modification of oxidizing LDL by lipid-peroxidation endproducts and modify LDL metabolism by macrophages.

When we decided to test for a possible effect of 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, the platelet-activating factor (PAF), we had speculations for a biological role of PAF in LDL oxidation. Lyso-PAF is produced in activated leukocytes by a phospholipase A_2 at considerable quantities from 1-hexadecyl-2-arachidonyl-PC and then converted to PAF which accumulates in subcellular membranes up to rather high concentrations (0.5 mol%), possibly high enough to influence vesicle fusion and biological properties of the membranes [32]. On the other hand, plasma contains PAF acetylhydrolase, 60–70% of its activity associated with LDL and 30–40% with HDL, which abolishes the biological activity of PAF by hydrolysis of the acetyl residue [29,30,33]. Therefore only low concentrations of this unusual phospholipid are found in plasma lipoproteins. Does PAF and its acetylhydrolase influence LDL oxidation? In the atherosclerotic lesion epitopes of ox-LDL were predominantly localized intracellularly in foam cells, where high concentrations of PAF might occur [5]. PAF might move from subcellular membranes to LDL and influence the rate of its modification.

We decided to test for a possible effect of PAF micelles on LDL oxidation by the addition of PAF to the LDL oxidation assay, in the presence of Cu(II) ions and in an assay system with linoleic acid. The effect of PAF on LDL stability was quantified in the presence of HDL to study the influence of HDL and a role of the HDL-associated PAF acetylhydrolase. Another aim of the study was then to consider the ranking of active analogues of PAF according to their activity in the Cu(II) ion-dependent LDL oxidation assay and to compare this effect with the LDL-stabilizing activity of the known LDL-protective antioxidant, probucol.

2. MATERIALS AND METHODS

2.1. *Materials*

Chemicals were purchased from E. Merck (Darmstadt, Germany), Sigma (Munich, Germany), and Aldrich Chemical Co. (Steinheim, Germany). Biochemicals were purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

2.2. *Synthesis of phospholipids*

1-Palmitoyl-2-acetyl-phosphocholine- and 1-*O*-palmityl-2-*sn*-glycero-3-phosphocholine analogues containing butyryl and heptanoyl groups at the *sn*-2 position were prepared from lyso-PAF and lysolecithin and the appropriate acid anhydride by the method of Gupta et al. [34]. 1-*O*-octadecyl-2-*O*-methyl-phosphatidylcholine (rac.) was generously provided by Dr. A. Hermetter (University of Graz, Austria). The products were purified by silica gel chromatography using chloroform/methanol/H_2O, 55:40:3 as solvent system. The concentration of the products was determined by TLC as described by Colarow [35]. Phospholipids in 20 mM Tris-HCl, pH 8.4, 150 mM NaCl (TN buffer) were sonicated for 15 s prior to use.

Table I

Substituent effects on LDL oxidation[a]

b–[a]–phosphocholine

Compound	Substituent		Duration of the lag phase (% of control)[b]	Slope of the curve during the propagation phase (% of control)[b]
	a	b		
L-PAF	1-*O*-hexadecyl	2-acetyl	157	71
D-PAF	1-*O*-hexadecyl	2-acetyl	143	72
Lyso-PAF	1-*O*-hexadecyl	-OH	106	98
4	1-*O*-hexadecyl	2-butyryl (rac)	167	68
5	1-*O*-hexadecyl	2-heptanoyl (rac)	163	63
6	1-*O*-palmitoyl	2-oleoyl	108	93
7	1-*O*-stearoyl	2-lauroyl	109	92
8	1-*O*-octadecyl	2-*O*-methyl (rac)	142	67

[a] Conditions for LDL oxidation as described in the legend to Fig. 1.
[b] Concentration of test compound was 50 μM.

2.3. *Isolation of LDL and LDL oxidation with Cu(II) ions as prooxidant*

Human plasma was obtained from healthy volunteers. EDTA (pH 7.4) was added to prevent blood clotting. LDL was isolated from pooled plasma by sequential flotation in an ultracentrifuge at preselected densities as described [36]. The concentration of LDL protein was determined with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, USA) and bovine albumin as standard [37]. LDL was kept sterile under nitrogen at 4°C in 20 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1 mM EDTA (TN buffer) up to two weeks and dialyzed against TN buffer prior to use.

LDL was oxidized at a concentration of 125 μg/ml (0.25 μM) in the presence of 10 μM copper(II) as prooxidant in TN buffer. Aliquots of 2 ml were analyzed at 42°C from 0 to 400 min. The oxidation reaction was followed at 234 nm as described by Esterbauer et al. [38] and the appearance of dienes at A_{234} was recorded. The so-called 'lag phase' (defined as the interval between the intercept of the tangent of the slope of the curve with the time-scale axis), and the maximal rate of A_{234} formation (calculated from the slope of the absorbance curve during the propagation reaction) was estimated.

2.4. *Oxidation of linoleic acid in presence of Cu(II)*

Linoleic acid was dispersed into TN buffer. Oxidation assays contained linoleic acid at a concentration of 1 mM, 100 μM Cu(II) with and without D-PAF at a concentration of 50 μM. The samples were then incubated at 42°C. The rate of oxidation was monitored by the increase of the conjugated diene level at 234 nm.

3. RESULTS

3.1. *Characteristics of the LDL oxidation reaction*

Incubation for oxidation of 0.25 μM LDL (125 μg/ml) in Tris-HCl buffer at pH 8 with Cu(II) at 42°C results in a slight increase of A_{234} during the lag phase and a rapid increase during the propagation phase before reaching a maximal value. When we analyzed 20 LDL samples from various healthy donors under our conditions we obtained values of A_{234} between 0.28 and 0.41/0.25 μM LDL protein at the beginning of the incubation and a mean increase of 0.05 O.D. units during the 'lag phase', which lasted 62–95 min (77 ± 8, mean ± S.D.); the maximal increases during the 'propagation reaction' were 0.24–0.3 O.D. units/10 min and the maximal values reached at the end of the propagation phases were 0.85–1.08 O.D. units. Assuming a molar extinction coefficient $e_M = 2.4 \times 104$/mol · cm for diene absorption the level of conjugated dienes was calculated to increase by 70 dienes per LDL during the lag phase and by ~900 dienes per LDL particle during the propagation phase.

3.2. *Effect of PAF and PAF analogues on the LDL oxidation reaction*

Stremler et al. have hypothesized that oxidation and fragmentation of phospholipids containing polyunsaturated fatty acid acyl residues should generate phospholipids with varying *sn*-2 residues and that PAF acetylhydrolase is sensitive to the length of the *sn*-2 residue [39]. Stimulated by their findings, we tested eight *sn*-glycero-3-phosphocholines, derivatives of platelet activating factor 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine in our assay (Table I). Some of the analogues clearly exhibit inhibitory activity on LDL oxidation at a concentration of 50 μM. L-PAF, D-PAF, compounds 4, 5 and 8 prolong the lag phase by 42–67%, and reduce the rate of the increase of the dienes during the propagation reactions by about 30%. The inhibitory effects are dependent on the concentration of the 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines in the assays as shown for L-PAF over a concentration range of 25–160 μM in Fig. 1. The inhibitory activity of L-PAF, D-PAF, compounds 4 and 5, appears to be rather insensitive to the variation of chain length at the *sn*-2 position and also compound 8 exhibited good inhibitory activity; however, the substitutions with longer alkyl chains such as oleoyl and lauroyl residues had only little inhibitory activity at a concentration of 50 μM. When compared with the effect of phenolic antioxidants like probucol on a molar basis, the LDL-stabilizing activity of the L-PAF, D-PAF, compounds 4, 5 and 8 appears rather weak. As published by us before [40], probucol prolonged at a much lower concentration of 2.5 μM the lag phase of the oxidizing LDL by about 60%, however, unlike the *sn*-glycero-3-phosphocholines, the phenolic antioxidant did not influence the rate of the propagation reaction.

3.3. *Effect of PAF and PAF analogues on LDL oxidation in presence of HDL*

We conducted experiments using 2 HDL particles per LDL particle (0.5 μM HDL and 0.25 μM LDL) and 50 μM PAF and PAF analogues. The results clearly show that concomittantly with HDL the prolongation of the lag phase of oxidizing LDL caused by PAF and PAF analogues in the Cu(II) assay is shortened and that the LDL is less effectively stabilized by PAF and its tested analogues (data not shown).

3.4. *Effect of PAF on the oxidation of linoleic acid (LA)*

Micelles containing 1 mM LA appeared to be unstable in the oxidation assay containing 100 μM Cu(II) and

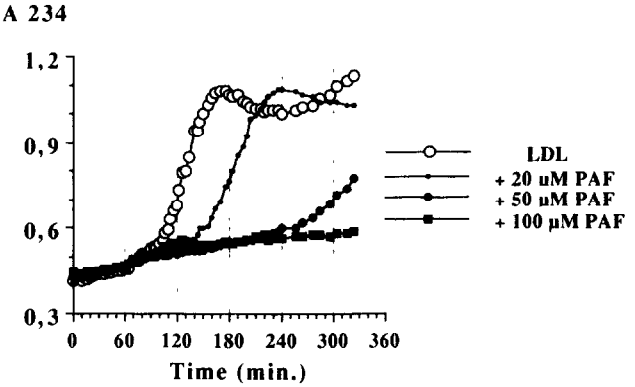

Fig. 1. Kinetics of the LDL oxidation in presence of Cu(II) and PAF as determined by formation of dienes (A_{234}). 0.25 μM LDL in 20 mM Tris-HCl, pH 8, 150 mM NaCl, 10 μM $CuCl_2$ and 0, 20 μM, 50 μM, 100 μM L-PAF was incubated at 42°C.

were oxidized over an incubation period of 5 h as indicated by a continuous increase of dienes at A_{234}. After a 6-h incubation period the A_{234} values reached a plateau. Addition of 50 μM PAF to the oxidation medium did not protect the linoleic acid from oxidation. In the presence of 50 μM PAF even a slight left shift of the diene curve was seen (Fig. 2). This phenomenon indicates a slightly faster oxidation in presence of PAF.

4. DISCUSSION

Incubations of LDL with 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor), and its *sn*-2 substituents 1-*O*-alkyl-2-butyryl-*sn*-glycero-3-phosphocholine and 1-*O*-alkyl-2-heptanoyl-*sn*-glycero-3-phosphocholine protected LDL, since they caused a prolonged stability of the LDL particles against reactive oxygen generated in presence of copper(II) ions. The D- and the L-isomers of platelet activating factor (PAF) exhibited a nearly equal effect on the lag phase of the diene curve. Lyso-PAF appeared to be less active than D- and L-PAF during the lag phase of the diene curve, however, it also decreased the rate of the hydrogen abstraction and diene formation during the propagation phase. The butyryl- and the heptanoyl substituents at the *sn*-2 position of PAF caused a slightly less prolongation of the lag phase when compared with D- and L-PAF, however, they both, like PAF, had only little effect on the propagation rate. PAF *sn*-2 long acyl chain substituents did neither significantly influence the lag period nor the propagation reaction of the oxidizing LDL particle. Exchange of the ester for an ether bond at the *sn*-1 position had no effect on the kinetics of LDL oxidation. Addition of HDL diminished the LDL protective effect of PAF and its analogues. PAF had no antioxidant effect and was not protecting linoleic acid against oxidation.

The reactions occurring in oxidizing LDL particles are very complex and not well understood. Therefore many possibilities remain. A few out of a long list could be addressed by our pilot studies. First, the antioxidant

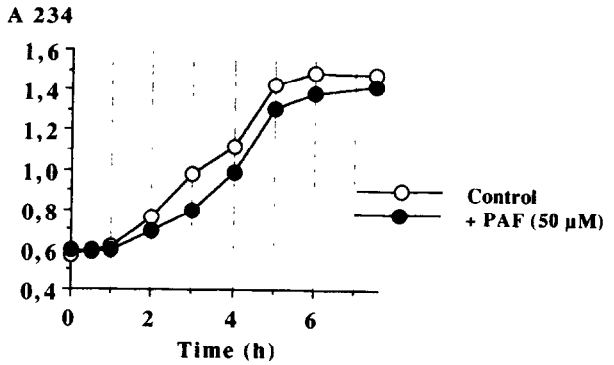

Fig. 2. Effect of 50 μM L-PAF on the Cu(II) initiated oxidation of 1 mM linoleic acid. Incubation was as described in the legend to Fig. 1.

content of LDL, and in particular the concentration of vitamin E as the predominant lipophilic phenolic antioxidant of LDL, are critical for LDL stability. We have no evidence that PAF has antioxidant activity even at rather high concentrations in the assay with linoleic acid and LDL. In these two assays the numbers of PUFAs are comparable. Antioxidants like probucol and β-estradiol are highly effectively inhibiting LDL oxidation at concentrations <1 μM [40]. However, the LDL antioxidants are not the only factor stabilizing LDL and there are reports of only a weak correlation and even a lack of correlation between the content of antioxidants and the duration of the lag phase of oxidizing LDL particles [41]. A second possibility is repair of LDL by PAF during the lag phase increasing stability of LDL in presence of copper(II) ions. It was shown recently that LDL particles, after reducing the hydroperoxide tone of the particle by combined treatment with glutathione peroxidase ebselen and glutathione, become highly resistant in presence of copper(II) ions. It was concluded that hydroperoxides carried by the LDL particles are necessary to generate Cu(I), an initiator of the Fenton reaction, and therefore are most important determinants of the duration of the lag phase [42]. Our data show that PAF reduces the hydroperoxide tone in LDL, since it delays formation of dienes, but we have no evidence for an active repair process. Third, PAF micelles could trap intermediate products released by oxidizing LDL and indirectly prolong the lag phase of LDL. HDL was suggested to protect LDL particles via an exchange of phospholipid hydroperoxides [28]. Parthasarathy et al. hypothesized that phospholipids from LDL might exchange phospholipids with unoxidized HDL. PAF micelles could act via an exchange of phospholipid hydroperoxides in our LDL oxidation assay; our results confirm that HDL was without effect on the formation of lipid peroxides as observed [28]. In the presence of PAF we observed no additive effect of HDL on the formation of dienes in the assay. Paradoxically HDL even reduced protection of LDL by PAF in the LDL oxidation assay. Thus, other mechanisms should be responsible for LDL stabilization by PAF. Fourth, the LDL-associated PAF acetyl hydrolase could influence the lag phase of the LDL in the Cu(II) assay. Stremler et al. [31] synthesized and tested the effect of various PAF *sn*-2 analogues and their effect on the purified enzyme. When they increased and extended the length of the *sn*-2 residue to 9 carbon atoms the catalytic effect of purified PAF acetylhydrolase was reduced dramatically [31]. Our results demonstrate that the prolongation of the lag phase in our LDL oxidation assay caused by the presence of PAF and analogues was also maximal with PAF and its short-chain *sn*-2 analogues and decreased when the *sn*-2 chain length was extended and substitution of the *sn*-2 position with a short chain ether had a comparable LDL stabilizing activity showing that also an ether bond has compara-

ble activity. This analogy makes it possible that PAF acetylhydrolase content of LDL is a critical determinant for the duration of the lag phase of LDL. If this is true, the observed influence of HDL on the PAF-induced prolongation of the LDL lag phase could be explained by the HDL associated PAF acetylhydrolase, which could reduce PAF concentration in the assay.

Thus, our experiments demonstrate that PAF and its *sn*-2 short chain analogues prolong the lag phase of LDL in presence of Cu(II). This effect of the test compounds could be caused by competitive inhibition of the LDL-associated PAF acetylhydrolase. These observations shed light on a role for the PAF acetylhydrolase in LDL oxidation. The concentration and activity of PAF acetylhydrolase could be a determinant of the duration of the lag phase and cause variability in the stability of LDL particles. Further analyses in our laboratories are on the way to test for the extent of variation in LDL stability caused by PAF acetylhydrolase associated with LDL from healthy people and patients with altered lipoprotein metabolism.

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