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Platelet Interaction with Bioactive Lipids Formed by Mild Oxidation of Low-Density Lipoprotein

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Key Words

Platelets · Lysophosphatidic acid · Lysophosphatidylcholine · Sphingosine 1-phosphate · Oxidized phosphatidylcholines

Abstract

Oxidation of low-density lipoprotein (LDL) generates pro-inflammatory and pro-thrombotic mediators that play a crucial role in cardiovascular and inflammatory diseases. Mildly oxidized LDL (mox-LDL) and minimally modified LDL (mm-LDL) which escape the uptake of macrophage scavenger receptors accumulate in the atherosclerotic intima. Oxidatively modified LDL is also present within the electronegative LDL fraction in blood, which is elevated in patients at high risk for cardiovascular diseases. Mox-LDL and mm-LDL, but not native LDL are able to induce platelet shape change and aggregation. LDL oxidation generates lipids with platelet stimulatory properties such as lysophosphatidylcholine, certain oxidized phosphatidylcholine molecules, F_2-isoprostanes and lysophosphatidic acid (LPA). Mox-LDL and mm-LDL are like a Trojan horse carrying these biologically active lipids and attacking cells through activation of physiological receptors and signaling mechanisms. LPA has been identified as the lipid responsible for platelet stimulation by mox-LDL, mm-LDL and also mox-HDL. These lipoproteins activate platelets by stimulating G-protein coupled LPA receptors and a Rho/Rho kinase signaling pathway leading to platelet shape change and subsequent aggregation. LPA-mediated platelet activation might contribute to arterial thrombus formation after rupture of atherosclerotic plaques and to the increased blood thrombogenicity of patients with cardiovascular diseases.

Introduction

Platelets are surrounded by low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in circulating blood. They also might interact with circulating oxidatively modified LDL [1–6]. Platelet interaction with LDL and oxidized LDL could explain the higher platelet aggregability associated with hypercholesteremia, diabetes and cigarette smoking [7–12]. Moreover, upon rupture of lipid-rich vulnerable plaques, platelets get exposed to oxidatively and enzymatically modified LDL and their degradation products accumulating in the plaque lipid-rich core [13], the most thrombogenic part of atherosclerotic plaques [14]. Platelet activation by this plaque material might be important in arterial thrombus formation leading to acute coronary syndromes (angina pectoris, myocardial infarction) or cerebral ischemia (TIA, stroke).

In contrast to the native lipoproteins, both LDL and HDL particles induce platelet activation, once they are

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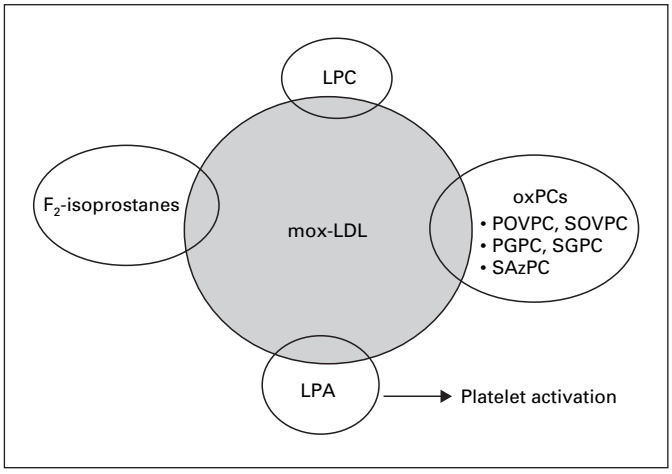

Fig. 1. Platelet-stimulatory lipids formed during mild oxidation of LDL. LPC = Lysophosphatidylcholine; LPA = lysophosphatidic acid; oxPCs = oxidized phosphatidylcholine molecules; POVPC = 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine; SOVPC = 1-stearoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine; PGPC = 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine; SGPC = 1-stearoylpalmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine; SazPC = 1-stearoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine.

oxidatively modified [15–21]. Several biologically active lipids are produced during LDL oxidation (fig. 1). They have often atherogenic actions on cells of the vessel wall (endothelial cells, smooth muscle cells and macrophages) and stimulate circulating blood cells including platelets. The first bioactive lipid produced by LDL oxidation found was lysophosphatidylcholine (LPC) [22, 23]. Other bioactive phospholipids formed during LDL oxidation are derived from oxidative degradation of mainly the 2-arachidonoyl group of 1-palmitoyl-PC and 1-stearoyl-PC, respectively (oxidized PC molecules) [24–26]. Also the F_2-isoprostanes are derived form oxidative transformation of arachidonoyl-containing phospholipids [27]. The most recent discovered bioactive lipid formed by oxidation of LDL is the lysophospholipid LPA [28]. LPA stimulates cells by activation of G-protein coupled surface receptors [29].

Sphingosine 1-phosphate (S1P) and shingosylphosphorylcholine (SPC) are lysosphingolipids present mainly in HDL, but also in LDL [30–32]. In contrast to the other bioactive lipids, the concentration of S1P decreases upon LDL oxidation [32]. Since S1P and SPC in HDL mediate certain cytoprotective actions of HDL on endothelial cells, these lysosphingolipids have been proposed as anti-atherogenic substances [32, 33]. S1P is much less potent in activating platelets than LPA (for review see [29]), and SPC even inhibits platelets [34]. Since S1P and SPC are apparently neither formed by lipoprotein oxidation, nor do they significantly activate platelets, they will not be considered further in this review.

The review discusses the different platelet – stimulatory lipids present in oxidatively modified lipoproteins and focuses on their role in mediating platelet activation by minimally and mildly oxidized LDL.

Mildly Oxidized Lipoproteins (LDL, HDL) Stimulate Platelets

Many studies have shown that native LDL (nLDL) particles themselves do not induce platelet activation, although they can after prolonged incubation sensitize platelets to other stimuli by specific mechanisms that have been unraveled only recently (see chapter Korporaal and Akkerman). In contrast to nLDL, oxidatively modified LDL induces rapid immediate platelet responses such as shape change and aggregation [16–19]. The platelet response appears to be dependent of the type of oxidation method used and the degree of oxidation of LDL. Using cupper-or SIN-1 induced oxidation, mild oxidation has been shown to produce LDL particles with higher platelet-activating potency than strong oxidation [17, 18]. Chemical characterization of mildly oxidized LDL (mox-LDL) showed no indication of protein modification and only a minor decrease of polyunsaturated fatty acids in mox-LDL, but protein modification and an almost complete disappearance of polyunsaturated fatty acids in ox-LDL [18, 35]. mm-LDL obtained by spontaneous oxidation of LDL had platelet activating properties similar to mox-LDL [19, 21, 28]. Both mox-LDL and Minimally modified LDL (mm-LDL) induced shape change of washed platelets at very low concentrations (5–50 μg/ml) [21, 36].

Platelet interaction with mm-LDL and mox-LDL might be – from a pathophysiological point of view – more relevant as compared to platelet interaction with more strongly oxidized LDL, since mm-LDL and mox-LDL (a) are likely to accumulate in the atherosclerotic intima due to its escape from scavenger receptor-mediated uptake by macrophages [37], (b) might circulate in blood, most probably within the fraction of electronegative LDL which is elevated in patients at high cardiovascular risk (familial hypercholesterolemia, hypertriglyceridemia, and diabetes mellitus) [6], and (c) enhance platelet activation induced by other platelet stimuli. Regarding

Table 1. Lipid and antioxidant composition of LDL (approximate molecules/LDL particle)

	Esterbauer et al. [42, 43] Hevonoja et al. [44]	Sanchez-Quesada et al. [6] Benitez et al. [66]	Corrinth and Siess* Siess et al. [28] Zhang et al. [79]
Protein (apoB100)	1	1	1
Cholesteryl ester	1,600		
Triglycerides	170		
Diglyceride	7		
Cholesterol	600		
Total phospholipids	700		490
PC	451	735	322
Lyso PC	80	28	15
SM	185	262	143
PE	10	133	10
LPA (bioassay)			0.05
Acyl-LPA (LC-MS)			0.1
Alkyl-LPA (LC-MS)			0.013
Free fatty acids	6	13	
Total fatty acids	2,700		
Total PUFAs	1,280		
Antioxidants			
α-Tocopherol	6	8	
γ-Tocopherol	0.5		
β-Carotene	0.3	0.17	
α-Carotene	0.1	0.05	
Lycopene	0.2	0.26	
Ubiquinol-10	0.1		

* Unpublished.

LPA = Lysophosphatidic acid; Lyso-PC = lysophosphatidylcholine; PC = phosphatidylcholine; PE = phosphatidylethanloamine; PUFA = polyunsaturated fatty acids; SM = sphingomyeline.

the latter point it has recently been reported, that the degree of oxidation of LDL is critical for its enhancement or attenuation of platelet activation induced by other stimuli. LDL, oxidized <30% enhanced as nLDL platelet function, but LDL oxidized >30% attenuated platelet function, as measured by TRAP-induced fibrinogen binding [38].

LDL oxidation can alter both its protein and lipid moieties [39]. It is of importance, that not only oxidized LDL, but also oxidized HDL, which has a protein moiety different than LDL, activates platelets in vitro [16, 20, 21]. From these observations, we have deduced that (a) lipids produced or altered during lipoprotein oxidation must be responsible for platelet activation, and that (b) the cell membrane receptors which bind the different protein constituents of LDL (apoB100) and HDL (apoE) are unlikely to mediate platelet activation by oxidized lipoproteins. LDL oxidation generates several lipids that can activate platelets as discussed in the following paragraphs (fig. 1).

Oxidized Phosphatidylcholines

General

Oxidized phosphatidylcholine molecules can mimic in vitro major events in the initiation and propagation of chronic inflammation such as to stimulate endothelial cells to bind monocytes, to secrete MCP-1 and IL-8, and to increase expression of atherogenic genes [25, 40, 41]. The current view is that these lipids act as pro-inflammatory agents in atherogenesis. On the other hand, oxidized phosphatidylcholines can also exert anti-inflammatory and cytoprotective effects by interfering with the binding of LPS to the toll-like receptor TLR4 [41]. This event is central for bacterially induced neutrophil activation and the subsequent acute inflammatory responses.

Formation by LDL Oxidation

Oxidation of LDL results in the modification of phospholipids, mainly of phosphatidylcholine (PC), the major phospholipid present in the outer layer of the LDL par-

ticle (table 1) [42–44]. PC oxidation is likely to be initiated by the seeding of LDL with fatty acid hydroperoxides generated by the 12/15-lipoxygenase of vascular cells [26, 45, 46]. Also myeloperoxidase expressed in human macrophages and the NADPH oxidase pathway of vascular cells are important in generating reactive oxygen species for LDL oxidation [47, 48]. Primarily, polyunsaturated fatty acids of phospholipids which are present in large amounts in LDL (table 1) are oxidized to hydroperoxides and subsequently undergo fragmentation to aldehydes. Interestingly, minimal oxidation of LDL predominantly modifies arachidonic acid and less linoleic acid esterified in *sn*-2 position of PC [49].

PC oxidation products exert different biological activities depending on the chemical bond at the *sn*-1 position [24, 50]. PCs containing alkyl residues (ether bond) at the *sn*-1 position become upon oxidative fragmentation of the unsaturated fatty acid at the *sn*-2 position (i.e. oxidized alkyl-PCs) agonists for the platelet-activating factor (PAF) receptor [50], whereas oxidized acyl-PCs activate cells via unknown receptors [41].

Many pro-inflammatory acyl-phosphatidylcholine oxidation products derived from 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine or 1-stearoyl-2-arachi donoyl-*sn*-glycero-3-phosphorylcholine have been identified in atherosclerotic lesions and mm-LDL. These are: 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phospho ryl-choline (POVPC), and 1-stearoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine and 1-stearoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine, and 1-palmitoyl-2-(5, 6-epoxyiso-prostane E2)-*sn*-glycero-3-phosphorylcholine [24, 51]. No information is available about their content in mm-LDL. It should be stressed that all these oxPC molecules are degraded by PAF-acetylhydrolase (PAF-AH), which is associated with LDL, and inhibitors of PAF-AH such as PMSF are normally included during LDL oxidation to allow the measurement of the oxPCs [24].

Platelet Receptors and Responses

Purified polar phospholipids derived from oxidized LDL have been shown to activate platelets via the PAF receptor [52]. Particularly, 1-O-hexadecyl-2-(5-oxovaleroyl)-*sn*-glycero-phosphocholine (alkyl-POVPC), an analog of PAF has been identified in human atheroma that induced aggregation of rabbit platelets through activation of the PAF receptor. In contrast its 1-acyl-analog POVPC stimulated in rabbits only platelet shape change, which was independent from the activation of the PAF receptor [53]. Very recently, three biologically active oxidative products of acyl-PC (1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine) were identified by electrospray ionization mass spectrometry which induced shape change of human platelets at low micromolar concentrations: 1-stearoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine, 1-stearoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine, and 1-stearoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine. All these compounds activated platelets independently of the PAF receptor and LPA receptors [54]. The concentrations of these platelet-stimulating alkyl-PC and acyl-PC products in mm-LDL and mox-LDL are not known.

F_2-Isoprostanes

F_2-isoprostanes are formed in situ on phospholipids by free radical-catalyzed peroxidation of arachidonic acid [55]. Oxidative stress and lipid peroxidation in vivo can be measured by enhanced plasma levels and urinary excretion of the hydrolysis product 8-iso-$PGF_2\alpha$ [56]. F_2-isoprostane formation is enhanced in association with cardiovascular risk factors such as cigarette smoking, hypercholesterolemia, and types 1 and 2 diabetes mellitus [57]. Cigarette smokers, who are exposed to free radicals present in cigarette smoke, showed elevated plasma levels of free and esterified F_2-isoprostanes in plasma and 8-iso-$PGF_2\alpha$ in urine, that fell significantly after cessation of smoking [57]. F_2-isoprostanes are not only formed in plasma, but also in LDL after exposition to oxidative stress [27]. Oxidation of LDL resulted in the subsequent depletion of the anti-oxidants ubiquinol-10, α-tocopherol, lycopene and β-carotene, and their consumption lead to the concomitant increase of lipid peroxides and esterified F_2-isoprostanes [27]. As reported in table 2 of reference [27] the increase of esterified F_2-isoprostanes during LDL oxidation was maximally 20 n*M* in LDL (0.2 mg LDL protein/ml), which corresponds to 0.1 nmol per mg protein or 0.05 mol/mol LDL. It is not known, how much the unesterified 8-iso-$PGF_2\alpha$ increases during LDL oxidation.

Since lipid peroxidation also occurs in human atherosclerotic lesions, it is not surprising that the content of F_2-isoprostanes (measured as 8-iso-$PGF_2\alpha$) was found to be higher in human atherectomy specimen as compared to vascular tissue devoid of atherosclerosis [58]. Immunohistochemical studies found that foam cells adjacent to the lipid necrotic core of plaques were markedly positive for 8-iso-$PGF_2\alpha$ indicating that these cells removed lipid

peroxidation products accumulating in the plaque core [58].

Unesterified, free 8-iso-$PGF_2\alpha$ (in former publications 8-epi-$PGF_2\alpha$) exposed to washed platelets induced at concentrations between 0.1 and 1 μM shape change, inositol phosphate production and cytosolic Ca^{2+} increase [59, 60]. Alone 8-iso-$PGF_2\alpha$ induced weak, reversible aggregation, but when added together with subthreshold concentrations of ADP, thrombin or collagen, it induced maximal irreversible aggregation [59, 60]. Although in one study platelet activation induced by 8-iso-$PGF_2\alpha$ could be prevented by the thromboxane receptor antagonist SQ29548, it was concluded on the basis of other results that 8-iso-$PGF_2\alpha$ did not activate either of the TXA2/EP (TP) receptor isoforms described in platelets [60]. This is contrast to studies of two other groups which described 8-iso-$PGF_2\alpha$ as partial agonist of platelet TP receptors with TP antagonistic properties [59, 61]. Own studies supported these findings: shape change induced by 8-iso-$PGF_2\alpha$ was blocked by the TP receptor antagonist BM13.505. Shape change induced by mox-LDL was, however, not inhibited after pretreatment of platelets with BM13.505 [36]. Together, these results indicate that F_2-isoprostanes are formed during mild oxidation of LDL, but they do not mediate shape change induced by mox-LDL.

Lysophosphatidylcholine

LPC Formation during Oxidation of LDL

LPC during LDL oxidation is generated mainly by phospholipase A_2-mediated degradation of PC [62]. This constitutively active plasma enzyme is primarily associated with LDL, whereas only a small portion (about 15–20%) is present in HDL [63]. The enzyme has been termed lipoprotein-associated PLA_2 or PAF-AH [63, 64]. Unlike other PLA_2, PAF-AH is specific for short acyl groups ($Cn < 6$) at the *sn*-2 position of phospholipid substrates. The enzyme is Ca^{2+} independent, and its highly restricted substrate specificity is apparently essential to prevent the continuous phospholipid hydrolysis of lipoproteins [63]. PAF-AH hydrolyzes effectively oxidized phospholipids such as the above-described oxidized PC molecules, and therefore prevents the accumulation of these bioactive PC molecules during LDL oxidation. Substances which inhibit PAF-AH such as PMSF have to be included in studies in vitro in order to observe the accumulation of oxidized bioactive PC molecules [24]. The biological function of PAF-AH in atherosclerosis is controversial [63], but evidence is now increasing to support a proatherogenic function [64]. Although it degrades pro-inflammatory bioactive oxidized PC molecules, it generates a new pro-inflammatory lipid mediator, LPC.

LPC is present already in nLDL at significant amounts (table 1). The mean values of LPC range between 2 and 5% LPC of total LDL phospholipids in most of the recent studies [65, 66] (Corrinth and Siess, unpublished observations). LPC increases 2–10-fold dependent on the degree of LDL oxidation. Electronegative LDL, found in the small dense fraction of plasma LDL, contains twice as much LPC as nLDL [66]. Mild oxidation also doubles the LPC content of nLDL (from 3.0 ± 1.2 to 5.9 ± 0.7% of LDL phospholipids). Strong oxidation results in a drastic increase of LPC to 28 ± 4% of LDL phospholipids (Corrinth and Siess, unpublished observation).

Action of LPC on Platelets

LPC has multiple effects on vascular cells [67, 68], and the reported effects on platelets are complex and on the first glance controversial. LPC can activate and inhibit platelets. An early very detailed study of the action of LPC on platelets found, that in platelet-rich plasma (PRP) LPC concentrations >100 μM caused instantaneous inhibition of stimulus-induced platelet aggregation which was found to be partially reversible over a period of 60–90 min [69]. LPC-induced potentiation could not be observed, neither in citrated nor in heparinized PRP. With washed platelets, inhibition was also observed at concentrations above 30 μM, but the inhibitory effect not only rapidly disappeared but was followed by transient potentiation of aggregation and serotonin release [69, 70]. Both inhibition and potentiation were observed at concentrations of LPC that did not cause a significant change in platelet shape or platelet lysis. It was concluded that the effects of LPC on platelet function were due to structural modification of the platelet membrane probably by its amphiphilic properties [69, 70]. In later studies, LPC-mediated platelet inhibition was related to the G-protein mediated activation of adenylate cyclase, or to the incorporation of LPC into the platelet membrane leading to changes of membrane fluidity [71, 72]. Another study found that incubation of washed platelets with low concentrations of LPC (10 μM) could stimulate P-selectin expression through a protein kinase C-dependent pathway [73]. Since LPC is known to bind to albumin, it seems that the action of LPC on platelets is critical dependent on the albumin concentration in the suspension medium; it might explain the different effects of LPC on PRP

which contains high concentrations of albumin (3–5%) and on washed platelet suspensions containing no or a low albumin concentration (0.1–3%). We observed no shape change after addition of LPC concentrations up to 30 μ*M* to platelet suspensions, provided the buffer contained albumin (0.5%) (Corrinth and Siess, unpublished observations). Some of the reported controversial effects of LPC on platelets might also be explained by different platelet isolation procedures.

For our considerations, the effect of LPC-enriched LDL on platelets is important. Native LDL which contains already significant amounts of LPC, did not activate the platelets directly, even if added at high concentrations to washed platelets (2 mg LDL/ml corresponding to 60 μ*M* LPC; Corrinth and Siess, unpublished observations) [18]. Treatment of LDL with bee-venom PLA_2, which resulted in a large selective increase of LPC not only failed to activate platelets, but it inhibited stimulus-induced activation of washed platelets [74]. In order to analyze the effect of the increased LPC concentrations present in mox-LDL, nLDL was spiked with LPC to reach the LPC content of mox-LDL, and then exposed to platelets. No direct activation of platelets was observed (Corrinth and Siess, unpublished observations). In a recent study, however, it has been observed that LDL isolated from insulin-dependent diabetic patients had a significant higher LPC content (9.62 ± 2.04% of LDL phospholipids) as compared to LDL of healthy controls (4.55 ± 1.86% of LDL phospholipids). The LDL of diabetic patients increased cytosolic Ca^{2+} concentrations and potentiated ADP-induced aggregation [65]. Whether this effect was due to the increased LPC content of LDL of diabetic patients or other mechanisms, remains an open question.

In conclusion, low concentrations of LPC (10–30 μ*M*) or LPC-enriched LDL, does not induce platelet activation, provided the platelet buffer contains minimal concentrations of albumin. Following exposure of platelets to high concentrations of LPC (100 μ*M*, added to plasma or buffer containing albumin) or LPC-containing ox-LDL (1 mg ox-LDL/ml corresponding to 200 μ*M* LPC final concentration) LPC is likely to intercalate into the platelet membrane and to inhibit and potentiate platelet function through its amphiphilic properties, not through binding to specific receptors [70]. The observation that a high LPC content in LDL is inhibitory might explain studies showing that strongly oxidized LDL activates platelets less than mildly oxidized LDL [18, 19, 38].

Lysophosphatidic Acid

LPA Induces Shape Change and Desensitizes Platelets to Subsequent Stimulation by mox-LDL, mm-LDL and mox-HDL

In studies of our laboratory it was observed that very low concentrations of mm-LDL and mox-LDL (5–200 μg/ml) induced reversible shape change and tyrosine phosphorylation of specific proteins in a manner similar to physiological stimuli [35, 36, 75]. Shape change induced by mm-LDL and mox-LDL showed homologous (mm-LDL/mm-LDL; mox-LDL/mox/LDL) and heterologous (mm-LDL/mox-LDL and vice versa) desensitization as observed for platelet stimuli that activate G-protein coupled receptors such as thrombin, TRAP, ADP, and the thromboxane analog U46619 [21, 28]. Moreover, mox-HDL (200–300 μg/ml) also induced reversible shape change which could not be re-induced by a subsequent addition of mox-HDL. Significantly mox-HDL and mm-LDL/mox-LDL induced shape change showed cross-desensitization [21]. We therefore postulated that (a) the platelet receptor activated by mox-LDL and mox-HDL are identical and is most likely a G-protein coupled receptors; (b) known LDL and HDL binding proteins can be excluded as receptors, since apoB100 of LDL and apoE of HDL bind to different surface receptors; (c) the active mediator generated by mild oxidation of LDL and HDL must be a lipid. Based on these postulates, the shape change desensitization assay was used and screened for lipids with platelet stimulatory properties, and within 2 days LPA was identified as the lipid that desensitized the shape change induced by mox-LDL, mm-LDL and mox-HDL [21, 28]. These results indicated that LPA is likely to be the active lipid mediating shape change by mox-LDL, mm-LDL and mox-HDL

LPA Formation during Oxidation of LDL and HDL

As expected by the desensitization experiments, LPA increased during mild oxidation of lipoproteins [28]. By using the shape change response as bioassay to quantify LPA after organic extraction of lipoproteins and TLC separation of polar phospholipids, it was found that LPA increased about 8-fold in mox-LDL and mm-LDL (from 0.1 nmol to 0.8 nmol/mg protein by using 1-palmitoyl-LPA for the standard curve) [28]. The biologically measured LPA content of mox-HDL was similar as in mox-LDL, it ranged between 0.5 and 1 nmol/mg protein [21].

LPA represents a class of different molecules. Depending on the type of fatty acid and the linkage of the fatty

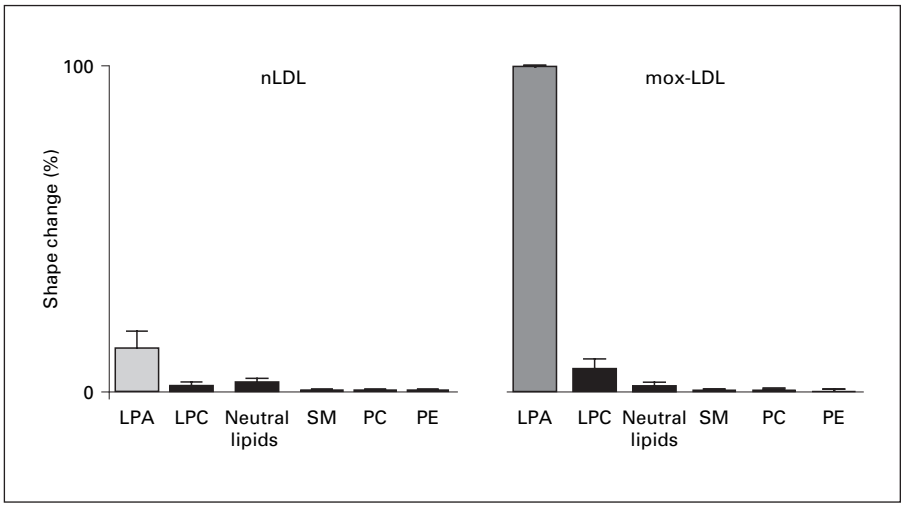

Fig. 2. LPA is the main platelet-stimulating lipid in mox-LDL and nLDL. Lipids were extracted from nLDL and mox-LDL by a two-step procedure, and separated by TLC. Lipids equivalent to 0.1 mg protein were added to platelet suspensions for measurement of platelet shape change. The highest activity found in the LPA-fraction of mox-LDL was set to 100%. The figure is based on data of table 1 of reference [28].

acid to the glycerol backbone (either in ester or ether bond), the different LPA molecules vary in their potency to activate platelets. The alkyl-LPA species are about 20 times more potent than the corresponding acyl-LPA species [76, 77], and it was recently found that of various acyl-LPA species, arachidonoyl-LPA was 7 times more potent than other saturated or unsaturated acyl-species [77, 78].

Measurements by LC-MS showed recently that mild oxidation of LDL increased the total amount of alkyl-LPA, but not acyl-LPA [79]. Levels of acyl-LPA were 0.19 and 0.18 nmol/mg protein in native and mox-LDL respectively. Alkyl-LPA increased about 6-fold from 0.025 to 0.15 nmol/mg protein, i.e. it accounted almost to 50% of total LPA in mox-LDL. Alkyl-LPA with saturated as well as unsaturated fatty acids (including 20:4) increased during oxidation, and the rank order of the different fatty acids of alkyl-LPA in mox-LDL was 18:1 > 18:0 > 18:2 > 16:0. This is in contrast to the acyl-LPA species: only acyl-LPA (18:0) increased, whereas acyl-LPA (18:2) and acyl-LPA (20:4) decreased during LDL oxidation [79]. These results indicate that peroxidation of unsaturated fatty acid occurs only on acyl-LPA, whereas alkyl-LPA must be protected against oxidative degradation. The LPA content of nLDL measured by platelet bioassay and LC-MS was in the same range (0.1 and 0.2 nmol/mg LDL, respectively). This amount of LPA was apparently not sufficient to confer platelet-stimulatory properties to nLDL.

The LPA content in mox-LDL measured by the platelet bioassay (0.8 nmol/mg protein) was higher as measured by LC-MS (total LPA: 0.32 nmol/mg protein), which is likely due to the increase of the more potent alkyl-LPA species in mox-LDL. The 8-fold increase of LPA measured by bioassay correlated well with the 6-fold increase of alkyl-LPA measured by LC-MS [28, 79].

Although the amount of LPA formed present in mox-LDL is relatively small (about 0.16 molecules per LDL particle; 0.03% of total LDL phospholipids), LPA is the main platelet-activating lipid of mox-LDL as shown by lipid fractionation studies. LPA was by far the most active lipid of mox-LDL; apart from some platelet-stimulatory activity in the LPC fraction, no other significant platelet activity was observed in the various lipid fractions of mox-LDL (fig. 2) [28]. LPA was also the main platelet-activating lipid of nLDL (fig. 2), but apparently without functional consequences: LPA was found not to be responsible for the platelet sensitizing effect of LDL [80].

The pathway by which alkyl-LPA is generated during mild oxidation of LDL or HDL is not known. Since LPA is produced by mild oxidation of both LDL and HDL, it is likely that LPA is formed directly by lipid oxidation. However, enzymatic pathways for LPA generation that are activated by oxidation cannot be excluded. In serum, LPA is mainly generated by two distinct steps: (1) PLA1 or PLA2 cleavage of PC, PE or PA, and (2) lyso-phospholipase D cleavage of the basic phospholipid headgroups choline or ethanolamine [81, 82]. Whether these enzymes are present in LDL and HDL is not known.

LPA Receptors and Antagonists

Platelets express all three EDG-family LPA receptors as revealed by RT-PCR analysis. [83]. LPA_4 mRNA could not be detected in a megakaryocytic cell line suggesting

that platelets do not express this receptor [84]. NPSerPA and NPTyrPA are LPA receptor ligands with both agonist as well as antagonist properties on platelets [28, 77, 85]. NPSerPA is an agonist of the three EDG family LPA receptors (LPA_{1-3}), whereas NPTyrPA activates LPA_2 and is an antagonist of LPA_3 [86]. Recently it was shown that DGPP 8:0, an antagonist of LPA-receptors of the EDG-family with preference to LPA_3 over LPA_1 without an effect on LPA_2 receptors, inhibited LPA-induced platelet activation suggesting that LPA_1 and LPA_3 mediate LPA-induced platelet activation [77, 87]. DGPP 8:0 and PA 8:0 were the only PA and LPA analogs that lacked agonistic activity on isolated platelets. Very recently, synthesis of various fatty alcohol phosphates, substances that lack a glycerol backbone and represent the minimal pharmacophore to interact with LPA receptors, provided LPA receptor ligands which activated all three LPA receptors (LPA_{1-3}), or only LPA_2, or were pan-antagonists, i.e they inhibited LPA_{1-3}. All these compounds inhibited the LPA-induced shape change, and, with the exception of the LPA receptor pan-antagonist, activated platelets with different potency [86]. Since platelet LPA receptors show a homologous desensitization 5 min after activation [28], it is possible that the agonistic fatty alcohol phosphates and fatty alcohol thiophosphates might exert their inhibition through desensitizing LPA receptors rather than receptor antagonism.

The effects of LPA-receptor agonists and LPA-receptor antagonists on platelets are not consistent with their pharmacological properties on the known EDG family receptors LPA_{1-3} [88]. Therefore it remains to be elucidated, whether all these LPA receptor antagonists exert their effect on platelets exclusively through the EDG family LPA receptors or in addition through an elusive platelet LPA receptor. In support of the heterogeneity of LPA receptors in platelets, Tokumura and colleagues noted that while platelets collected from all donors responded to acyl LPA 18:1, some of them did not respond to alkyl-LPA 16:0 [78]. In this study, the authors found that platelets from a donor with platelets non-responsive to alkyl-LPA expressed mRNA to $LPA_2 > LPA_3 > LPA_1$ receptors. DGPP 8:0 and NPSerPA inhibited both acyl-LPA and alkyl-LPA responses [77]. Thus, the possibility remains that LPA responses in platelets are mediated via yet unidentified receptor(s).

Platelet Responses to LPA and mox-LDL: Inhibition by Albumin and LPA-Receptor Antagonists

The type of response of washed platelets to LPA and mox-LDL is very critical dependent on the type of platelet isolation procedure. After pretreatment of PRP with aspirin, washed platelets can be obtained which respond with shape change upon exposure to low (1–10 n*M*) concentrations of acyl-LPA or 5–50 μg/ml concentrations of mox-LDL or mm-LDL [36, 75, 77]. LPA with 16:0, 18:0 or 18:1 fatty acid in ester bond show similar potency in inducing shape change [77]. Even after high concentrations of acyl-LPA (1–10 μ*M*), only shape change, but no aggregation or secretion is observed in these platelets [77, 89].

Shape change induced by LPA or mox-LDL, mm-LDL or mox-HDL was completely inhibited by LPA receptor antagonists, indicating that LPA is the platelet-activating compound in all three oxidized LDL preparations (fig. 3) [21, 28, 77]. The LPA-receptor antagonists tested were NPSerPA, NPTyrPA as well as DGPP8:0.

In washed platelets, low nanomolar concentrations of LPA induced shape change (EC50 of acyl-LPA 16:0: 2–20 n*M*). In contrast , much higher concentrations were required to induce shape change in PRP (EC50: 4–12 μ*M*) and in blood (EC50: 4 μ*M*) [77, 89, 90]. Also for mox-LDL much higher concentrations were required to observe shape change of PRP (EC50 $\geq$ 2 mg/ml) as compared to washed platelets (EC50: 5–50 μg/ml) [90]. Preincubation of washed platelets with albumin inhibited dose-dependent LPA or mox-LDL-induced shape change (IC50 of albumin 6 μ*M*) [89]. These observations might be explained by the high affinity binding of LPA to albumin [91]. Albumin in plasma might compete with platelet LPA receptors for LPA binding. Alternatively, albumin might also block binding of LPA to certain platelet LPA receptors. Indeed, studies on Sf9 cells expressing the individual LPA receptors showed that albumin interfered at lower concentrations with LPA activation of LPA_3, as compared with LPA activation of the other two LPA receptors [92].

Aggregation of washed platelets upon stimulation by low concentrations of LPA (0.1–1 μ*M*) can be observed, when platelets are separated from plasma by centrifugation and are resuspended in buffer without further washing and without addition of apyrase [89, 93]. These platelets are, however, often preactivated (i.e. they are not discoid) [90]. LPA-induced platelet aggregation in these platelet preparations is likely due to synergism of LPA with extracellular ADP artificially present in the medium [77, 89]. Indeed, addition of apyrase or ADP-receptor antagonists completely blocked the LPA-induced aggregation in this platelet preparation [89, 93]. Also mox-LDL (1–2 mg/ml) could induce platelet aggregation of washed platelets, when apyrase was omitted

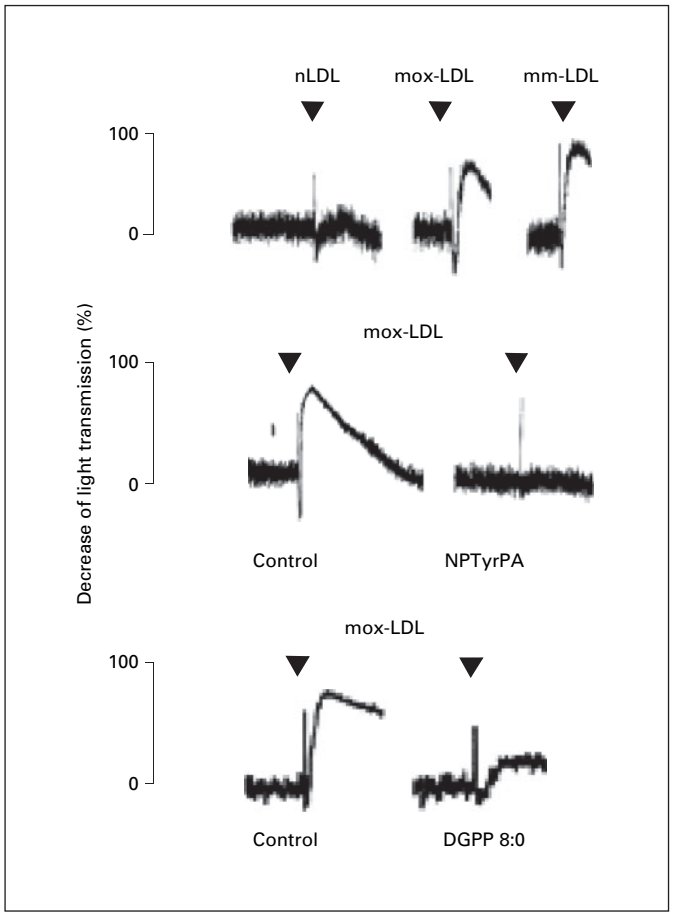

Fig. 3. Induction of shape change by mox-LDL and mm-LDL, but not nLDL. Inhibition by LPA-receptor antagonists. Suspensions of washed platelets were exposed to nLDL, mox-LDL or mm-LDL (0.2 mg/ml), or preincubated for 10 min with solvent (control), or the LPA-receptor antagonists NPTyrPA (10 μ*M*) and DGPP 8:0 (5 μ*M*) before stimulation with mox-LDL. Data are from references [28, 77].

during platelet isolation and in the platelet resuspension buffer [18, 21]. Aggregation induced by mox-LDL was completely inhibited by LPA receptor desensitization or NPTyrPA, indicating that LPA is also the compound of mox-LDL responsible for stimulation of platelet aggregation [21].

We have recently followed a platelet preparation protocol, which used PGI_2 during the isolation procedure [94]. A preparation of discoid platelets was obtained, which showed shape change to low concentrations of LPA (0.1–1 μ*M*), and platelet aggregation to higher concentrations of LPA (>1 μ*M*) [95]. Addition of fibrinogen was required to observe platelet aggregation. Upon high concentration of LPA (>10 μ*M*), maximal irreversible platelet aggregation was observed which was associated with secretion. LPA-induced aggregation was almost completely inhibited by either antagonist of the two ADP receptors $P2Y_1$ and $P2Y_{12}$, but not by the cyclooxygenase inhibitor aspirin. Similar results were obtained upon LPA-induced platelet aggregation in whole blood [95]. These results indicate LPA synergized with ADP to induce platelet aggregation [77]. Where did the ADP originate from? In washed platelet preparations containing apyrase, ADP can only derive from dense granules of stimulated platelets. Indeed LPA concentrations inducing aggregation of washed platelets (>3 μ*M*), evoked a significant, but minute dense granule secretion (as measured by the release of serotonin). In blood, ADP could derive from dense granules of activated platelets and/or red cells [89].

In blood, LPA induced platelet aggregation at low concentrations, the EC50 for alkyl-LPA (16:0) was 0.3 μ*M*, and for acyl-LPA (16:0) 5 μ*M* [95]. These LPA concentrations are close to LPA plasma levels: the reported concentrations of acyl-LPA are between 0.1 and 0.6 μ*M* [96–98]. No studies have been done concerning the effect of mox-LDL or mm-LDL on platelets in whole blood.

Signaling Pathways Stimulated by LPA and mox-LDL

LPA activates the small GTP-binding protein Rho via stimulation of the heterotrimeric G_{12} and G_{13} proteins. By performing conditional gene knock-out experiments in mice, it has been recently shown, that in stimulated platelets only G_{13}, but not G_{12} mediates Rho-dependent shape change [99]. These results suggest that in platelets LPA will activate Rho through G_{13} stimulation. During LPA- and mox-LDL induced shape change, Rho-mediated activation of Rho-kinase (p160ROCK) leads to the reorganization of the actin cytoskeleton underlying platelet shape change [100–102]. We have recently found that LPA induced a rapid and reversible increase of Thr696 and Thr853 phosphorylation of myosin phosphatase targeting subunit during shape change (Pandey et al, unpublished observations). Phosphorylation of myosin phosphatase targeting subunit inhibits the binding and activity of the myosin phosphatase to dephosphorylate myosin light chain (MLC). As consequence the phosphorylation of MLC is enhanced [100, 101]. Specific inhibition of the Rho kinase with the inhibitor Y-27632 or the new specific, structurally unrelated Rho-kinase inhibitor H-1152 inhibited myosin phosphatase targeting subunit and MLC phosphorylation, and in parallel the LPA- and mox-

LDL induced shape change (Pandey et al., unpublished observations) [100, 101].

Recent studies in our laboratory have shown that Rho-kinase not only controls MLC phosphorylation, but also the F-actin increase during LPA-induced shape change. Rho-kinase led to rapid Thr508 phosphorylation of LIM-kinase 1. However, the LIM-kinase substrate cofilin did not show an increased phosphorylation, probably because of simultaneous dephosphorylation by a cofilin phosphatase (Pandey et al., unpublished observations).

In addition to the Rho/Rho-kinase pathway, both LPA and mox-LDL stimulate a different pathway during shape change, i.e. the activation of the Src-family tyrosine kinases and the subsequent activation of the tyrosine kinase Syk [75], which possibly mediates the exposure of fibrinogen-binding sites on the fibrinogen receptor during shape change [103], a prerequisite for platelet aggregation. It is not known which G-protein is involved in the activation of this pathway.

High concentrations of LPA (>1 μM) were needed to stimulate in platelets a small increase of cytosolic Ca^{2+}. LPA as well as mox-LDL (0.5 mg/ml) increased the cytosolic Ca^{2+} concentration mainly through the stimulation of Ca^{2+} entry across the plasma membrane, much less by cytosolic Ca^{2+} mobilization from intracellular stores [75, 77]. LPA and mox-LDL showed cross-desensitization of the cytosolic Ca^{2+} increase (LPA/mox-LDL and mox-LDL/LPA), and the mox-LDL evoked Ca^{2+} increase was inhibited by LPA receptor antagonists [75]. These results indicate that the mox-LDL induced Ca^{2+} response in platelets is also completely dependent on LPA receptor activation.

Conclusion and Perspective

Although LDL oxidation generates numerous lipids with platelet stimulatory properties, it emerges that LPA is the only lipid responsible for platelet stimulation by mox-LDL and mm-LDL. These lipoproteins activate platelets by stimulating G-protein coupled LPA receptors and a Rho/Rho kinase signaling pathway leading to platelet shape change and subsequent aggregation. Notably alkyl-LPA is not only the LPA-species which is formed by mild oxidation of LDL, but also the LPA species with high platelet-activating potency. In whole blood, submicromolar concentrations of alkyl-LPA induced platelet shape change and, synergistically with ADP, aggregation. Alkyl-LPA might be present in circulating electronegative and oxidized LDL, which is elevated in patients at high cardiovascular risk, and acyl-LPA as well as alkyl-LPA are known to accumulate in atherosclerotic plaques and to mediate platelet activation by the plaque lipid-rich core [28, 77]. It is therefore possible that LPA-mediated platelet activation contributes to the increased blood thrombogenicity of patients with cardiovascular diseases and participates in arterial thrombus formation after rupture or erosion of atherosclerotic plaques. The platelet LPA receptor which remains to be identified might present a promising new anti-thrombotic target for the prevention of ischemic cardio- and cerebro-vascular diseases.

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