Bioactive lipids as key regulators in atherosclerosis

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3 Atherosclerotic Lesion Progression Changes Lysophosphatidic Acid Homeostasis to Favor Its Accumulation

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Submitted

Abstract

Objective: Lysophosphatidic acid (LPA) accumulates in the lipid core of human atherosclerotic plaques and is the primary platelet-activating lipid constituent of plaques. Here, we aimed to delineate the metabolic regulation of LPA homeostasis in atherosclerotic lesions at various stages of disease progression.

Methods and Results: Atherosclerotic lesion formation in carotid arteries was accomplished by perivascular collar placement in LDL receptor-deficient mice. At 2-week intervals after collar placement, lipids and RNA were extracted from the plaques. Enzymatic and LC-MS based lipid profiling revealed progressive accumulation of LPA species in atherosclerotic tissue preceded by an increase in lysophosphatidylcholine, a building block in LPA synthesis. Plaque expression of cytoplasmic phospholipase A_2IVA (cPLA_2IVA) and calcium-independent PLA_2VIA (iPLA_2VIA), which generate LPA, was gradually increased while that of LPA acyltransferase α, which hydrolyzes LPA, was quenched. Increased expression of cPLA_2IVA and iPLA_2VIA in advanced lesions was confirmed by immunohistochemistry. Moreover, LPA receptors 1 and 2 were 50% decreased and 7-fold upregulated, respectively, while the intracellular LPA receptor peroxisome proliferator-activated receptor γ and its effector CD36 showed a 93% reduction at later stages of plaque formation.

Conclusions: During atherogenesis expression of key proteins in LPA homeostasis is increasingly perturbed favoring intracellular LPA production within the plaque, which concurs with the observed progressive accumulation of this thrombogenic, pro-inflammatory lipid in the plaque. Combined with the changed signal transduction through LPA receptors, this indicates a key role for LPA in plaque development. This renders intervention in the enzymatic LPA production an attractive measure to normalize intraplaque LPA content and thereby to reduce plaque development and thrombogenicity.

Introduction

Cardiovascular diseases continue to be one of the major causes of death in Western society. They are often attributable to the development of vulnerable atherosclerotic lesions in the large arteries consisting of a lipid core and an overlying thin fibrous cap[1,2]. Upon rupture of the fibrous cap, the thrombogenic content of the plaque will be exposed to the blood circulation and trigger the coagulation cascade, leading to thrombus formation and acute coronary syndromes[3,4]. One of the major thrombogenic constituents of the lipid core was demonstrated to be lysophosphatidic acid (LPA)[5-7].

LPA is a bioactive lipid that was originally known as a key intermediate in *de novo* lipid synthesis, but has emerged as an intra- and intercellular phospholipid messenger with a variety of biological activities[8]. LPA has multiple effects on blood cells and various cell types of the vessel wall and evidence is accumulating that this lipid can aggravate cardiovascular diseases by virtue of its athero- as well as thrombogenic activity[7,9]. LPA mediates multiple cellular processes that are instrumental in atherogenesis, including smooth muscle contraction[10,11], endothelial/leukocyte interaction[12], platelet aggregation[5] and cell proliferation[13,14].

LPA exerts its effects through specific G-protein-coupled receptors (GPCRs), such as LPA receptor 1, 2 and 3 (LPA_{1-3}), which belong to the Endothelial Differentiation Gene family, and the genetically more distant LPA_4 (GPR23) and LPA_5 (GPR92)[15-18]. Furthermore, studies have revealed a direct role for LPA as peroxisome proliferator-activated receptor (PPAR)γ agonist, regulating the expression of genes that contain PPAR Response Elements (PPRE)[7]. The CD36 promoter was found to contain such PPRE, rendering it sensitive to LPA induced transcriptional upregulation of CD36 in macrophages. Indeed, Zhang *et al.* have shown that LPA induces neointima formation via PPARγ activation and CD36 upregulation[19].

LPA is formed during mild oxidation of low-density lipoprotein (LDL) and was found to be the main platelet-activating lipid constituent of LDL[5,20]. It is in part directly deposited in the plaque by mildly oxidized LDL retained in the subendothelial matrix[21]. However, LPA can also be formed *in situ* from phosphoglycerides by macrophages and smooth muscle cells that are present in the developing atherosclerotic lesion[22]. Therefore, in addition to LDL-mediated influx of LPA into the lesion, a dysbalance in cellular LPA homeostasis may also result in progressive build-up of LPA in atherosclerotic lesions. Overall, LPA accumulates progressively in the lipid-rich core of atherosclerotic plaques and is considered the primary platelet-activating lipid constituent of the plaque. Thus, it is conceivable that LPA is an important risk factor of intra-arterial thrombus formation in later stages of atherogenesis[7,9].

In this study, we aimed to delineate the intracellular metabolic regulation of LPA homeostasis within atherosclerotic lesions of Western type diet fed LDL receptor deficient ($LDLr^{-/-}$) mice. We are the first to demonstrate that also in $LDLr^{-/-}$ mice, LPA accumulates in carotid artery plaques as has previously been shown for human atherosclerotic tissue. RNA analysis of plaque material from these mice provided evidence that LPA homeostasis indeed is altered during atherosclerotic lesion development favoring intracellular LPA accumulation and we believe that intervention in the LPA metabolism could be an effective new strategy in reducing plaque thrombogenicity.

Methods

Animals
All animal work was approved by the regulatory authority of Leiden University and performed in compliance with Dutch government guidelines. Male LDLr$^{-/-}$ mice, obtained from Jackson Laboratories and bred in our local animal breeding facility, were fed a Western type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Sussex, UK) two weeks prior to surgery and throughout the experiment. To determine the LPA content of and gene expression levels in mouse plaques (respectively n=16 and n=20), atherosclerotic carotid artery lesions were induced by perivascular collar placement as described previously[23]. During the experiments, total serum cholesterol levels were quantified colorometrically by enzymatic procedures using Precipath (Roche Diagnostics, Mannheim, Germany) as internal standard. Total plasma cholesterol levels between groups did not differ.

Tissue harvesting
LPA content and gene expression profiles of carotid artery plaques in LDLr$^{-/-}$ mice were determined at 0 to 8 weeks after perivascular collar placement[23]. Hereto, a subset of 4 mice was sacrificed by perfusion through the left cardiac ventricle with phosphate buffered saline (PBS). Subsequently, both common carotid arteries were excised and snap-frozen in liquid nitrogen for optimal RNA and lipid preservation. The specimens were stored at -80°C until further use. For immunohistochemistry animals were sacrificed at 6 weeks after collar placement. Mice were subjected to *in situ* perfusion-fixation with PBS and subsequently 4% Zinc Formalfixx (Shandon Inc). The carotid arteries were removed and after overnight fixation stored at -20°C until further use. Transverse 5 µm cryosections were prepared in a proximal direction from the carotid bifurcation and mounted in order on a parallel series of slides.

Lipid extraction from plaque material
Lipids were extracted from carotid artery segments containing the plaque by a modified Bligh and Dyer protocol[24] and a subsequent 1-butanol extraction step for optimal recovery of the lysophospholipids as described previously[25,26]. In short, two to three carotid artery plaque samples were pooled and homogenized in 0.5 mL distilled, nitrogen-flushed water using a mechanical potter and used for lipid extraction. All solvents were evaporated under nitrogen and extracted lipids were dissolved in PBS + 0.1% bovine serum albumin (fatty acid free, Sigma, Zwijndrecht, The Netherlands) by sonication (MSE Soniprep 150, 18 amplitude microns) for 20 seconds on ice and under argon.

LPA quantification
LPA content of the plaque lipid extracts, from 0, 2, 4 and 8 weeks after collar placement, was determined in a highly sensitive radio-enzymatic assay as described previously[27], which is based on recombinant rat LPAAT catalyzed conversion of LPA and alkyl-LPA into [^{14}C]PA in the presence of [^{14}C]Oleoyl-CoA. Resulting lipid mixture was subjected to two-dimensional thin-layer chromatography and the radiolabeled products identified by co-migration with unlabeled lipid and subsequent visualization by iodine staining. [^{14}C]PA spots were scraped and the silica pools were counted for

radioactivity after suspending in scintillation cocktail, after which the LPA content (in picomoles) was calculated from the radioactivity[27].

Mass spectrometry
Mass spectrometric analysis was performed essentially as described earlier[28]. Prior to mass spectroscopy, lipid molecular species were separated by high-performance liquid chromatography. For this the lipid extracts were dissolved in methanol / acetonitrile / chloroform / water (46:20:17:17). Separation was performed on a Synergi 4 µm MAX-RP 18A column (250 × 3 mm; Phenomenex, CA, USA). Elution was performed with a linear gradient of water in methanol / acetonitrile (60:40 v/v) decreasing from 12.5% to 0% in 25 min, followed by further isocratic elution for another 25 min. The flow rate was kept constant at 0.425 mL • min^{-1} and 1 µM serine and 2.5 mM ammonium acetate were used in all solvents as additives.
Mass spectrometry of lipids was performed using electrospray ionisation (ESI), on a 4000 QTRAP system (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Source temperature was set to 450°C and nitrogen was used as curtain gas. LPA was measured in negative mode, the optimal ionization energy, declustering potential and collision energy were empirically determined at -4500 V, -110 V and -50 V, respectively. Lysophosphatidylcholine (LPC) was measured in positive mode, and the optimal ionization energy, declustering potential and collision energy were set at 5000 V, 110 V and 45 V, respectively. Both molecular species were measured in multiple reaction monitoring mode (MRM), monitoring for 34 head group specific mass transitions with a total dwell time of 20 msec • $transition^{-1}$.

RNA isolation
Two to three carotid artery segments carrying the plaque from 0, 2, 4, 6 or 8 weeks after collar placement were pooled for each sample and homogenized by grounding in liquid nitrogen with a pestle. Total RNA was extracted from the tissue homogenates using Trizol reagent according to manufacturer's instructions (Invitrogen, Breda, The Netherlands). RNA was reverse transcribed by M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, St. Leon-Rot, Germany) and used for quantitative analysis of gene expression with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems, Foster City, CA) as described previously[29], with murine hypoxanthine phosphoribosyltransferase (HPRT) and cyclophilin A as standard housekeeping genes (Table 1).

Immunohistochemistry
For immunofluorescence sections were blocked in 0.1% Triton-X100 and 2% goat normal serum for 4 hours and incubated overnight at 4ºC with rabbit polyclonal antibodies against $cPLA_2$ (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) or $iPLA_2$ (1:500, Cayman Chemical, Ann Arbor, MI) together with rat monoclonal antibody against monocytes + macrophages (MOMA-2, 1:50, Serotec Inc, Toronto, ON). After washes in PBS, sections were incubated for 1 hour at room temperature with a biotinylated goat anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) combined with a goat anti-rat rhodamine-conjugated secondary antibody (1:100, Jackson ImmunoResearch Laboratories), followed by 1 hour incubation with fluorescein-conjugated streptavidin (1:500, Molecular Probes,

Eugene, OR). Following several washes with PBS, cover-slips were mounted on the sections with DAPI containing mounting medium (Vector Laboratories Inc, Burlingame, CA).

Statistical analysis

Data are expressed as mean ± SEM. Comparison of lesion development related changes between groups was done by ANOVA in case of normal distributed values followed by Dunnet post-hoc testing or, in case of a non-Gaussian distribution, by Kruscal-Wallis test with Dunn's post-hoc testing. To determine significance of the relative mRNA expression levels, statistical analysis was performed on ΔCt values. A level of $P<0.05$ was considered significant.

Gene	Source	forward primer (5'-3')	reverse primer (5'-3')
LPA_1	NM_010336	TGTCCTGGCCTATGAGAAGTTCT	TTGTCGCGGTAGGAGTAGATGA
LPA_2	NM_020028	CTCACTGGTCAATGCAGTGGTATAT	GAAGGCGGCGGAAGGT
LPA_3	NM_022983	GGGACGTTCTTCTGCCTCTTTA	GAAAGTGGAACTTCCGGTTTGT
GPR23	NM_175271	GATGGAGTCGCTGTTTAAGACTGA	TGTTTGATCACTAACTTCCTCTTGGATA
LPAATα	BC009651	TCCCTCGACCTGCTTGGA	CATAGTAGCTCACGCTTGGCAAT
LPAP	AF216223	AAATGGCCCCCATTTGCT	TGCACAAACCACTCCTTAGATTCTT
PLD_1	XM_130807	GACTCTGCCTGTGACCGTGAT	CCAGATGCATAAATGAACCTAAGAAC
PLD_2	NM_008876	GCCAGCAAACAGAAATACTTGGA	GGCGTGGTAATTGCGATAGAA
LPP1	AY247795	CATGCTGTTTGTCGCACTTTATCT	AAACTGGAGCATGGGTCGTAA
FABP1	BC009812	GAACTTCTCCGGCAAGTACCAA	GGCAGACCTATTGCCTTCATG
FABP2	BC013457	AGCAACGCTGAAGAGCTAAGCT	CCAGTGCTGATAGGATGACGAA
FABP3	BC002082	ACTCGGTGTGGGCTTTGC	TATCCCCGTTCTTCTCGATGAT
FABP4	BC054426	GCGTGGAATTCGATGAAATCA	CCCGCCATCTAGGGTTATGA
FABP5	BC002008	GGAAGGAGAGCACGATAACAAGA	GGTGGCATTGTTCATGACACA
$cPLA_2IVA$	NM_008869	GGATGAGCATGACCCTGAGTAGTT	GAGACACGTGAAGAGAGGCAAAG
$iPLA_2VIA$	NM_016915	TCCATGAGTACAATCAGGACATGAT	AGAAACGACTATGGAGAGTTTCTTCAC
PPARγ	NM_011146	CATGCTTGTGAAGGATGCAAG	TTCTGAAACCGACAGTACTGACAT
CD36	NM_007643	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGTT
CD68	NM_009853	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTTGA
α-actin	NM_007392	TCCCTGGAGAAGAGCTACGAACT	GATGCCCGCTGACTCCAT
NM-MHC	AK040822	TTTGGATGGCAGGTTATAAGGAA	GATAACGACCATGCATCCTACAGT
HPRT	NM_013556	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Cyclophilin A	AK010338	CCATTTCAAGAAGCAGCGTTT	ATTTTGTCTTAACTGGTGGGTCTGT

Table 1. RT-PCR primer sequences and sources.

Results

LPA quantification

To accelerate atherosclerotic lesion formation, slightly constrictive perivascular col-

lars were placed at the carotid arteries of male Western type diet fed LDLr$^{-/-}$ mice and at 0 to 8 weeks after collar placement, plaques were isolated and entrapped lipids, extracted from these plaques, were analyzed. Intact carotid artery segments contained very little, if any, LPA (0.95 ± 0.95 pmol/mg tissue). LPA levels in advanced lesions were found to increase during atherogenesis via 19 ± 9 and 31 ± 13 (at 2 and 4 weeks, respectively) to 38 ± 17 pmol/mg tissue in advanced lesions at 8 weeks after collar placement ($P<0.05$, Figure 1). These data indicate that in LDLr$^{-/-}$ mice LPA is abundantly present in atherosclerotic lesions but not in intact arteries. Moreover, LPA content of advanced mouse plaques was essentially similar to those observed in human atherosclerotic tissue specimens from high-grade stenotic carotid arteries[5,6], suggesting that it is legitimate to use this animal model to further delineate the metabolic regulation of LPA homeostasis in plaques.

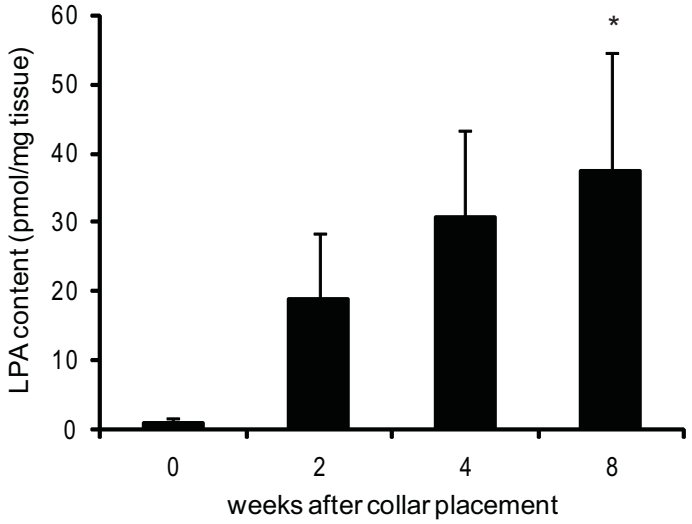

Figure 1. LPA accumulation in carotid artery plaques. LPA quantification in lipid extracts of carotid artery lesions from LDLr$^{-/-}$ mice shows that already at two weeks after collar placement LPA starts accumulating in vessel wall and continues during plaque development. *$P<0.05$.

Quantitative LC-MS analysis of LPA molecular species in mouse atherosclerotic plaques

Rother *et al.* have shown that the platelet-activating effect of the lipid-rich core of atherosclerotic plaques is determined not only by the total amount of LPA but is also highly dependent on the alkyl-acyl ratio and the actual chemical composition of LPA[6]. Therefore, we set out to identify and quantify the LPA molecular species present in mouse atherosclerotic plaques by LC-MS analysis. In keeping with the enzymatic LPA assay, LC-MS analyses confirmed the progressive accumulation of multiple LPA species during atherosclerotic lesion development, some of which known to have potent platelet-activating capacity such as the highly unsaturated, long-chain acyl-LPA species 18:2 LPA, 20:2 LPA ($P<0.05$), 20:3 LPA, and 22:4 LPA ($P<0.05$) (Figure 2). In advanced lesions (8 weeks after collar placement) the major LPA species were 16:0 LPA and 18:1 LPA contributing 55% and 21 % of total LPA, respectively, of

which 18:1 LPA, surprisingly, showed a 2-fold decrease rather than increase during lesion development. However, the relative amounts of the most potent thrombogenic species 18:2 LPA, 20:3 LPA, and 22:4 LPA increased from less than 0.2% in the normal arteries to 6.5%, 9.1%, and 4.2%, respectively, in advanced atherosclerotic lesions, implying that this LPA pool may, although present at lesser amounts, still contribute significantly to the platelet-activating potential of the lipid-rich core.

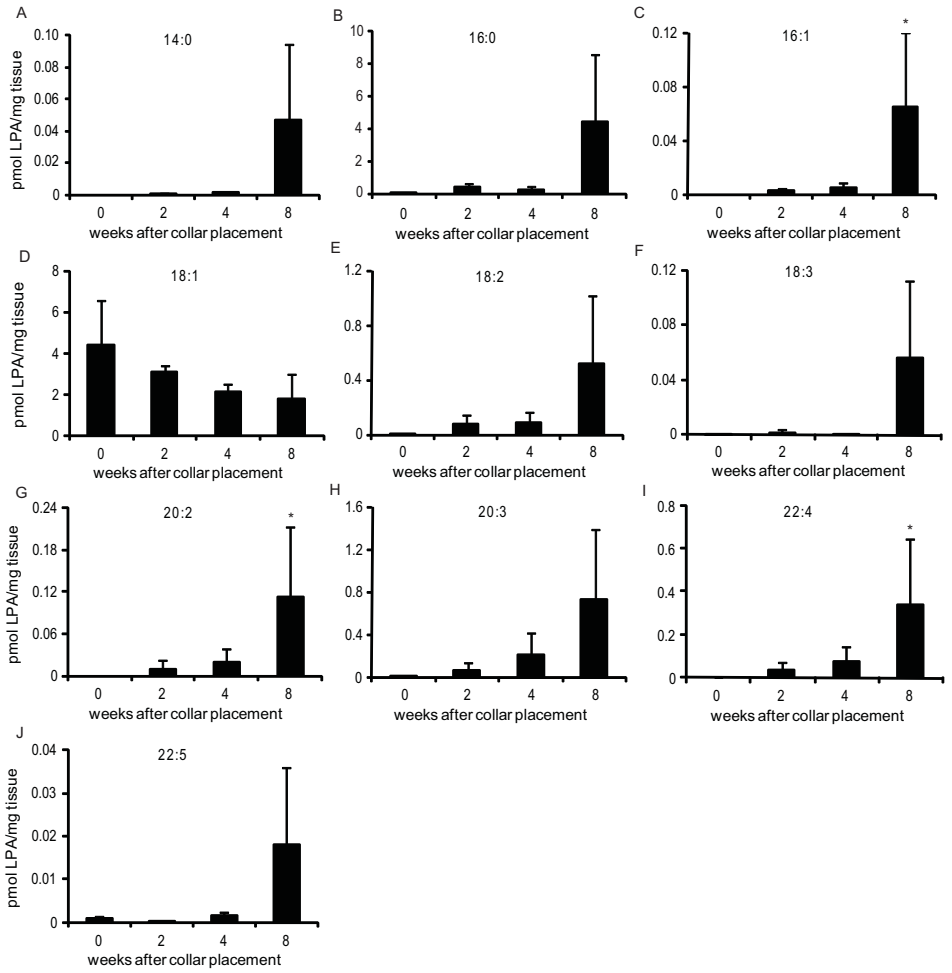

Figure 2. Accumulation of LPA species during collar-aided plaque development in carotid arteries of LDLr$^{-/-}$ mice. MS-analysis shows accumulation of highly unsaturated long-chain LPA species in plaque lipid extracts. *P<0.05.

Interestingly, we found, preceding the LPA accumulation, already at 4 weeks after lesion initiation, a remarkable accumulation of multiple LPC species, that have been attributed a pro-inflammatory role in atherosclerosis (Figure 3A-J)[30]. In addition, also phosphatidylcholine (PC), which is a major component of cell membranes, was increased at 4 weeks of lesion development (Figure 3K, 3L), possibly reflecting an increased cellularity and thus influx of leukocytes in the plaque at that stage.

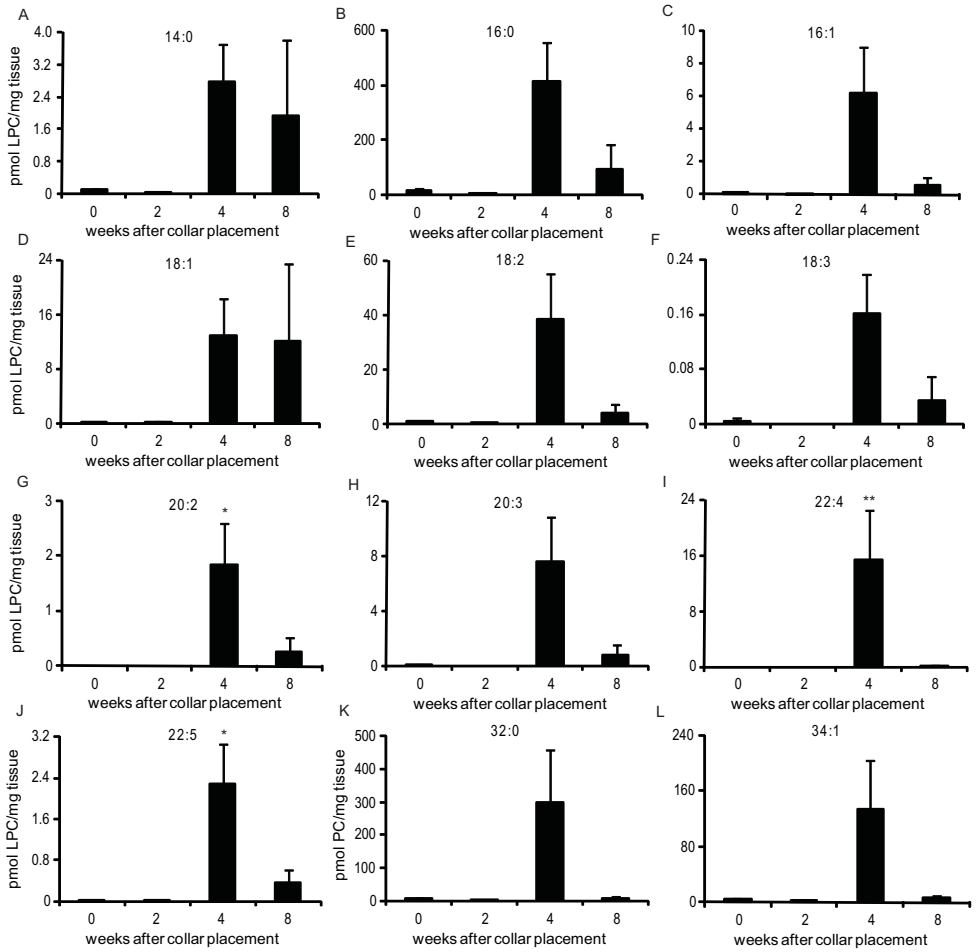

Figure 3. Accumulation of LPC (panels A-J) and PC (panels K,L) species during collar-aided plaque development in carotid arteries of LDLr$^{-/-}$ mice. MS-analysis shows strong accumulation of multiple LPC species in plaque lipid extracts. *P<0.05, **P<0.01.

Expression profiling

In the next step, we investigated whether the increased LPA accumulation correlates with altered expression of important genes in LPA homeostasis and effectors of LPA in the atherosclerotic plaque. Induction of atherosclerotic lesion development is reflected by an increase in CD68 expression, which peaks at 4 weeks after collar placement indicating an influx of macrophages (Figure 4A). The CD68 expression pattern essentially concurs with the increase in phosphatidylcholine measured by LC-MS. No changes were shown in expression over time for phospholipase D $(PLD)_1$ and PLD_2, which hydrolyze glycerophospholipids under formation of phosphatidic acid (PA, data not shown). Interestingly, cytosolic phospholipase A_2 group IVA ($cPLA_2$IVA) and calcium-independent phospholipase A_2 group VIA ($iPLA_2$VIA), which both may be involved in the intracellular conversion of PA into LPA, were upregulated up to

2-fold during atherogenesis (P<0.01 and NS, respectively, Figure 4B, 4C). Gene expression of LPA acyltransferase (LPAAT)α, which converts LPA into PA, progressively decreased by up to 75% (P<0.05, Figure 4D). No change was seen in the expression of lipid phosphate phosphatase 1, which converts LPA to monoacylglycerol (MAG, Figure 4E). Expression of lysophosphatidic acid phosphatase (LPAP), which regulates mitochondrial lipid metabolism via hydrolysis of LPA to monoacylglycerol, was 2-fold downregulated during lesion progression (P<0.01, data not shown).

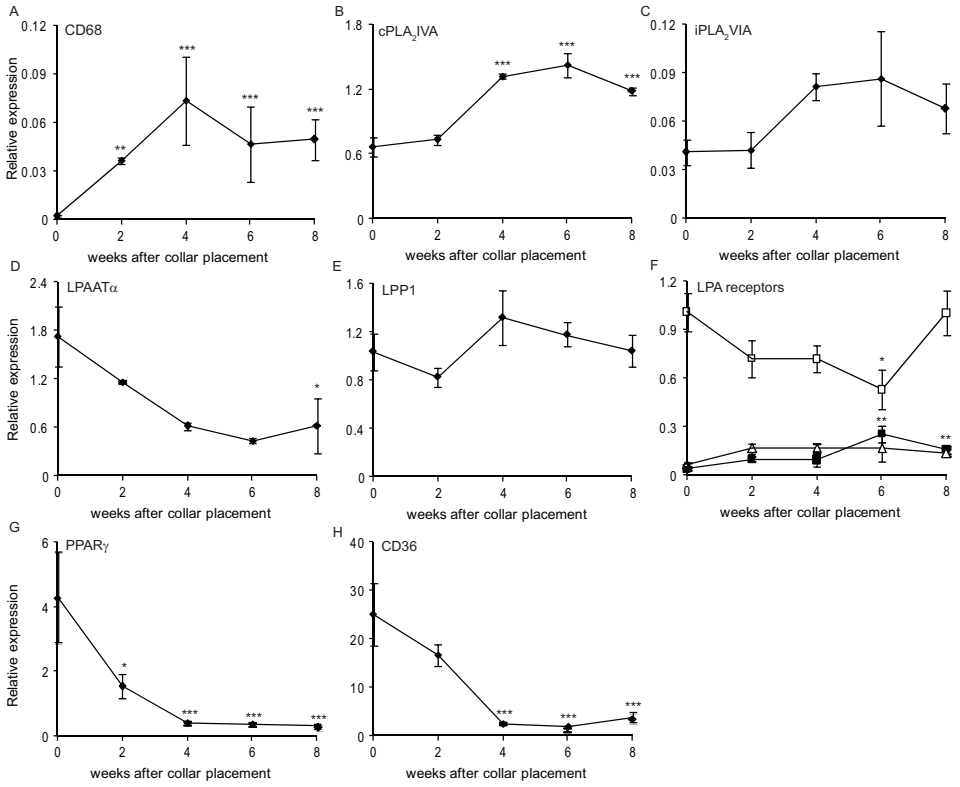

Figure 4. Gene expression profile of LPA metabolizing enzymes and LPA receptors. (A) Induction of atherosclerotic lesion development is reflected by an increase in CD68 expression, indicating an influx of macrophages. (B) $cPLA_2IVA$, involved in PA conversion to LPA, is highly significantly upregulated in atherosclerotic lesions compared to non-atherosclerotic artery tissue. (C) $iPLA_2VIA$, is also 2-fold upregulated during lesion progression. (D) The gene expression pattern of LPAATα, which converts LPA into PA, revealed a downregulation during atherogenesis. (E) No changes were seen on the gene expression of LPP1, which converts LPA to MAG. (F) Expression of LPA_1 (□), decreases, while LPA_2 (■) shows significantly increased gene expression during atherogenesis. LPA_3 does not show any significant changes (Δ). (G) PPARγ, the nuclear receptor known to be activated by LPA, and its effector CD36 (H) illustrated ,respectively, a 14- and 13-fold reduction in relative expression. *P<0.05, **P<0.01, ***P<0.001.

Signal transduction mediated by LPA in the plaque might also be altered during atherogenesis, which prompted us to study the expression of the different LPA receptors. LPA_1 and LPA_2 both displayed altered gene expression during atherogenesis (50% reduction, P<0.05, and 7-fold increase, P<0.01, respectively, Figure 4F).

Expression of LPA_3 and GPR23 did not show any significant changes during lesion progression (Figure 4F and data not shown). PPARγ, a nuclear transcription factor known to be activated by LPA, and its effector CD36 showed a 14- and 13-fold decrease in expression, respectively (P<0.01, Figure 4G, 4H).
LPA is believed to be transported in part by fatty acid binding proteins (FABPs), which belong to a family of intracellular lipid binding proteins and some of these FABPs, including liver FABP, have already been shown to be able to bind LPA in the micromolar range in mitochondria. Although the exact role of FABPs in LPA transport from the endoplasmatic reticulum and the mitochondria to the cytoplasm remains to be determined, the net activity of LPA might be related to the expression pattern of FABPs. Therefore, expression of FABPs during atherogenesis was measured. Expression of FABP1, -2 and -5, also known as L(liver)-, I(intestine)- and E(epidermal)-FABPs, was upregulated over time (Figure 5A, 5B, 5E). However, 8 weeks after collar placement the expression of FABP1 and -2 displayed a sharp decline, which concurred with that of the vascular smooth muscle cell marker α-actin and non-muscle myosin heavy chain (NM-MHC, data not shown). FABP3 and -4, the H(heart)- and A(adipocyte)-FABP, respectively, both illustrated a decreased expression during atherogenesis by up to 96% compared to non-atherosclerotic tissue (P<0.01, Figure 5C, 5D).

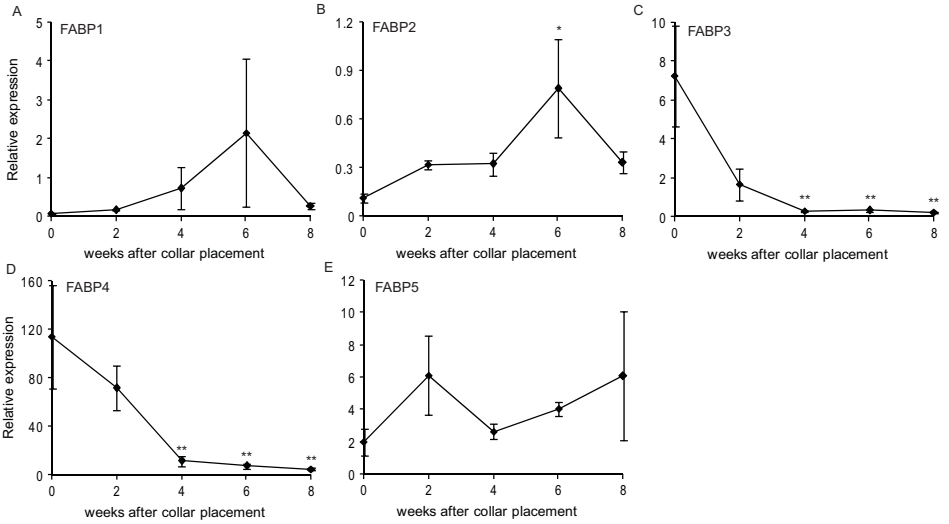

Figure 5. Expression pattern of the FABPs in atherosclerotic plaques. (A) The expression profile of FABP1 did not change significantly during atherogenesis. (B) The expression profile of FABP2 reveals a significant increase in relative expression up to week 6, after which a sharp decrease in expression is observed. (C, D) Gene expression of FABP3 and -4 is significantly downregulated during atherogenesis up to 95% in advanced plaques compared to week 0 (non-atherosclerotic tissue). (E) The expression profile of FABP5 did not change significantly during atherogenesis. *P<0.05, **P<0.01.

Immunofluorescence

Verification of increased expression at the protein level was performed for both $cPLA_2$IVA and $iPLA_2$VIA. Increased expression of $cPLA_2$ and $iPLA_2$ was observed by immunofluorescence in advanced atherosclerotic lesions (Figure 6A, 6D). The increased expression was mainly localized in the central atheroma rather than the cap

of the lesions suggesting that both enzymes are produced by macrophages. Double-immunofluorescence labelling indicated that $cPLA_2$ and $iPLA_2$ expressing cells in these plaques are in fact MOMA-2 positive macrophages (Figure 6C, 6F).

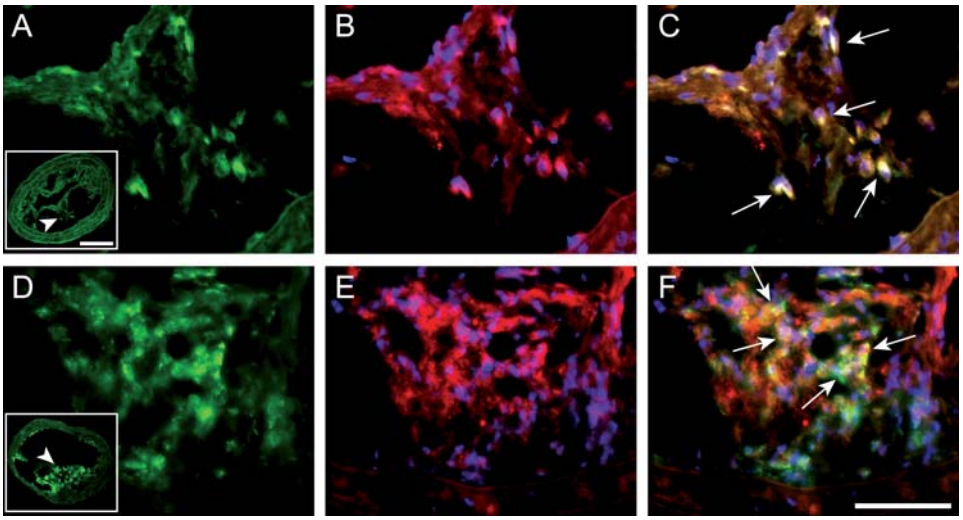

Figure 6. Immunohistochemically stained cryosections of advanced atherosclerotic lesions. $cPLA_2$ (A) and $iPLA_2$ (D) (insert shows total vessel area, scale bar 150 µm; arrow indicates the magnified area) both colocalize with the monocyte/macrophage specific marker MOMA-2 (B and E, respectively; merge C and F, respectively, arrows indicate double-positive areas, scale bar 50 µm).

Discussion

Acute coronary syndromes as unstable angina and myocardial infarction are thought to be caused by erosion or rupture of atherosclerotic plaques and superimposed thrombosis. Rupture-prone plaques are characterized by a lipid core covered by a thin fibrous cap[1-4]. One of the most thrombogenic lipid plaque constituents, lysophosphatidic acid, was previously shown to accumulate in these lipid-rich regions of human atherosclerotic lesions[5,6]. We show here that LPA progressively accumulates in mouse atherosclerotic lesions as well, at concentrations that (particularly at 8 weeks after collar placement when advanced atherosclerotic lesions have developed) are essentially similar to those observed in human atherosclerotic tissue specimens from high-grade stenotic carotid arteries[5,6]. Furthermore, we demonstrate accumulation of highly unsaturated, long-chain acyl-LPA species, which have particularly potent platelet-activating capacity. Finally, this study is the first to establish altered LPA homeostasis in the developing plaque favoring LPA production.

Rother *et al.* have shown that the platelet-activating effect of the lipid-rich core of atherosclerotic plaques is determined not only by the total amount of LPA, but is also dictated by the alkyl-acyl ratio and the chemical composition of LPA species in the plaque[6]. Our study confirms that during development of mouse atherosclerotic lesions multiple LPA species are accumulating, of which the highly unsaturated, long-chain acyl-LPA species 18:2 LPA, 20:3 LPA, and 22:4 LPA have particularly

potent platelet-activating capacity. Remarkably, we also observed accumulation of LPC, which has been implicated in modulation of platelet function as well[20]. In low concentrations LPC (<30 μM) appears not to influence platelet activation, but upon stimulation with higher concentrations of LPC (100 μM) or LPC-containing oxLDL it can cause a rapidly disappearing inhibitory effect on platelet function followed by a transient potentiation[20]. These effects are thought to occur by the amphiphilic properties of LPC, through which it can intercalate into the platelet membrane, and not by binding to specific receptors[20]. This means that upon rupture of the atherosclerotic lesion LPC could first act as inhibitor, but will eventually lead in potentiation of the aggregatory response. In this study, the observed LPC accumulation appeared to precede that of LPA, suggesting that LPC may serve as source of LPA production. Such process would require lysophospholipase D (LysoPLD, Autotaxin) enzyme activity, which can convert LPC directly into LPA[31], inside the atherosclerotic lesion. Indeed increased serum LPC concentrations observed in rabbits upon high fat diet feeding concurred with increased LysoPLD activity and serum LPA concentration, pointing to a role of LysoPLD in LPA homeostasis and thus in atherosclerotic lesion development[32]. As lysoPLD is an extracellular enzyme present mostly in blood, its plaque expression pattern during atherogenesis may not be very informative of plaque LPA homeostasis.

As these data clearly show that LPA progressively accumulates in the intima during atherosclerotic lesion progression, the question remains to what extent plaque LPA is deposited by infiltrated modified LDL or synthesized *in situ*. Already at 2 weeks after collar placement, when initial lesions start to develop, there is considerable build-up of LPA in the vessel wall. It is conceivable that the main contributor of LPA at this stage is delivery via modified LDL. At later stages, cellular metabolism may become more important (Figure 7).

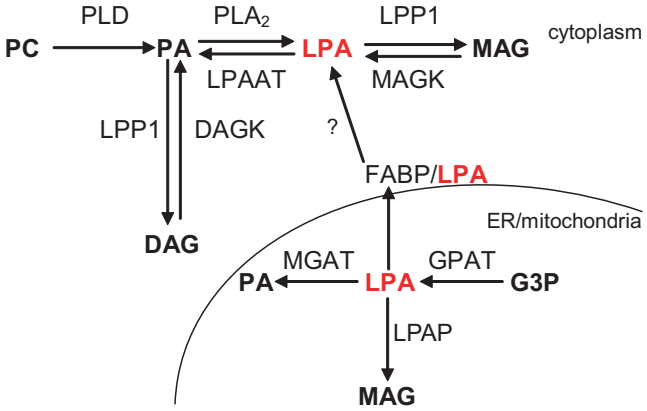

Figure 7. Intracellular pathways of LPA production and degradation. LPA can be produced from either PA or monoacylglycerol (MAG) by PLA_2 and MAG-kinase, respectively, whereas it is degraded by LPAATα and lipid phosphate phosphatase (LPP)1. The link between the FABPs and transport of LPA to the cytoplasm has not been proven yet. DAG(K), diacylglycerol (kinase); ER, endoplasmic reticulum; MGAT, monoacylglycerophosphate acyltransferase; GPAT, glycerophosphate acyltransferase; G3P, glycero-3-phosphate.

Therefore, we have delineated the metabolic regulation of LPA at different stages of lesion development. Taken together, our expression data point toward an increased LPA synthesis in atherosclerotic plaques. The induced expression of $cPLA_2$IVA and $iPLA_2$VIA, the two major intracellular PLA_2 enzymes in humans[33], could indirectly translate in an increased LPA production. The induced expression was corroborated on protein level by immunohistochemical analysis of plaque cryosections, which showed a markedly increased macrophage expression of both $cPLA_2$IVA and $iPLA_2$VIA in advanced atherosclerotic lesions. While the relative contribution of these PLA family members to the total LPA production remains controversial[34], our findings support such a role in the context of atherosclerosis. Indications for strong involvement of these PLA family members in LPA production have been furnished by Tang *et al.*, amongst others, which have shown that $iPLA_2$VIA has a preference for conversion of 1,2-dipalmitoyl phosphatidic acid to lysophosphatidic acid[35]. In our study we show an increased presence of palmitoyl (16:1) LPA in advanced atherosclerotic lesions underpinning an important role for $iPLA_2$VIA in LPA accumulation during lesion development. In addition, both $cPLA_2$IVA and $iPLA_2$VIA have been implicated in LPA production by peritoneal mesothelial cells involved in metastasizing ovarian cancer[36]. Furthermore, cytosolic PLA_2 is involved in vascular smooth muscle cell and macrophage apoptosis[37,38] and in the production of pro-inflammatory prostaglandins via arachidonic acid, pointing to a pro-atherogenic and plaque destabilizing activity of this enzyme. Finally, in our study we observed downregulation of the LPA-converting enzyme LPAATα[39].

Not only was LPA homeostasis perturbed during atherogenesis, LPA signal transduction also seemed to be changed. A significant modulation was shown for LPA_1, which was downregulated, and for LPA_2, which was upregulated, reflecting an altered balance in LPA signaling. Selective antagonists of both LPA_1 and LPA_3 on platelets were shown to quench plaque lipid induced platelet activation[6]. Thrombogenic capacity was decreased after blocking of both these receptors, suggesting that alterations in LPA receptor expression may be accompanied by a changed risk of thrombosis. We also observed profound downregulation of the intracellular LPA receptor PPARγ, and its downstream effector, CD36 in atherosclerotic plaques. PPARγ activation has been associated with reduced atherosclerosis and regulates the expression of many genes involved in inflammation and lipid homeostasis[40], implying that downregulation may act pro-atherogenic.

A third group of critical proteins in LPA homeostasis are the FABPs, which can bind intracellular LPA and have been shown to play a role in atherosclerosis[41]. Absence of the adipocyte-specific FABP4 (aP2) in macrophages attenuates atherosclerosis in hypercholesterolemic mice[42,43]. Expression analysis revealed a downregulation of FABP4 during plaque development, suggesting that FABP4 may contribute to the initiation of atherosclerotic lesion development. Recently, Makowski *et al.* described a role for aP2 in macrophage cholesterol trafficking and inflammatory activity[44]. Our data on cardiomyocyte-specific FABP3 expression during atherogenesis are in keeping with earlier reports in rabbits[45,46], demonstrating decreased FABP3 activity in atherosclerotic aortas upon high cholesterol feeding. However, the effects of FABP3 downregulation on atherogenesis have not yet been elucidated.

In conclusion, LPA accumulates during atherogenesis, already starting in the initial stage of atherosclerotic lesion development. The observed accumulation of potent

platelet-activating LPA species and pro-inflammatory LPC species in advanced atherosclerotic lesions suggests conversion towards a high risk plaque phenotype on the lipid level during lesion development. Although we only have verified the temporal expression profiling for two critical enzymes in LPA metabolism by immunohistochemistry, the shifts in gene expression profiles will favor accumulation of LPA and changed LPA receptor signal transduction during plaque progression. As such, this study provides a panel of potentially relevant candidate genes for further investigation with respect to atherosclerotic lesion development on the one hand and thrombogenicity on the other hand and leads us to suggest that intervention in the LPA metabolism can be an effective new therapeutic entry in the reduction of plaque thrombogenicity.

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