LPA Administration Changes Atherosclerotic Plaque Phenotype in ApoE-/- Mice By Affecting Vascular Smooth Muscle Cell Content

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Abstract

Objective: Migration and proliferation of dedifferentiated vascular smooth muscle cells (VSMCs) is considered one of the early hallmarks of atherosclerotic lesion development. Lysophosphatidic acid (LPA), a bioactive lipid, has been demonstrated to induce VSMC dedifferentiation, proliferation and migration. In this study, we investigated the effect of in vivo LPA administration on atherosclerotic lesion development and morphology in ApoE deficient mice.

Methods and Results: We show here that systemic supplementation of unsaturated 18:1 LPA in a mouse model for collar-induced atherosclerosis can modulate lesion morphology by increased α-actin positive smooth muscle content, which is most likely dependent on the mitogenic effects of LPA, and decreased collagen content.

Conclusions: Enhancement of the systemic unsaturated 18:1 LPA availability can significantly alter atherosclerotic plaque phenotype by increasing the smooth muscle cell content. Further research into the exact mechanisms, by which LPA acts in atherosclerotic lesion development, is warranted as different expression and activation patterns of the multiple LPA receptors could be crucial for the diverse modifications.
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Introduction

Lysophosphatidic acid (LPA) is a family of bioactive lipids that was originally mainly regarded as key intermediate in de novo lipid synthesis, but soon emerged as an intra- and intercellular phospholipid messenger with a variety of biological activities. LPA was reported to have a thrombogenic effect on blood platelets as well as multiple effects on all cell types of the vessel wall and evidence is accumulating that it contributes to cardiovascular diseases by virtue of its athero- as well as thrombogenic activity. For instance, LPA mediates smooth muscle contraction and proliferation, endothelial-leukocyte interaction, and platelet aggregation, all processes which are instrumental in atherogenesis.

LPA acts through specific G-protein-coupled receptors (GPCRs), such as LPA receptor 1, 2 and 3 (LPA1-3), which belong to the Endothelial Differentiation Gene family, and the genetically more distant LPA4 (GPR23) and LPA5 (GPR92). Studies have revealed a direct role for LPA as peroxisome proliferator-activated receptor (PPAR) agonist, regulating the expression of genes that contain PPAR Response Elements (PPRE), including CD36. Zhang et al. have shown that unsaturated 20:4 LPA induces neointima formation via PPAR activation and subsequent CD36 upregulation. Moreover, other unsaturated LPA species, i.e. 16:1 LPA, 18:1 LPA, and 18:2 LPA, were demonstrated to promote phenotypic dedifferentiation of vascular smooth muscle cells (VSMCs) by activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38MAPK) and ensuing upregulation of epiuregulin. Furthermore, the same ERK/p38MAPK pathway appeared to be responsible for 18:1 LPA-mediated vascular remodeling and neointima formation in vivo.

LPA has been demonstrated to be present within the blood circulation in the low-density lipoprotein (LDL). As VSMCs play a major role in atherogenesis throughout the disease process and given that LPA, in particular the unsaturated species, has been demonstrated to profoundly affect VSMC migration and proliferation, we sought to investigate whether increased availability of 18:1 LPA may affect atherosclerotic lesion development in ApoE-/- mice. Here, we demonstrate that repeated three-weekly intraperitoneal injections for 5 weeks leads to marked changes in plaque morphology as shown by increased smooth muscle content but decreased collagen fiber content. Therefore, enhanced plasma LPA levels will modulate fibrous cap composition and thereby plaque stability.

Methods

LPA kinetics

All animal work was performed in compliance with the Dutch government guidelines. Male ApoE-/- mice were obtained from the local animal breeding facility. LPA pharmacokinetics after intraperitoneal (i.p.) injection was assessed as follows. Mice received 3 i.p. injections of [3H]LPA (1-oleoyl[oleoyl-9,10-3H(N)]-LPA), specific activity of 47 Ci/mmol, PerkinElmer, Groningen, the Netherlands) in PBS at 2-3 day intervals and blood samples were collected and measured for LPA-associated radioactivity by liquid scintillation counting. For distribution analysis of intraperitoneally injected LPA...
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Over plasma lipoprotein and protein pools, plasma samples were subjected to gel exclusion chromatography using a superose 6 column equipped Smart™ micro FPLC system (Pharmacia, Uppsala, Sweden). For each fraction, total cholesterol levels were quantified colorimetrically by enzymatic procedures using Precipath (Roche Diagnostics, Mannheim, Germany) as internal standard and the radioactivity of the pooled fractions containing the different lipoprotein subsets was measured. 24 hours after the last injection the animals were anesthetized and organs were extracted for determination of LPA-associated radioactivity distribution by liquid scintillation counting. Organs were homogenized with Solvable (PerkinElmer).

Collar placement and LPA administration
Male ApoE−/− mice were fed a Western type diet, containing 0.25% cholesterol and 15% cocoa butter (SDS, Sussex, UK). Atherosclerotic carotid artery lesion formation was induced in a shear stress-induced and lipid-dependent model of atherosclerosis by perivascular collar placement as described previously[21]. Mice were anesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, The Netherlands), fentanyl citrate and fluanisone (1.26 mg/kg and 2 mg/kg respectively, VetaPharma Ltd, Leeds, UK). One week after collar placement all animals were injected intraperitoneally 3 times a week with either 18:1 LPA (50 μg/kg, Sigma, Zwijndrecht, the Netherlands) or PBS as a control. Total plasma cholesterol levels were quantified as described above and showed no differences between treatment groups. Six weeks after collar placement and 5 weeks after start of LPA treatment the mice were anesthetized and in situ fixation through the left cardiac was performed[21], after which the carotid artery lesions were analyzed for size and composition.

Histology
Cryosections of the carotid arteries (5 μm thick) were prepared and stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica, Darmstadt, Germany). Monocytes/macrophages were stained immunohistochemically with antibodies directed against mouse macrophages (monoclonal mouse IgG2a, clone monocye + macrophage antibody-2 [MOMA-2], dilution 1:50; Serotec, Kidlington, Oxford, UK). Vascular smooth muscle cells were stained immunohistochemically with antibodies directed against mouse vascular smooth muscle α-actin (ASMA, monoclonal mouse IgG2a, clone 1A4, dilution 1:50; Sigma). Sections were stained for collagen using Sirius Red (Sigma). Mast cells were visualized by staining cryosections with aqueous toluidin blue (Sigma). Neutrophils were stained with naphthol AS-D chloroacetate esterase (Sigma). Iron staining was performed according to Perl’s method. Apoptosis was visualized using a terminal deoxytransferase dUTP nick-end labeling (TUNEL) kit (Roche Diagnostics).

Morphometry
Morphometric analysis (Leica Qwin image analysis software) was performed on hematoxylin-eosin stained sections of the carotid arteries at the site of maximal stenosis[21]. MOMA-2, ASMA, collagen positive areas were quantified by Leica Qwin image analysis software and TUNEL positive nuclei were counted manually. Toluidin blue stained sections were used for histological examination for the presence of mast cells, while naphthol AS-D chloroacetate esterase stained sections were used for
histological analysis of neutrophils. Neutrophil numbers, mast cells numbers and the extent of mast cell degranulation were assessed manually. All morphometric analyses were performed by two blinded independent operators (M.B./I.B.).

**Differential blood cell analysis**

Differential blood cell analysis on blood, lymph nodes, spleen and peritoneal leukocytes was performed by flow cytometry (FACSCalibur, BD Biosciences, Breda, The Netherlands) for (activated) T lymphocytes (CD4, CD8, CD69, CD45RA), B lymphocytes (CD19), monocytes (CD11b), neutrophils (CD11b, GR1), macrophages (F4/80), mast cells (CD117) and (activated) dendritic cells (CD11c, MHCII, CD86). Monoclonal antibodies for flow cytometry were obtained from eBioscience, Halle-Zoersel, Belgium and BD Biosciences. For each FACS staining 2*10^6 cells were incubated with antibody dilutions (0.25 μg for each antibody) in PBS (150 mmol/L NaCl, 1.5 mmol/L NaH_2PO_4, 8.6 mmol/L Na_2HPO_4, pH 7.4) plus 1% mouse serum at 4°C.

**Cell culture**

Aortic vascular smooth muscle cells were isolated by collagenase digestion of aortas isolated from male C57Bl/6 mice as described previously\(^{22}\). The resulting cells were cultured onto 0.1% (w/v) gelatin-coated plates in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin. RAW 264.7 macrophage cells were cultured in DMEM containing 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin.

For proliferation experiments cells were seeded in 24-well dishes at a density of 1*10^5 cells/mL in DMEM containing 1% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin for 24 hours to synchronize cell cycle. Subsequently, fresh serum-free DMEM containing 0.2% BSA and 18:1 LPA at various concentrations was added to the cells and cultured for 40 hours without medium change. 10% FBS was used as a positive control. After 16 hours \([^{3}\text{H}]\text{thymidin} (5.0 \mu\text{Ci/well}; \text{GE Healthcare, Eindhoven, The Netherlands}) was added and cells were incubated for a further 24 hours. Thereafter, cells were washed 3 times with PBS, lysed with 0.1 mol/L NaOH, and cell-associated radioactivity was determined by liquid scintillation counting.

For collagen synthesis experiments VSMCs were seeded in 24-well dishes at a density of 3*10^4 cells in DMEM containing 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin for 24 hours. RAW 264.7 macrophages were cultured in DMEM containing 0.5% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 18:1 LPA at various concentrations. After 24 hours, supernatant of the RAW 264.7 cells was collected and used for incubation of VSMCs. The RAW 264.7 cells were washed, lysed in 0.1 mol/L NaOH and the amount of protein present was determined by BCA protein assay (Thermo Scientific, Perbio Science, Etten-leur, the Netherlands) to correct for possible effect on cell number. VSMCs were washed and DMEM containing 0.5% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 18:1 LPA at various concentrations or the collected supernatant of RAW 264.7 cells was added to the cells and they were cultured for another 24 hours. Thereafter, cells were washed with PBS and analyzed for collagen synthesis and protein content as described above. Intracellular colla-
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gen was assessed in cells fixated for 1 hour in methanol. Cells were subsequently washed with deionized water and stained with 0.1% Sirius Red for 1 hour. Unbound dye was removed by washing twice with 0.01 mol/L HCl and cells were lysed using 0.1 mol/L NaOH. Absorbance measurements were performed in a microplate reader at an optical density of 550 nm. The amount of collagen was calculated using gelatin (Sigma) as internal standard.

Statistical analysis
Data are expressed as mean ± SEM. A 2-tailed Student’s t-test was used to compare individual groups. Non-Gaussian distributed data were analyzed by Mann-Whitney U test. A level of P<0.05 was considered significant.

Results

LPA Kinetics
First, we have determined the pharmacokinetics of 18:1 LPA after intraperitoneal injection into mice. Figure 1A showed that plasma LPA levels rapidly increase after each injection to reach peak values of 2-3% of the injected dose (i.d.), with peak values slightly increasing after each subsequent injection.

Figure 1. Pharmacokinetics of [3H]LPA in ApoE−/− mice. (A) Blood samples were collected to determine LPA-associated radioactivity by scintillation spectrometry. The average recovery of [3H]LPA derived radioactivity in plasma was 2.35% of the injected dose (i.d.), corresponding to 52 nmol/L of 18:1 LPA. (B) Lipoprotein analysis was performed to assess association of plasma [3H]LPA with lipoproteins. Clearly, [3H]LPA did not comigrate with lipoproteins, so no integration of intraperitoneally injected LPA occurs. (C) Organs were isolated to measure organ association of [3H]LPA derived radioactivity showing predominant accumulation in adipose tissue, preputial gland, testes, adrenals and gallbladder.
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LPA decay was rather slow with an estimated plasma half life of approximately 70 hours. A dosage of 50 μg/kg was seen to establish sustained average plasma LPA levels of 2.35% i.d., or 52 nmol/L 18:1 LPA. In mice normal total LPA concentrations measured in plasma are about 170 nmol/L. In healthy human individuals 5-10% of total LPA consists of 18:1 LPA, which means if extrapolated to mice that the pursued dosage regimen of 50 μg/kg will increase plasma LPA 18:1 levels by approximately 3-fold (17 versus 70 nmol/L 18:1 LPA). LPA is also an integral part of lipoprotein particles, and LDL in particular. Lipoprotein analysis however showed that only a minute fraction of the 3H-labeled LPA was incorporated into the lipoprotein particles (Figure 1B). Most radioactivity could be detected in the low molecular weight fractions of gel exclusion chromatography, probably representing association to plasma albumin. Analysis of the biodistribution profile of 18:1 LPA showed particularly high accumulation in lipid-rich and/or endocrine compartments such as adipose tissue, preputial gland, testes, adrenal and gallbladder (Figure 1C).

Plaque morphology
Morphometric analysis of the lesions did not reveal any differences in plaque size between control and LPA-treated animals after 6 weeks of lesion development (83 ± 11*10^3 μm^2 versus 61 ± 8*10^3 μm^2 respectively, Figure 2A). Also no differences were found in the medial surface area and percentage of artery stenosis. Plaque macrophage content did not differ between groups (12.85 ± 3.57% versus 11.88 ± 1.83% in control animals, Figure 2B). However, smooth muscle α-actin stained plaque area was increased by 70% (1.65 ± 0.15% versus 0.99 ± 0.15% in control animals, P<0.01, Figure 2C). Sirius Red staining measured with bright-field microscopy showed a minor, non-significant decrease in plaque collagen content (15.7 ± 2.1% versus 18.6 ± 2.4% in control animals, P=0.35, Figure 2D), whereas measurements under polarized light revealed a sharp reduction in birefringent collagen fiber content of 68% (0.31 ± 0.11% versus 0.98 ± 0.23% in control animals, P<0.05, Figure 2E). As LPA was reported to affect mast cell and neutrophil recruitment and degranulation, adventitial resting and activated mast cell and neutrophil numbers were measured. No differences were seen in number or percentage of degranulated adventitial mast cells in LPA-treated mice (Figure 3A, 3B). Similarly naphthol AS-D chloroacetate esterase staining did not reveal any differences in the number of perivascular (Figure 3C) or intimal neutrophils (0.30*10^4 ± 0.08*10^4 cells/μm^2 plaque area versus 0.14*10^4 ± 0.04*10^4 cells/μm^2 plaque area in control animals). Finally, we did not observe any differences in the number of TUNEL positive apoptotic cells (Figure 3D).

Differential blood cell analysis
As LPA is known to be a potent immunomodulator, we performed differential blood cell analysis by flow cytometry. To our surprise and despite supraphysiological levels of LPA in blood and organs (including spleen and thymus), and a significant decrease in thymic weight by 40% (31.8 ± 2.0 mg versus 52.5 ± 2.8 mg in control animals), long-term systemic LPA treatment did not have any significant effects on leukocyte subsets or activation status in peritoneum, blood, lymph nodes or spleen (data not shown).
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Figure 2. Morphological analysis of atherosclerotic carotid artery lesions. (A) Intimal surface area of control and LPA-treated animals showed no change in lesion area. (B) Also no differences were seen on intimal macrophage content between groups. (C) The LPA-treated animals showed a significantly increased percentage of α-actin positive VSMCs (ASMA). Representative ASMA-stained sections of control (middle panel) and LPA-treated (right panel) animals. (D) Bright-field microscopy of Sirius Red stained slides showed no change in plaque collagen content. (E) Polarization microscopy with Sirius Red stained slides demonstrated a sharp reduction in plaque birefringent collagen fiber content in the LPA-treated group. (F) Representative Sirius Red stained sections of control (upper panel) and LPA-treated animals (lower panel) imaged with polarization microscopy. *P<0.05. **P<0.01.
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Figure 3. Adventitial mast cell and neutrophil numbers and intimal TUNEL positive cells in atherosclerotic carotid artery lesions. (A,B) Mast cell number and percentage of degranulated mast cells did not differ between control and LPA-treated animals. (C) Adventitial neutrophil content between control and LPA-treated animals did not differ. (D) Also no changes were seen on the percentage of TUNEL positive cells, thus no change in apoptosis.

VSMC and macrophage proliferation
LPA has previously been demonstrated to induce proliferation of human, rat and rabbit VSMC. In keeping with this notion, we show here that also in murine VSMCs LPA acts mitogenic in a dose-dependent manner (Figure 4A). Next, we extended these studies to a macrophage subset, as LPA promotes macrophage survival of serum-starved murine peritoneal macrophages and proliferation of the human monocytic cell line THP-1. Indeed, LPA induced a dose-dependent proliferative response in RAW 264.7 murine macrophage cells (Figure 4B).

VSMC collagen production
To further elucidate the effect of LPA on VSMCs, we investigated the influence of LPA on collagen synthesis by VSMCs. We show that LPA slightly but significantly stimulated collagen production by VSMCs (Figure 5A). Incubation with conditioned medium of LPA-stimulated RAW 264.7 cells was seen to increase VSMC collagen content to a similar extent as that of unstimulated RAW 264.7 cells (Figure 5B).
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Figure 4. Effect of LPA on murine VSMC and RAW 264.7 proliferation. Cells were grown in DMEM plus 10% FBS and serum-starved before each experiment to rule out possible interference of mitogenic serum components. Cells were challenged with different LPA concentrations (0.5-7.5 μg/mL). [3H]thymidin incorporation into DNA was assessed as reported in the Methods section. Data are reported as mean ± SEM. **P<0.01, ***P<0.001, as reported from student’s t-test in respect to untreated cells (control). (A) A dose-dependent increase in proliferation is seen in murine VSMCs in response to LPA. (B) Duplicate of figure 4, chapter 5. LPA dose-dependently increased the proliferation of RAW 264.7 murine macrophages. dpm, disintegrations per minute.

Figure 5. Effect of LPA on collagen content of murine VSMC. Cells were grown in (A) DMEM plus 0.5% FBS and challenged with different LPA concentrations (2.5 or 5 μg/mL) or (B) supernatant of RAW 264.7 cells previously challenged with LPA. Collagen synthesis was assessed by Sirius Red staining as reported in the Methods section. Data are reported as mean ± SEM. **P<0.01 and ***P<0.001 in respect to untreated VSMCs (control). (A) LPA slightly, but significantly, stimulated collagen synthesis in murine VSMCs. (B) Incubation with medium of unstimulated RAW 264.7 already increased VSMC collagen synthesis, which was not further stimulated by LPA incubation of the RAW 264.7 cells.
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Discussion

Early stage atherosclerosis is characterized by proliferation, dedifferentiation and migration of medial vascular smooth muscle cells. LPA has been demonstrated to affect phenotypic VSMC dedifferentiation, as well as proliferation of human, rat and rabbit VSMCs. With respect to LPA-induced migration of VSMCs some controversy exists as Gennero et al. show inhibition of migration in response to LPA, while other groups have shown a stimulatory effect of LPA on migration. Combined with its potent immunomodulatory activity and the relative abundance of LPA in oxidized lipoproteins, this points to a key role of this bioactive lipid in the pathophysiology of atherosclerosis. Therefore, we investigated whether supraphysiological levels of unsaturated 18:1 LPA as achieved by repeated injection affects a flow-induced atherosclerotic plaque formation in the carotid artery of ApoE-/- mice.

We demonstrate that supraphysiological plasma and organ concentrations of unsaturated 18:1 LPA do not noticeably affect the immune status of ApoE-/- mice, but can modulate atherosclerotic plaque morphology. On the one hand LPA induced a beneficial increase of VSMC content, while on the other hand a detrimental lowering of plaque collagen fiber content was observed. So the net effect on plaque stability remains to be elucidated.

Pharmacokinetic analysis showed that LPA decay was rather slow with an estimated plasma half-life of approximately 60-70 hours, which indicated that a three-weekly regimen of injections would give a relatively stable increase of 18:1 LPA bioavailability during 5 weeks of treatment. A dosage of 50 μg/kg was seen to establish sustained average plasma LPA levels of 52 nmol/L, which results in an approximately 3-fold increase in bioavailability of 18:1 LPA (17 versus 70 nmol/L). Most radioactivity could be detected in the low molecular weight fractions of gel exclusion chromatography, probably representing association to plasma albumin, which has previously been demonstrated to act as a major LPA-binding plasma protein. Organ distribution analysis of LPA showed vast percentages of [3H]LPA in lipid-rich and/or endocrine compartments such as adipose tissue, preputial gland, testes, adrenal and gallbladder. This concurs with previous data demonstrating that LPA abundantly localizes in testes, where it plays a crucial role in spermatogenesis. Furthermore, LPA was seen to distribute to adipose tissue, which is as expected as LPA mediates growth and function of adipocytes. Accumulation in the preputial and adrenal glands may be consistent with the observed role of LPA in endocrine function, such as adrenal catecholamine secretion.

Systemic LPA treatment for 5 weeks led to supraphysiological levels of unsaturated 18:1 LPA, but had surprisingly little effects on circulating and resident (splenic, lymph node, and peritoneal) hematopoietic cells. Both numbers and activation status of the major subsets remained unaltered. LPA did not have any effect on atherosclerotic lesion size either, nor did it influence plaque macrophage content even though LPA could dose-dependently induce macrophage proliferation in vitro. Conversely, systemic LPA treatment did increase the α-actin positive VSMC content of the intima by 70%. This concurs with previous studies on LPA-induced proliferation of VSMC of human, rat and rabbit. Furthermore, unsaturated species of LPA (18:1 and 20:4) have been implicated in neointima formation in rats. This suggests that LPA might be a driving factor in fibrous cap formation and that it, when applied systemically, has
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the potential to stabilize the fibrous cap. As it has been demonstrated that excreted growth factors, such as LPA, from mature adipocytes and perivascular adipose tissue stimulate VSMC proliferation⁴³, we could even speculate that distribution of the systemically delivered LPA to perivascular fat tissue is a possible pathway of the observed VSMC proliferation in our study.

Another phenotypic change we observed upon LPA treatment was the paradoxical 68% decrease in plaque collagen fiber content, despite the higher abundance of VSMC. We can exclude this to result from an LPA-induced reduction of VSMC extracellular matrix production as collagen synthesis analysis even revealed a moderate increase in intracellular collagen content of VSMCs upon LPA exposure (either direct or indirect). Alternatively, the reduction in collagen may be explained from LPA induced matrix metalloproteinase (MMP) activity, similar as described before in different cell types such as endothelial cells, T cells and cancer cells⁴⁴-⁴⁶. However, the exact mechanism of decreased collagen in atherosclerosis remains to be elucidated.

LPA treatment did not change immune status in circulation, spleen, or lymph nodes, nor did it have any influence on adventitial mast cell numbers and activation, and on neutrophils at the site of atherosclerotic lesion development. This is in seeming contrast to previous studies showing that mast cell and neutrophils can both be attracted and activated by LPA²⁶-³⁰. It also implies that the reduced collagen fiber content is not caused by the potent proteases released by these leukocyte subsets upon activation. With its beneficial increase of VSMC content and detrimental lowering of plaque collagen fiber content, the net effects of LPA on plaque stability remain difficult to interpret.

In conclusion, we demonstrate that supraphysiological plasma and organ concentrations of unsaturated 18:1 LPA do not noticeably affect immune status of ApoE⁻/⁻ mice, but can contribute significantly to atherosclerotic plaque morphology. On the one hand these changes could be considered stabilizing by increasing α-actin positive smooth muscle content, while on the other hand decreased collagen fiber content indicates plaque and fibrous cap destabilization. As differences in LPA receptor expression and activation patterns could be essential for the diverse modifications in atherosclerotic lesion development, further research into the exact mechanisms is warranted.
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