Bioactive lipids as key regulators in atherosclerosis

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9 General Discussion and Perspectives

Introduction

Atherosclerosis is a chronic inflammatory disease of medium- to large-sized arteries, with extensive intimal lipid accumulation as its most prominent feature. It has long been undisputed that dyslipidemia is instrumental in atherogenesis at all stages of disease progression[1]. In addition to circulating lipids, intimal lipids were also regarded as key determinants of the biomechanical stability of atherosclerotic plaques and the size of intimal lipid deposits is in fact viewed as important criterion for plaque stability[2]. The last decade of research has culminated in the recognition not only that lipids contribute to the disease as major constituents of the neointima, but also that specific lipids in the circulation as well as in plaques can independently modulate processes that are instrumental in disease initiation and progression. In this thesis, I have focused on the two major bioactive phospholipids that were recently shown to be potentially important mediators in atherogenesis: lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P). While structurally unrelated, these lysolipids both act as agonists of G-protein-coupled receptor family members expressed on the surface of all vascular wall cell types involved in atherosclerosis. Furthermore they are complementary in their mode of action. LPA may be held accountable for the pro-atherogenic and pro-thrombotic actions of low density lipoprotein (LDL)[3-5], while S1P, on the other hand, proved to be mainly associated with high-density lipoprotein (HDL), in which it contributed to its anti-atherogenic effects[6-8].

Lysophosphatidic Acid Accumulation in Atherosclerotic Lesions

Evidence is accumulating that LPA, by virtue of its multiple effects on blood cells and cells of the vasculature, is potentially athero- and thrombogenic and may aggravate cardiovascular disease[5,9,10]. In humans, plaque intima has been demonstrated to contain more LPA than normal arterial tissue and it was shown to be one of the most thrombogenic constituents in the lipid core of atherosclerotic lesions[3,11]. As major bioactive component of mildly oxidized LDL (moxLDL), LPA is most likely directly deposited via moxLDL entering the arterial wall, during early lesion formation, with subsequent uptake by subendothelial macrophages. During lesion progression, LPA may still be delivered through LDL, however intraplaque formation of LPA from its precursors will become increasingly important. Accumulation of LPA and other lipids in the plaque may lead to cell death due to necrosis of lipid-laden macrophages (foam cells). It is plausible that progressive build-up of LPA will enhance the thrombogenicity of the plaque and may help to prime platelets toward coagulation upon rupture of the plaque[11]. This could increase the risk of thrombotic complications following plaque rupture.

In chapter 3, the suitability of the LDLr$^{-/-}$ atherosclerotic mouse model for evaluation of LPA homeostasis was established. First, we describe that in LDLr$^{-/-}$ mice LPA accumulates in the intima during lesion progression to a similar extent as in advanced human lesions[3]. In addition, by means of liquid chromatography coupled with mass spectrometry (LC-MS), we confirmed in mouse lesions the accumulation of highly-unsaturated long-chain LPA species, which have high platelet-activating capacity[11]. Furthermore, we have investigated LPA accumulation and the regulation and expression of genes involved in LPA metabolism in the vascular wall, during diet induced lesion formation in LDLr$^{-/-}$ mice. To determine whether or not the metabolism of LPA by plaque cells is disturbed during atherosclerotic lesion progression, mRNA expression levels were analyzed of enzymes involved in LPA conversion. During atherosclerotic lesion development the expression pattern of intracellular enzymes in LPA homeostasis appears to shift to favor LPA synthesis, as enzymes involved in synthesis are upregulated (cytoplasmic phospholipase A_2IVA [cPLA$_2$IVA] and calcium-independent phospholipase A_2VIA [iPLA$_2$VIA]), whereas a key enzyme involved in degradation (LPA acyltransferase [LPAAT]α) was downregulated. The induced expression of cPLA$_2$IVA and iPLA$_2$VIA was corroborated on protein level by immunohistochemical analysis of plaque cryosections, which show a markedly increased macrophage expression of both these enzymes in advanced atherosclerotic lesions. The induced expression of cPLA$_2$IVA and iPLA$_2$VIA, two major intracellular PLA$_2$ enzymes in humans[12], could indirectly translate in an increased LPA production[13,14]. Furthermore, cytosolic PLA$_2$ is involved in vascular smooth muscle cell and macrophage apoptosis[15,16] and in the production of pro-inflammatory prostaglandins via arachidonic acid, pointing to a pro-atherogenic and plaque destabilizing activity of this enzyme. In analogy, downregulation of LPAATα as seen in lesion tissue may also result in cytotoxic effects in dedifferentiated or dysregulated cells in the plaque, since inhibition of LPAATβ induced cytotoxicity in various tumor cell types, while in most non-tumor cells it affected growth arrest and quiescence[17]. Further study is warranted to address these hypotheses.

In chapter 4, a series of LPA species were localized within the atherosclerotic lesions by time-of-flight secondary ion mass spectrometry (TOF-SIMS) imaging and we determined colocalization with other constituents of the atherosclerotic lesion, and the lipid core in particular. We demonstrate the prominent presence of LPA species in the acellular non-nucleated regions of the atherosclerotic lesion, which are considered to be lipid-rich necrotic cores, and as expected colocalization of these lysolipids with cholesterol, but remarkably also with S1P. The atherosclerotic plaques showed phosphate and phosphocholine localization in the cellular parts of the lesion, which have been previously described to indicate cellular contributions such as vascular smooth muscle cells[18]. Thus, by chemical evaluation of analytes in the atherosclerotic lesion we could specify the non-nucleated compartments as lipid rich necrotic debris or large foam cells, visualized as a single nucleus surrounded by a large acellular region, while nucleated regions consist of cellular contributions.

Altogether these data demonstrate that LPA, and in particular highly-unsaturated long-chain LPA species, indeed accumulates during atherosclerotic lesion progression. However, the relative contribution of intraplaque LPA synthesis versus LDL mediated delivery still remains to be determined. Apparently, a significant amount of LPA has accumulated in the plaque in the first two weeks after collar placement.

At this time point, only fatty streaks have developed and we believe that here the delivery via modified LDL is contributing most to the LPA pool. Upon further lesion progression, local synthesis of LPA may become more important. As demonstrated by immunohistochemistry, $cPLA_2$IVA and $iPLA_2$VIA localize mainly in macrophages, which indicates that this cell type contributes to both pathways of LPA accumulation. On the one hand, macrophages take up the modified LDL, while on the other hand they are considered to be the main cell type responsible for increased intracellular LPA production. Therefore, macrophages are a promising target for correction of LPA content at later stages of lesion development. By correction of the expression of one of the key enzymes in LPA metabolism, the LPA content in the plaque might be normalized resulting in a concomitant reduction in plaque thrombogenicity.

Lysophosphatidic Acid in Atherosclerotic Lesion Stability

In addition to its thrombogenic effects, LPA has multiple effects on various cell types involved in atherosclerosis. One of these cell types, the mast cell (in particular when activated), has been implicated in atherosclerotic lesion development and lesion destabilization[19,20]. As studies have suggested a role for oxidized low-density lipoprotein (oxLDL) particles in mast cell activation[21,22], LPA could well be the sought-after endogenous trigger for mast cell activation and degranulation *in vivo*, by virtue of its presence in oxLDL[3] and its activating effects on mast cells[23-26].

To emulate enhanced focal exposure to LPA that occurs during plaque development on mast cell activation and concomitant plaque stability, we performed a local LPA challenge on the adventitia of carotid artery lesions induced by perivascular collar placement in $ApoE^{-/-}$ mice (chapter 5). *In vitro* studies confirmed LPA-induced mast cell activation consisting primarily of tryptase release. We further demonstrate that focal LPA-induced mast cell activation in the adventitia of advanced carotid artery plaques enhances microvascular leakage and macrophage recruitment, and results in increased incidence of intraplaque hemorrhage and iron deposits. The enhanced microvascular leakage by LPA-induced mast cell activation was partly mediated by either LPA_1 or LPA_3 or a combination of these receptors, as it could be partly inhibited by an $LPA_{1/3}$ antagonist. Due to enhanced microvascular leakage upon mast cell activation, LPA can contribute to increased macrophage recruitment as increased leakage allows the influx of hematopoietic subsets such as monocytes[19,27]. In addition, mast cell activation can directly stimulate macrophage recruitment by triggering monocyte arrest via release of the CXCR2 ligand, KC[28,29]. Furthermore, LPA has direct effects on macrophages, such as increased survival and proliferation and reduced macrophage emigration[30-32]. These LPA-induced effects all act in concert to contribute to the increased macrophage content seen in atherosclerotic lesions. Importantly, the adverse events of LPA-induced mast cell activation could be inhibited by the mast cell stabilizer cromolyn.

Besides its effects on mast cells, LPA mediates multiple cellular processes that are instrumental in atherogenesis, including smooth muscle contraction and proliferation[33-38], endothelial/leukocyte interaction[39] and macrophage survival, proliferation and plaque emigration[30-32]. Of particular interest are the vascular smooth muscle cells (VSMC), as previous studies have shown that LPA is capable of inducing dedif-

ferentiation of VSMCs[40,41] and *in vivo* of neointima formation[42,43]. To investigate long-term effects of increased plasma LPA concentrations on atherosclerotic lesion development and morphology, we have induced carotid artery lesions in ApoE$^{-/-}$ mice that were treated systemically with LPA or PBS as a control by repeated intraperitoneal injections for 5 weeks (Chapter 6). We demonstrate that supraphysiological levels of unsaturated 18:1 LPA do not affect atherosclerotic lesion size but can contribute significantly to atherosclerotic plaque morphology rearrangements. Systemic LPA treatment increased the percentage of α-actin positive smooth muscle cells in the intimal lesion area by 70%. *In vitro*, LPA dose-dependently increased murine VSMC proliferation indicating that systemic LPA treatment may stabilize the fibrous cap of atherosclerotic lesions by increased proliferation. Paradoxically, the atherosclerotic lesion showed 68% decrease in birefringent collagen fiber plaque content, despite a higher abundance of VSMCs. LPA-induced negative modulation of VSMC extracellular matrix production can be excluded as collagen synthesis analysis revealed a mild, but significant, increase in collagen production by VSMCs upon LPA exposure. Alternatively, the contradictory reduction in collagen content may be explained by an induction of matrix metalloproteinase (MMP) activity, as LPA has been demonstrated to induce MMP activity in different cell types such as endothelial cells, T cells and cancer cells[44-46]. However, the exact mechanism of decreased collagen in the atherosclerotic lesions remains to be further elucidated. Remarkably, LPA does not affect macrophage content of the atherosclerotic lesions, even though LPA could induce macrophage proliferation *in vitro*.

In conclusion, LPA has multiple effects, which affect plaque stability. Increased vascular leakage, increased intraplaque hemorrhage and iron deposition and decreased collagen fiber content point towards plaque destabilization. These effects suggest that intervention in LPA bioavailability and thereby either reducing mast cell activation or preventing the reduction in collagen content could be an effective new therapeutic entry in the prevention of acute coronary syndromes. Conversely, increased smooth muscle content is considered favorable for high lesion stability, which indicates that part of the LPA bioavailability could also work beneficially. Therefore, 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 awaited.

Sphingosine 1-Phosphate in Atherosclerosis Development

In contrast to LPA, *in vivo* evidence documenting an involvement of S1P in the development and progression of atherosclerosis is scarce. Neither intimal S1P accumulation nor expression of S1P receptors in atherosclerotic lesions have been examined to date. In addition, no studies have been published on the effects of S1P receptor knockout or overexpression on the development of atherosclerotic lesions in animal models. However, numerous *in vitro* studies suggest that S1P, a bioactive lysosphingolipid associated with high-density lipoproteins, may account at least partly for the potent anti-inflammatory properties of HDL and, thereby, contributes to the anti-atherogenic potential attributed to high-density lipoproteins[6-8].

In chapter 7, we have investigated whether modulation of S1P signaling by the syn-

thetic sphingosine analogue FTY720, which in its phosphorylated form (FTY720-P) acts as a high-affinity agonist for $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$, affects atherosclerosis in $LDLr^{-/-}$ mice[47]. We demonstrated that FTY720 preferentially distributes into HDL, inhibits the development of atherosclerotic lesions and almost completely blunts necrotic core formation in $LDLr^{-/-}$ mice on a cholesterol-rich diet. FTY720 was previously shown to produce lymphopenia by sequestering lymphocytes from blood into lymph nodes, thereby preventing their recruitment into sites of inflammation[48,49]. In our study, FTY720 also induced lymphopenia in $LDLr^{-/-}$ mice and, in addition, reduced $CD3^+$-cell infiltration into atherosclerotic lesions. As lymphocytes are crucially involved in the propagation of inflammatory processes within the arterial wall, and T-cell deficiency has been repeatedly reported to attenuate atherogenesis[50-52], it is conceivable that the decreased lymphocyte availability partly accounts for the reduction of atherosclerosis. As atheroprotective effects of this compound were even apparent at low doses of FTY720 that only slightly affected blood lymphocyte counts and T-cell subset patterns, altered T-cell trafficking and sequestration cannot be held fully accountable for the reduced atherosclerosis. FTY720-treated animals also showed decreased splenocyte proliferation and lymphocyte cytokine patterns reflective of lymphocyte T helper (Th)1 polarization, such as interferon-γ, interleukin (IL)-12 and RANTES, even at low doses. This suggests that FTY720 attenuates lymphocyte function and Th1 immune responses, as was previously shown for S1P[53-56]. Because Th1 cells are considered to represent a particularly pro-atherogenic subset within the $CD4^+$ T-cell population[57], FTY720 could in addition impair atherogenesis by attenuating Th1 responses and skewing the immune response toward Th2.
Analogous to lymphocytes, FTY720 did modulate macrophage function *in vivo* but without changing the plaque macrophage content. Plasma levels of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, TNF-receptor, and IL-6, which are abundantly secreted by activated macrophages, are markedly reduced in $LDLr^{-/-}$ mice treated with low and high doses of FTY720. In addition, peritoneal macrophages from FTY720-treated animals displayed a decreased response to LPS, an established inducer of classical (M1)-type macrophage activation, while it enhanced the IL-4-elicited production of IL-1 receptor antagonist (IL-1RA), a marker of alternative (M2)-type macrophage activation. Collectively, these observations suggest that the FTY720-induced polarization of lymphocytes towards a Th2 response is paralleled by a complementary M1→M2 switch in macrophages. A rich source of anti-inflammatory cytokines[58], M2 macrophages will dampen inflammatory responses within the vascular wall and, thereby, counteract the formation of atherosclerotic lesions.
In conclusion, these results demonstrate that FTY720 inhibits atherosclerosis by modulating lymphocyte and macrophage function.

In chapter 8, we further explored the effects of altering S1P signaling on atherogenesis and performed a study on increased endogenous S1P availability. Previous studies have demonstrated critical involvement of S1P and its cognate receptor, $S1P_1$, in maintaining proper lymphocyte egress from lymphoid organs[59-62]. In fact, S1P gradients between lymphoid organs (low S1P concentration) on the one hand and circulation (high S1P concentration) on the other hand are driving forces in lymphocyte fluxes from lymphoid organs to the periphery[63-65]. As intracellular S1P levels are tightly controlled by S1P lyase, encoded by *Sgpl1*, we investigated the effect of

hematopoietic absence of S1P lyase on leukocyte homeostasis and atherosclerosis.

Similar to S1P analogues[47,66], enhanced S1P levels as apparent in hematopoietic *Sgpl1*-deficiency, ameliorates atherogenesis and leads to decreased T cell influx into the lesion, while leaving plaque macrophage content unaltered. Several mutually non-exclusive mechanisms may account for the diminished atherosclerosis development observed under conditions of enhanced endogenous S1P production. Obviously, the most dramatic effects of S1P lyase-deficient bone marrow transplantation were noticed on lymphocyte counts and distribution. Decreased lymphocyte availability could partly account for the reduced atherogenic response seen in S1P lyase-deficient animals. The profound lymphopenia was a likely consequence of S1P gradient disruption[64], which is crucial for normal lymphocyte egress from lymphoid organs and therefore for proper immune effector function. Indeed, effector functions in the *Sgpl1*$^{-/-}$ transplanted animals appeared to be hampered. Hematopoietic *Sgpl1* deficiency rendered T lymphocytes non-responsive to other chemotactic signals, such as CCL19. In addition, hematopoietic *Sgpl1* deficiency demonstrated a reduction in plasma levels of IL-12 and TNF-α upon CCL19 stimulation, decreased splenocyte proliferation and ablated CCL19-stimulated IL-2 and IL-4 responses *in vitro*, all pointing towards reduced effector functions. This once more confirms that S1P signaling attenuates lymphocyte function and Th1 immune effector functions[53-56], which are crucial in atherosclerotic lesion development. Hematopoietic *Sgpl1* deficiency to our surprise also affected the myeloid lineage by inducing mild monocytosis and a pro-inflammatory macrophage phenotype, while decreasing monocyte chemoattractant protein (MCP)-1-dependent trafficking. *Sgpl1*$^{-/-}$ macrophages displayed increased excretion and/or expression of IL-6, IL-12, TNF-α, and IL-1α and lowered expression of IL-10, IL-1RA and arginase 1 reflective of M1-shifted macrophage polarization. This pro-inflammatory M1 polarization in the *Sgpl1*$^{-/-}$ chimeras was in sharp contrast with the FTY720 study described above in which S1P agonism led to an M1→M2 shift[47] and the study by Hughes *et al.* in which S1P clearly induced an anti-inflammatory macrophage phenotype[67]. However, the reduction in MCP-1 levels seen in the *Sgpl1*$^{-/-}$ chimeras were consistent with previous data on S1P agonism, which resulted in reduced plasma MCP-1 levels and macrophage content in the lesion observed in ApoE$^{-/-}$ mice treated with FTY720[66]. MCP-1 is a major chemokine not only driving stromal egress but also monocyte migration to inflammation sites such as atherosclerotic lesions in the vasculature[68,69]. Therefore, reduced MCP-1 production may also underlie the unchanged macrophage content in atherosclerotic lesions of hematopoietic S1P lyase-deficient animals despite the modest monocytosis in these mice. Overall, hematopoietic S1P lyase deficiency exerts rather contradictory effects on the monocyte subset favoring pro-inflammatory monocytosis and M1 polarization, but reducing MCP-1-dependent influx. In addition to the effects seen on T lymphocytes and macrophages, the *Sgpl1*$^{-/-}$ chimeras showed a severely blunted hyperlipidemic response to Western type diet feeding and these mice presented a considerably less atherogenic lipoprotein profile. The attenuated hyperlipidemic response to high cholesterol diet in S1P lyase deficient mice could be ascribed to disturbed intestinal fat absorption as manifested by decreased plasma cholesterol uptake upon oral cholesterol delivery and indicated by morphological features of the intestine showing an aberrant microvilli architecture. Apparently S1P or analogues

can directly or indirectly intervene in intermediary lipid metabolism as previous studies have shown a dose-dependent inhibition of intestinal fat adsorption by S1P receptor-activating phytosphingolipids and concomitant reduction of plasma cholesterol and triglycerides in ApoE*3-Leiden mice[70]. In addition, S1P homeostasis has been demonstrated to be pivotal in normal intestinal regeneration and function[71,72]. In summary, hematopoietic S1P lyase is essential for maintenance of S1P gradients and its absence has profound effects not only on lymphoid surveillance but also on lymphocyte activity, stromal monocyte release and intestinal and hepatic lipid homeostasis. Importantly, we also show that S1P lyase deficiency renders lymphocytes as well as monocytes refractory to chemotactic and signaling pathways. The pro-inflammatory effects on *Sgpl1*$^{-/-}$ chimera macrophages apparently are insufficient to reverse the aforementioned atheroprotective effects on T lymphocytes and lipid profile. Collectively, these effects translate in a reduced atherogenic response in S1P lyase deficiency.

In conclusion, induction of S1P signaling can affect lymphocyte and macrophage function and trafficking, and lipid homeostasis. Although these effects are not in every case anti-inflammatory, the net effect results in inhibition of atherosclerosis, and are consistent with the notion that S1P contributes to the anti-atherogenic potential of HDL.

Perspectives

This thesis presents an overview of the contribution of two lysolipids, LPA and S1P, in the development and progression of atherosclerosis. We have demonstrated that thrombogenic LPA, and specifically highly-unsaturated long-chain species, accumulates particularly in the non-nucleated lipid rich necrotic core of the atherosclerotic lesions. Furthermore, we have established that during atherogenesis expression of key proteins in LPA homeostasis is increasingly perturbed favoring intracellular LPA production and altered signal transduction. As macrophages appear to be the main cell type for LPA accumulation, macrophages are considered a promising target for correction of LPA content in atherosclerotic lesion development, rendering intervention in their enzymatic LPA production an attractive measure to normalize the intraplaque LPA content. In addition, LPA was seen to negatively influence plaque stability by its effects on vascular leakage, intraplaque hemorrhage, iron deposition and collagen, supporting the notion that intervention in LPA bioavailability could be an effective strategy in the prevention of acute coronary syndromes. Conversely, LPA-induced increase in smooth muscle content of the atherosclerotic lesions is considered favorable in lesion stability, which indicates that part of the LPA bioavailability could also work beneficially. Since differences in LPA receptor expression and activation patterns could be essential for the diverse modifications in atherosclerotic lesion development, further research into the cell-specific receptor expression and LPA-induced effects is warranted. These studies have led to a better understanding of pathways possibly involved in accumulation of thrombogenic LPA in the lipid-rich necrotic cores of atherosclerotic lesions. Furthermore, we have demonstrated that LPA, besides its detrimental thrombogenic ability, also negatively affects lesion sta-

bility. Future studies should provide evidence for the major contributing pathway leading to the increase in intraplaque LPA bioavailability to efficiently reduce LPA accumulation and thereby intervene in the mechanism of reduced plaque stability.

Furthermore, we investigated the role of S1P signaling in atherosclerosis. FTY720, which upon phosphorylation is a synthetic S1P mimetic, inhibited atherosclerotic lesion development by modulating lymphocyte and macrophage function. Treatment with FTY720 caused a T-cell skewing towards a Th2 response while favoring M2 polarization of macrophages, effects which are both considered anti-atherogenic. Absence of hematopoietic S1P lyase, which increases endogenous S1P bioavailability, also inhibits atherosclerotic lesion formation. In this case, increased S1P bioavailability disrupted S1P gradients necessary for normal lymphocyte egress from lymphoid organs, thereby hampering normal lymphocyte trafficking. In addition, *Sgpl1*$^{-/-}$ chimeras showed a severely blunted hyperlipidemic response to Western type diet feeding and these mice presented a considerably less atherogenic lipoprotein profile, which could be ascribed to disturbed intestinal fat absorption. Furthermore, *Sgpl1*$^{-/-}$ chimeras showed hampered macrophage trafficking, while the inflammatory status of the macrophages was considered pro-inflammatory. However, the latter effect appears to be insufficient to reverse the atheroprotective effects shown for T lymphocytes and lipid profile. Both S1P studies demonstrate that intervention in S1P signaling can modulate atherogenesis and are consistent with the concept that S1P contributes to the anti-atherogenic potential of HDL. Therefore, these studies warrant further evaluation of S1P-receptor agonists as potential HDL surrogates and modulators of atherosclerotic cardiovascular disease. However, the contradictive data on macrophages indicate that S1P signaling does not definitively act anti-atherogenic demanding additional research with respect to S1P-mediated macrophage polarization and its contribution to cardiovascular disease. In addition, S1P signaling proved to be important in regulating intestinal lipid uptake and lipid homeostasis, which indicates that further studies on S1P homeostasis with respect to lipid metabolism could possibly lead to new (dietary) strategies in the battle against atherosclerosis.

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Chapter 9