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Bot, M.

Citation

Bot, M. (2009, January 15). *Bioactive lipids as key regulators in atherosclerosis*. Retrieved from <https://hdl.handle.net/1887/13407>

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

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Lysophospholipids: Two-Faced Mediators in Atherosclerosis

Martine Bot¹, Jerzy-Roch Nofer², Theo J.C. van Berkel¹, Erik A. L. Biessen¹

¹Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, the Netherlands, ²Institute of Clinical Chemistry and Laboratory Medicine and Leibniz Institute for Arteriosclerosis Research, University of Münster, Münster, Germany.

Future Lipidology 2007;2:341-356.

Abstract

As risk factors for atherosclerosis and major constituents of atherosclerotic lesions, lipids have long been regarded as instrumental in the development of this disease. Apart from their structural contribution to lesion development, lipids may also modulate processes, both in circulation and in the plaque, which are instrumental in this vasculopathy. This review focuses on two major bioactive phospholipids, lysophosphatidic acid and sphingosine 1-phosphate, which were recently shown to be potentially important mediators in atherogenesis. These lysolipids display a dysregulated biosynthesis during atherosclerosis and are rather complementary in their mode of action. They both act as agonists of G-protein-coupled receptor family members on all vascular wall cell types involved in atherosclerosis, rendering them attractive targets for therapeutic intervention. This review will outline the current knowledge on their homeostasis, their physiological activity in various inflammatory and vascular wall cells and the implications of this for atherosclerosis.

Lipids and Atherosclerosis

Atherosclerosis is widely understood to be 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. In addition to circulating lipids, intimal lipids were also regarded as prominent determinants of the biomechanical stability of the atherosclerotic plaques and are in fact used as an important criterion for plaque stability. The last decade of research has culminated in the recognition that lipids not only 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 review, we shall focus on the two major bioactive phospholipids that were recently shown to be potentially important mediators in atherogenesis: lysophosphatidic acid (monoacyl-sn-glycerol-3-phosphate [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, and are complementary in their mode of action. This review will present the current knowledge on their homeostasis and their physiological activity in the context of atherosclerosis.

Lysophosphatidic Acid: General Features

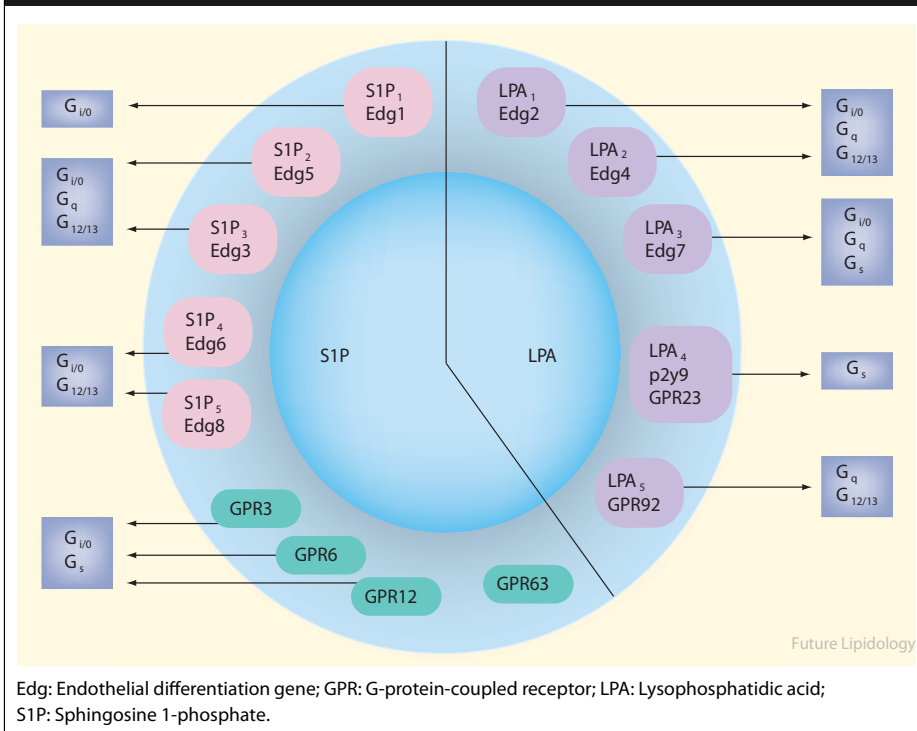
LPA, a member of the glycerol backbone-containing phospholipid subfamily, was originally described as a key intermediate in *de novo* lipid synthesis. In 1990, it was identified as an intercellular phospholipid messenger, which is involved in a wide variety of biological activities such as cell proliferation¹⁻³, smooth muscle contraction⁴, platelet aggregation^{5,6}, and cytoskeletal reorganization⁷. Its pleiotropic activity is mediated by at least three types of G-protein-coupled receptors specific for LPA, endothelial differentiation gene (Edg)2/LPA₁, Edg4/LPA₂ and Edg7/LPA₃, which all belong to the so-called Edg family (Figure 1)⁸⁻¹⁰. Of these receptors, LPA₁ is also a nuclear receptor¹¹. Recently, two additional G-protein-coupled LPA receptors have been discovered, p2y₉/G-protein-coupled receptor (GPR)23 and GPR92, which are genetically distant from the Edg receptors (Figure 1)^{12,13}. Apart from interacting with lysophospholipid receptors, LPA also binds to and activates peroxisome proliferator-activated receptor (PPAR) γ ¹⁴. As most cells express several types of LPA receptors, the cellular action of LPA will critically depend on the profile and level of expression of the different LPA receptors (Table 1).

Sphingosine 1-Phosphate: General Features

A second bioactive phospholipid involved in atherosclerosis is S1P, which is derived from sphingosine, the common backbone of most sphingolipids. It was originally believed to be formed intracellularly either as intermediate in sphingosine detoxification pathways or as an intracellular second messenger. Later studies revealed that S1P

is present in plasma and other body fluids, where it acts in an autocrine or paracrine fashion to regulate important physiological and pathophysiological processes. With regard to cellular signaling, S1P functions as a natural ligand of various Edg family members, also known as S1P receptors¹⁵. In addition, S1P proved to be an agonist for four former orphan receptors, albeit some with very low affinity^{16,17}. To date, nine S1P receptors have been identified: S1P₁ (Edg1), S1P₂ (Edg5), S1P₃ (Edg3), S1P₄ (Edg6), S1P₅ (Edg8), GPR3, GPR6, GPR12 and GPR63 (Figure 1). In addition to S1P, other lysosphingolipids in plasma, such as sphingosyl phosphorylcholine, were shown to signal via S1P receptors. S1P receptors are ubiquitously expressed and couple to various G proteins that regulate numerous downstream signals (Table 1). This endows S1P with the ability to regulate diverse physiological processes, including cell proliferation, motility, apoptosis, angiogenesis, wound healing and immune response.

Figure 1. Overview of lysophosphatidic acid and sphingosine 1-phosphate receptors and accessory G-protein signaling partners.



Formation of LPA and S1P

LPA is produced and released from activated adipocytes, fibroblasts, some epithelial cells and tumor cells. Furthermore, LPA is rapidly produced in serum following platelet activation. Thus, the serum LPA concentration can be as high as 10 μ M due to the release of secretory phospholipases A₁ and A₂ (sPLA_{1/2}) from activated platelets,

while, interestingly, plasma LPA levels are much lower and may range from 80 to 700 nmol/L^{18,19}. In the circulation, LPA mainly associates with albumin and lipoproteins, which appear to protect LPA from rapid degradation, but may also influence its bioavailability. Moreover, LPA has also been detected in other biological fluids, such as saliva, follicular fluid, seminal plasma and malignant effusions/ascetic fluid from ovarian cancer patients²⁰.

LPA can be generated both extra- as intracellularly²¹⁻²³. Extracellular LPA production mainly occurs through the conversion of lysophospholipids to LPA involving autotaxin (ATX)/lysophospholipase D/nucleotide pyrophosphatase/phosphodiesterase-2, an extracellular protein that is upregulated in various malignancies, including breast and lung cancer, and potently stimulates cell proliferation, cell motility and angiogenesis. ATX can produce LPA from lysophosphatidylcholine originating from lipoprotein particles, cellular plasma membranes or indirectly following sPLA₂-mediated phosphatidylcholine hydrolysis^{21,22}. An alternative pathway involves the conversion of phosphatidic acid (PA) by sPLA₂ released from activated platelets²³. Moreover, LPA was reported to be formed in a nonenzymatic manner by mild oxidation of low-density lipoprotein (LDL)⁵. At the catabolic site, LPA can be inactivated by reacylation and subsequently converted into PA or it can be degraded pericellularly by lipid phosphate phosphatases (LPP)-1/2/3, transmembrane proteins with an extracellular catalytic domain to focally regulate the bioavailability of LPA²⁴.

The synthesis of intracellular LPA is even more complicated and tightly regulated. Intracellular LPA is partly derived from *de novo* lipid synthesis in the endoplasmic reticulum or mitochondria as an intermediate product in the conversion of glycerol-3-phosphate into PA²⁵. Secondly, LPA is produced in the cytoplasmic compartment by monoacyl glycerol kinase-catalyzed phosphorylation of monoacyl glycerol and from PA by the conversion of cytoplasmic PLA₂ (cPLA₂) type IV or calcium-independent PLA₂²⁶. A third potential route for LPA synthesis proceeds in the cytoplasm through phospholipase (PL)D-mediated reduction of lysophosphatidylcholine²⁶. LPA acyltransferase (α and β) catabolizes LPA into PA²⁷, while LPP1-3 on the luminal side of the intracellular membranes can degrade LPA into monoacyl glycerol²⁴. It remains to be established whether LPA is shuttled between the two sites of synthesis, although the fatty acid binding proteins, and possibly the intracellular fatty acid translocase CD36, may very well act in this capacity.

S1P is present in the plasma and serum at nanomolar levels (200 and 500 nmol/L, respectively)²⁸⁻³⁰. Its concentrations are slightly higher in men than in women and tend to decrease with age³⁰. Approximately 40 to 60% of plasma S1P partitions into high-density lipoprotein, while the remainder likely associates with albumin²⁹. S1P is formed both intra- and extracellularly. Intracellular S1P is generated by mammalian sphingosine kinases (SphK)1 and 2. Both enzymes are ubiquitously expressed, however, their diverging kinetic properties and temporal expression patterns may point to a distinct cellular function³¹. SphK1 is predominantly located in the cytosol, but it will translocate to the cell membrane upon exposure of cells to hormones, growth factors and other signaling molecules³². The membrane-associated form was shown to exhibit enhanced enzymatic activity. In addition, SphK1 is actively exported from vascular endothelial cells to the extracellular milieu, thus establishing a local S1P concentration gradient³³. SphK2, on the other hand, is mainly associated with

Table 1. Expression pattern of lysophosphatidic acid and sphingosine 1-phosphate receptors in cell types relevant to atherothrombosis.

Human	SIP ₁	SIP ₂	SIP ₃	SIP ₄	SIP ₅	GPR3	GPR6	GPR12	GPR63	LPA ₁	LPA ₂	LPA ₃	LPA ₄	LPA ₅
Endothelial cells	+	+	+	+	nd	+	-	+	nd	+	+	+	nd	nd
SMCs	+	+	+	+	nd	nd	nd	nd	nd	+	-	+	nd	nd
Platelets	nd	nd	+	+	nd	nd	nd	nd	nd	+	+	+	nd	nd
Macrophages	+	+	+/-	+/-	-	nd	+	nd	nd	+	+	+	nd	-
Dendritic cells*	+	+	+	+	+	nd	nd	nd	nd	+	+	+	nd	nd
CD4 ⁺ T cells	+	+	+ [§]	+	-	nd	nd	nd	nd	+ [¶]	+	-	nd	-
CD8 ⁺ T cells	+	+/-	-	+	+	nd	nd	nd	nd	-	+	-	nd	+
NK cells	+	-	-	+	+	nd	nd	nd	nd	-	-	-	nd	nd
B cells	+	-	-	+	-	nd	nd	nd	nd	-	+	-	nd	-
Neutrophils	+	-	-	+	+	nd	nd	nd	nd	-	+	-	nd	nd
Mast cells	+ [‡]	+ [‡]	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	nd

+ mRNA and/or protein expression; +/- Trace amounts of mRNA or protein expression; - No mRNA and protein expression.

*Both immature and mature dendritic cells.

‡Expression on murine cells.

§Not on activated CD4⁺ T cells.

¶On activated CD4⁺ T cells.

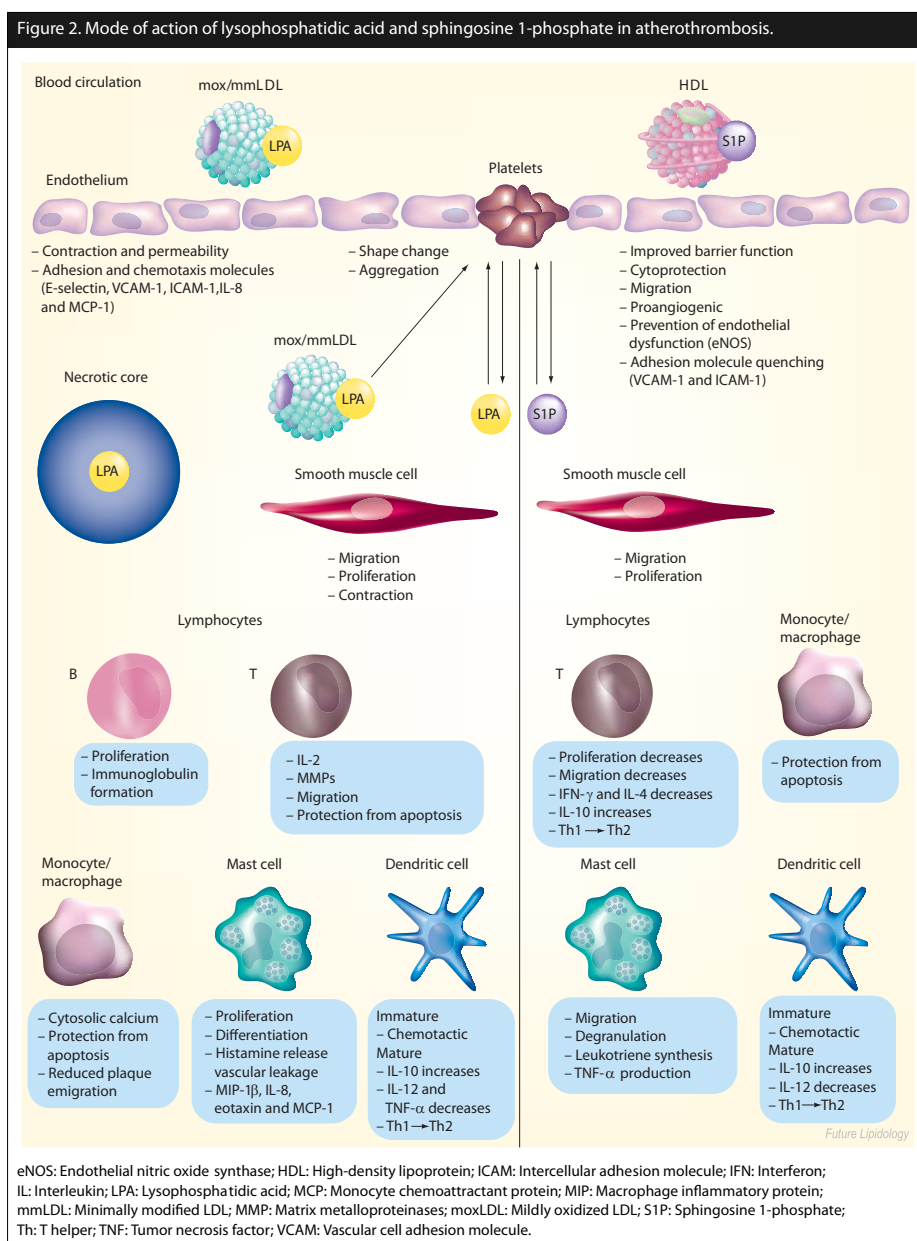
GPR: G-protein-coupled receptor; LPA: Lysophosphatidic acid; nd: Not determined; NK: Natural killer; SIP: Sphingosine 1-phosphate; SMC: Smooth muscle cell.

the plasma membrane and internal organelle membranes, and is present in the cytosol at much lower levels³². The SphKs appear to have opposite effects on cell survival as SphK1 activity promotes cell growth and inhibits apoptosis, while SphK2 activity is associated with inhibition of cell growth and acts pro-apoptotically. These paradoxical effects may reflect differences in the intracellular ceramide levels, which are decreased by SphK1 and increased by SphK2³². Furthermore, the capacity of ATX to convert sphingosine phosphorylcholine to S1P could contribute to extracellular S1P homeostasis³⁴.

The degradation of S1P is accomplished either by S1P lyase, which cleaves S1P into phosphoethanolamine and fatty acid aldehyde, or by S1P phosphatases (SPP)1 and 2, which belong to the family of type 2 lipid phosphate phosphohydrolases³⁵. Both S1P lyase and SPPs are ubiquitously expressed. Interestingly, S1P lyase was found to be absent in platelets. As platelets possess high SphK activity³⁶, they accumulate large amounts of S1P, which are released upon activation. In addition, other cells of hematopoietic origin, such as mast cells, neutrophils, erythrocytes and mononuclear cells, were reported to store S1P. Erythrocytes were recently identified as the main blood cell population capable of incorporating, protecting, storing and releasing S1P into the plasma³⁷. It has been suggested that S1P secretion from hematopoietic cells does not proceed through conventional exocytosis. In fact, S1P externalization occurs by transbilayer transfer across the plasma membrane and very recently the ATP-binding cassette transporter C1 (multidrug related protein 1), has been proposed to mediate this process³⁸.

LPA in Atherosclerosis

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 (Figure 2). In humans, plaque intima was shown to contain more LPA than normal arterial tissue⁵. As a major bioactive component of mildly oxidized LDL (moxLDL), LPA is most likely directly deposited via moxLDL entering the arterial wall, although substantial experimental proof for this notion is still lacking. Furthermore, intimal LPA may be produced *in situ* by sPLA₂ type IIA and V, which are induced by pro-inflammatory cytokines or a high-lipid diet, respectively³⁹. Intriguingly, *Chlamydia pneumoniae*, a pathogen that has been implicated in cardiovascular disease, harbours proteins with PLD-like activity and can promote the formation of LPA from lysophosphatidylcholine present in oxidized LDL (oxLDL) and in the outer leaflet of cellular plasma membranes. In addition, LPA is readily formed after platelet activation, thus linking LPA to atherothrombosis^{21,22}. Finally, the intimal LPA content depends on the inflammatory status, as local vascular leakage⁴⁰ and cell activation will affect the permeation of circulating LPA into tissue and focal LPA production, respectively. All these pathways potentially augment the formation of LPA in atherosclerotic lesions⁴¹. Since LPA is the primary platelet-activating lipid, rupture of the LPA-containing plaque increases the risk of intra-arterial thrombus formation leading to myocardial infarction and stroke⁵. Siess and colleagues have demonstrated that LPA extracted from both moxLDL and human atherosclerotic lesions can induce platelet shape change, while lipids from native LDL only lead to a



weak platelet response. Given that LPA represents a class of related, but chemically distinct, lipid species, it is not unconceivable that the potency of LPA to activate platelets depends on actual fatty acid composition and on the chemical nature of linkage of the fatty acid to the glycerol backbone (either ester or ether bond). Indeed, the biological potency of alkyl-LPA species, present in both moxLDL and human atherosclerotic lesions, are approximately 20-times higher than the corresponding acyl-esterified analogue^{6,42-44}. Furthermore, it was found that arachidonoyl-LPA (20:4) had

a higher platelet-activating potency than other saturated or unsaturated acyl-LPA species (16:0/18:1). These results imply the existence of additional LPA receptors, at least on platelets, as the ligand preference of platelets to alkyl-LPA does not match that of any of the currently known LPA receptors.

In addition to its thrombogenic activity, LPA exerts several pro-inflammatory effects that are associated with atherogenesis. In the early phase of atherosclerosis, exposure of endothelial cells to LPA will increase the permeability of the endothelium and thus result in barrier dysfunction⁴⁵. Furthermore, LPA can prime endothelial cells by activation of nuclear factor- κ B and subsequent upregulation of pro-inflammatory adhesion molecules and cytokines, such as E-selectin, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, interleukin (IL)-8, and monocyte chemoattractant protein (MCP)-1^{46,47}. The fact that immune cells critical to atherosclerosis, such as T lymphocytes, macrophages, dendritic cells (DCs) and mast cells, all express several LPA receptors, as well as PPAR γ , an LPA sensitive nuclear receptor regulating lipid homeostasis and inflammatory status^{48,49}, suggests that this lipid mediator has an important role in innate and adaptive immune responses involved in atherosclerosis. Preliminary data suggest that mononuclear phagocytes, dedicated dendritic subsets, mast cells and B cells, but not T cells, are able to generate LPA⁴⁸, perpetuating the LPA-mediated regulation of inflammatory status. In the next section, we will elaborate on the effects of LPA on specific immune cells and their implications for the atherogenic process.

Lymphocytes and Macrophages

LPA can protect T cells from apoptosis through combined LPA₁₊₂ signalling cascades, thus sustaining the ongoing inflammatory response⁴⁸. In unstimulated T cells, which predominantly express LPA₂, LPA was reported to induce cell migration and the expression of matrix metalloproteinases (MMPs) but to inhibit the T helper (Th)1 cytokine IL-2 production, possibly in a nuclear factor of activated T cells (NFAT)-dependent manner. Mitogen activation of T cells leads to a downregulation of LPA₂ and a concomitant upregulation of LPA₁ expression, translating to an impaired migratory capacity and activation of IL-2 production. This cytokine will in turn cause proliferation of activated T cells. Furthermore, LPA exerts a stimulatory influence on activated immunoglobulin (Ig)-secreting B cells by inducing their proliferation and Ig formation⁴⁸.

Monocytes can be activated by nanomolar concentrations of LPA, while at higher concentrations it acts as a survival factor to murine macrophages^{48,50}. In addition, in human monocytic THP-1 cells, LPA at micromolar concentrations is able to stimulate proliferation, the production of reactive oxygen intermediates and the release of arachidonic acid metabolites (e.g. prostaglandin E₂ and leukotriene B₄)⁵¹.

Robust monocyte recruitment into the arterial wall was initially thought to be the main trigger for atherosclerotic plaque expansion. Recently, egress of monocytes from advanced plaques was also shown to be impaired as a consequence of an altered integrin and CC chemokine receptor expression pattern^{52,53}. As LPA may lower the capacity of monocyte-derived cells to emigrate from the vessel wall, LPA could in this way also contribute to lesion progression. Furthermore, oxLDL can, by virtue of entrapped LPA, stimulate lipid accumulation in human monocytes by direct PPAR γ activation and subsequent CD36 upregulation, thus promoting lesion progression¹⁴.

Dendritic Cells

DCs are dedicated antigen-presenting cells. They form focal clusters, for example subendothelially at the known arterial predilection sites for lesion development⁵⁴. These subendothelial DCs increase in number during lesion progression, become activated and appear to be particularly abundant in the shoulder region of a plaque. Furthermore, the proportion of activated DCs was shown to be increased in vulnerable plaques, and DCs were generally colocalized with activated T cells and natural killer T cells in the shoulder region of these plaques⁵⁴. LPA may be one of the chemotactic factors attracting immature human DCs to the plaque. Remarkably, in mature DCs, LPA enhances the secretion of IL-10, while inhibiting that of IL-12 and tumor necrosis factor (TNF)- α to favor a Th2 rather than Th1 response. Therefore, the activity pattern of LPA on DCs is rather inconclusive; while LPA may facilitate the recruitment of DCs to the site of inflammation, it also seems to restrict the capability of mature DCs to amplify a Th1 immune response^{55,56}. However, recent findings by Chen and colleagues have demonstrated that LPA modulates Toll-like receptor-mediated regulation of the maturation of human myeloid DC, rather than altering the ability of DCs to influence Th cell proliferation itself⁵⁷.

Mast Cells

Vasa vasorum-derived microvessels nurture the atherosclerotic plaque and are regulated via an organized system of sympathetic and hormonal stimuli. These vasa vasora-derived neovessels represent a permanent communication route between circulation and the central atheroma, allowing the influx of detrimental agents and hematopoietic subsets, such as monocytes and erythrocytes⁴⁰. Mast cells are particularly abundant in the perivascular adventitia and near the neovessels of atherosclerotic lesions, and are regarded as a major source of a plethora of angiogenic and pro-inflammatory mediators and histamine⁵⁸. Their activation will cause vascular leakage, chemotaxis to the atheroma and angiogenesis and apoptosis in the atheroma, all of which are adverse features in disease development. As a potent inducer of histamine release from mast cells, LPA can increase plasma exudation and vascular leakage, which potentially aggravates lesion progression⁵⁹. Furthermore, Bagga and colleagues demonstrated that LPA accelerates human mast cell proliferation and differentiation via LPA_{1/3}- and PPAR γ -dependent pathways, which can further influence plaque stability⁴⁹. As mentioned previously, LPA triggers the release of a wide range of mediators, among which the pro-inflammatory chemokines macrophage inflammatory protein-1 β , IL-8, eotaxin and MCP-1 can provoke further recruitment of inflammatory cells into the arterial wall⁶⁰.

Vascular Smooth Muscle Cells

Vascular smooth muscle cells (VSMCs) are responsive to LPA, as reflected by the massive rise in intracellular Ca²⁺ upon LPA exposure¹, translating to an increase in proliferation in rat as well as human VSMCs¹⁻³. Moreover, LPA, and in particular the unsaturated LPA species, can induce a phenotypic shift in VSMCs in an extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK)-dependent manner⁶¹. Migration studies on VSMCs have shown rather contradictory results. Ai and colleagues⁶² reported that LPA induced migration by myosin light chain phosphorylation via the Rho-Rho kinase pathway, whereas an inhibitory effect

on VSMC migration was shown by Gennero and colleagues¹. Furthermore, LPA induces tissue factor (TF) expression through G α protein and activation of MAPK and ERK1/2⁶³. Elevated LPA levels, as seen in the atherosclerotic plaque, may therefore also indirectly enhance the plaque thrombogenicity by inducing the expression of TF.

LPA in Vascular Remodeling

Involvement of LPA in vascular remodeling may be inferred from recent studies on its capacity to activate PPAR γ ¹⁴. As described above, LPA can act as an agonist of PPAR γ , a key regulator of genes that contain PPAR response elements such as CD36. LPA upregulates CD36 in macrophages, which results in cholesterol accumulation and foam cell formation in atherosclerotic lesions¹⁴. In a rat model of injury-inflicted vascular remodeling, LPA aggravated neointima formation⁶⁴ and this effect could be prevented by the PPAR γ antagonist GW9662 and mimicked by PPAR γ but not PPAR α agonists. Conceivably, vascular remodeling is mediated mainly by naturally occurring unsaturated LPA species *in vivo*, and these agents have already been shown to potently induce smooth muscle cell dedifferentiation in a p38 MAPK-dependent manner⁶⁵.

S1P in Atherosclerosis

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 the expression of S1P receptors in the atherosclerotic lesion have been examined to date. In addition, no studies on the effects of S1P receptor knockout or overexpression on the development of atherosclerotic lesions in animal models have been conducted. Two very recent studies demonstrated reduced atherosclerosis in apolipoprotein E^{-/-} or low-density lipoprotein receptor^{-/-} mice treated with a synthetic S1P analogue, FTY720^{66,67}, which works as a high-affinity agonist on S1P₁, S1P₃, S1P₄ and S1P₅. Furthermore, the interaction of S1P with various cells relevant to the formation of atherosclerotic plaque has been thoroughly examined *in vitro*. The overall impression emerging from these studies is that, *in vivo*, S1P could be regarded as an anti-atherogenic modulator mainly by affecting inflammatory cell function (Figure 2). Interestingly, a recent study showed that FTY720 displayed an unexpected direct inhibitory effect on cPLA₂, independently of S1P receptors. cPLA₂ is rate limiting in the synthesis of arachidonic acid from glycerol phospholipids, and as such essential for the production of immunomodulatory eicosanoids⁶⁸.

Lymphocytes and Macrophages

Experiments *in vitro* have demonstrated the influence of S1P on various aspects of lymphocyte physiology. In high physiological concentrations, S1P suppresses lymphocyte proliferation and migration towards chemotactic factors via activation of S1P₁ receptor⁶⁹. In addition, signaling through S1P₁ induces marked suppression of interferon- γ , whereas the production of IL-4 is primarily inhibited via S1P₄. More-

over, signaling through the latter receptor induces production of IL-10, a cytokine with potent anti-atherogenic effects. The changes in the type of cytokines secreted by lymphocytes in the presence of S1P suggest that the integrated activities of the S1P/S1P-receptor axis promote the switch from a Th1 phenotype towards a less atherogenic Th2 functional phenotype. Recent results from *in vivo* studies accounts for this contention⁷⁰.

In addition to modulation of CD4⁺ cytotoxic T cells, S1P is required for the optimal function of CD4⁺CD25⁺ regulatory T cells (Treg), which inhibit diverse activities of autoimmune effector CD4⁺25⁻ T cells⁷¹. Wang and colleagues demonstrated that CD4⁺25⁺ T-cell suppression of proliferation and IL-2 generation by CD4⁺25⁻ T cells stimulated with anti-T cell receptor antibodies was enhanced significantly by S1P⁷². These levels of S1P also enhanced IL-10 generation by CD4⁺25⁺ T cells. The pertinence of these observations to the pathogenesis of atherosclerosis gains weight in light of recent findings by Ait-Oufella and colleagues demonstrating that depletion of Treg cells in mouse models aggravates atherosclerosis and that, conversely, transfer of CD4⁺25⁺ T cells to Treg-depleted animals brings the development of atherosclerosis to a halt⁷³.

In addition to modulation of functional responses, S1P exerts a potent influence on lymphocyte migration, tissue homing and recirculation, which are mediated exclusively by S1P₁⁷⁴. In current models, the concentration of S1P in blood effectively suppresses the chemokine-driven movement of T cells into peripheral tissues and a 1.5-fold increase in the plasma concentration of S1P promotes lymphoid-organ sequestration of lymphocytes⁷⁵. Furthermore, S1P appears to negatively regulate the efflux of lymphocytes from peripheral lymph organs by trapping lymphocytes on the abluminal site of the sinus-lining endothelium⁷⁶. The integrative effect of S1P on the processes of lymphocyte migration is the reduction of circulating lymphocyte pools available for infiltration of arterial wall. In fact, treatment of atherosclerosis-prone mice with FTY720 dramatically reduces the number of CD3⁺ lymphocytes present in the atherosclerotic lesions⁷⁷.

Little efforts have been devoted to better understanding the interactions between S1P and macrophages. S1P₁, S1P₂ and S1P₄ were reported to be expressed in monocytes and macrophages, whereas S1P₃ was induced during macrophage differentiation⁷⁸. Early studies documented that exogenous S1P inhibits apoptosis in monocytes and bone marrow-derived macrophages⁷⁹ via the activation of the protein kinase Akt and upregulation of the anti-apoptotic protein Bcl-XL. More recently, the survival effect associated with the ingestion of apoptotic cell debris by macrophages was demonstrated to strictly depend on enhanced production and release of S1P, which acts in an autocrine loop to stimulate Akt activation and to upregulate the expression of anti-apoptotic proteins Bcl-XL and Bcl-2⁸⁰. These observations are potentially of great importance as an abundance of apoptotic cells, which potently stimulate S1P production and secretion by macrophages, was repeatedly demonstrated in the lesion area⁸¹.

Dendritic Cells

S1P, like LPA, is a chemotactic factors that attracts immature human DCs to atherosclerotic plaques. S1P stimulates a pertussis toxin-sensitive Ca²⁺ increase, actin polymerization and chemotaxis. These responses are lost in lipopolysaccharide (LPS)-

matured DC. This maturation results in the inhibition of IL-12 secretion while IL-10 secretion is enhanced, suggesting a reduced capacity to generate an allogeneic Th1 response and an increased capacity to generate a Th2 response⁵⁶. Therefore, the activity pattern of S1P on DCs is also rather inconclusive; whilst it may facilitate the recruitment of DCs to the site of inflammation, it also seems to restrict the capability of mature DCs to amplify a Th1 immune response. Furthermore, S1P dose dependently influences DC survival⁶².

Mast Cells

Upon antigen (Ag)-directed cross-linking of the high-affinity receptor for IgE (FcεRI) on mast cells, both SphK1 and 2 are activated. SphK1 activation appears to be important in the regulation of the levels of circulating S1P, extrinsically affecting mast cell responsiveness⁸³. Sphk2 was found to be an intrinsic regulator of mast cells influencing S1P production, calcium influx, activation of protein kinase C, cytokine production and degranulation⁸³. Extracellular S1P can, in an autocrine manner, enhance mast cell function by binding and transactivating its receptors (S1P₁ and S1P₂). Transactivation of S1P₁ is required for the chemotaxis of mast cells towards low concentrations of Ag, whereas S1P₂ is important for degranulation responses. As mast cells approach higher concentrations of Ag, a shift in the expression of S1P receptors (enhanced S1P₂ expression) resolves migration while promoting degranulation⁸⁴. A recent study showed that, in contrast to S1P, both FTY720 and FTY720-P, (the active form with agonist activity on S1P receptors) do not affect mast cell degranulation⁶⁸.

Endothelial Cells

Endothelial dysfunction has been shown to be instrumental in early-stage atherogenesis and this process is tightly regulated by S1P. Endothelial cells express S1P₁ and to a lesser extent S1P₂ and S1P₃⁸⁵. Initial studies revealed increases in transendothelial electrical resistance across endothelial cells suggestive of improved endothelial barrier function⁸⁶. In addition, S1P, as well as the S1P analogue FTY720, reversed the reduction of transendothelial resistance elicited by pro-atherogenic factors^{86,87}. S1P-mediated barrier modulation involves activation of the small G protein Rac-1, which coordinately regulates diverse processes related to endothelial integrity^{86,88}. S1P₁ appears to be the primary route to Rac-1 activation in endothelial cells, illustrating the major importance of S1P₁ in endothelial function and silencing of S1P₁ expression prevented the S1P-induced increase in barrier integrity⁸⁹. In addition, S1P₁ could be demonstrated to participate obligatorily in the enhancement of endothelial barrier function by activated protein C and high-molecular-weight hyaluronan⁹⁰.

In addition to improving endothelial integrity, S1P also exerts pro-angiogenic and cytoprotective effects on endothelial cells in a S1P₁- and S1P₃-dependent manner⁹¹. Several studies demonstrated that S1P suppresses caspase-3 activation and thereby prevents endothelial apoptosis induced by growth factor deprivation or oxLDL⁹². Once again, the anti-apoptotic effect of S1P was mediated via S1P₁ and to a lesser extent S1P₃ signaling. S1P also contributes to injury repair by promoting endothelial migration via S1P₁ and S1P₃⁹³. Conversely, S1P₂ activation impairs cell migration and this effect was critically dependent on phosphatase and tensin homologue de-

leted on chromosome 10 (PTEN), a tumor suppressor phosphatase important for cell motility and invasion⁹⁴. Interestingly, both S1P₁ and S1P₃, but not S1P₂, activation resulted in Rho activation in endothelial cells and blunting Rho prevented S1P-induced endothelial motility⁹⁵. S1P may also facilitate endothelial injury repair in an indirect manner by stimulating the functional capacity of progenitor cells, a process which was reported to involve CXC chemokine receptor 4-dependent signaling via S1P₃⁹⁶. As injury repair, endothelial cell proliferation and migration are all crucial early steps in neovessel formation, it is not surprising that S1P induces endothelial tube and capillary formation^{97,98}. The identity of S1P receptors involved in the S1P-induced angio- and vasculogenic responses remains a matter of debate, although S1P₁ and S1P₃ seem to be the most likely candidates⁹⁹.

Finally, S1P may prevent endothelial dysfunction in response to atherogenic stimuli. Early studies revealed stimulatory effects of S1P on endothelial nitric oxide synthase (eNOS) activation and nitric oxide (NO) release, which were mediated via S1P₁ and dependent on the sequential activation of phosphoinositide 3-kinase and Akt¹⁰⁰. The eNOS-activating effects of S1P could be recapitulated in intact vessels, as both S1P and its synthetic analogue FTY720 induced NO-dependent relaxation of isolated precontracted aortas¹⁰¹. Vasodilatory effects of S1P and FTY720 appeared to be attributable to S1P₃ rather than to S1P₁ since these effects were completely abolished in aortas from S1P₃-deficient animals¹⁰¹.

In contrast to the atheroprotective effects of S1P on endothelium, several studies have demonstrated that S1P also upregulates adhesion molecules such as E-selectin, ICAM-1 and VCAM-1. However, this effect was generally only observed at supraphysiological S1P concentrations¹⁰². In keeping with its alleged atheroprotective action, S1P at physiological levels was shown to quench TNF- α -induced ICAM-1 and VCAM-1 expression¹⁰³. The notion that S1P may be protective against monocyte-endothelial interactions gained much strength from recent studies demonstrating that this compound prevents adhesion of monocytes to aortic endothelium after TNF- α exposure or in mice suffering from Type 2 diabetes mellitus^{103,104}.

Vascular Smooth Muscle Cells

S1P receptors are abundantly present in VSMCs, but the expression pattern appears to strictly depend on the phenotype⁸⁵. Whereas both S1P₂ and S1P₃ are expressed in adult and foetal/intimal phenotypes, the expression of S1P₁ appears to be restricted to neointimal VSMCs in the plaque. S1P₁ activation in VSMCs was shown to account for the S1P-induced cyclin D expression and cell proliferation⁸⁵. Nevertheless, S1P was repeatedly demonstrated to induce proliferation of adult VSMCs expressing small amounts of S1P₁, also indicating an S1P₁-independent mechanisms¹⁰⁵. Transactivation of platelet-derived growth factor (PDGF)- β and epidermal growth factor receptors in VSMCs may be at least partly responsible for the growth stimulating effects of S1P, and interestingly a direct crosstalk between S1P₁ and PDGF- β receptors via macromolecular complexes has been reported¹⁰⁶.

In addition to a mitogenic response, S1P was found to exert potent motogenic effects in intimal SMCs, which were attributed to the S1P₁ receptor⁸⁵, and were prevented by an inverse S1P₁ receptor agonist¹⁰⁷. While S1P₁ serves as a chemotactic sensor not only to endothelial cells but also to VSMCs, S1P₂ has been uniformly characterized as a chemorepellant receptor in various cell lines due to G α_q - and Rho-dependent

inhibition of Rac-1, which is critically involved in lamellapodia formation¹⁰⁸. Conceivably, as S1P₂ expression level in adult VSMCs far exceeds that of S1P₁, while the contrary is true for intimal VSMCs, this may explain the impaired migration repeatedly shown in normal VSMCs by S1P and stimulation shown in intimal VSMCs^{108,109}.

Eicosanoid-Mediated Effects of S1P

Eicosanoids (prostaglandins and leukotrienes) are well-established mediators of many inflammatory responses. A recent study on FTY720 showed an unexpected effect on eicosanoid synthesis, as it was shown to directly inhibit cPLA₂ independently of S1P receptors. cPLA₂ is rate limiting in arachidonic acid synthesis, which is an essential building block in eicosanoid synthesis⁶⁸. Both S1P and FTY720-P were shown to be ineffective.

Interestingly, S1P can also upregulate cyclooxygenase (COX)-2, an inducible key enzyme in the synthesis of prostaglandins (PGs), which plays an important role in vasorelaxation and platelet aggregation^{110,111}. In response to cytokines, such as IL-1 β and TNF- α , S1P and ceramide 1-phosphate synergistically induce PGE₂ by COX-2 induction and cPLA₂ activation and translocation, respectively¹¹¹. In macrophages, COX-2-dependent PGE₂ production and the ensuing cyclic AMP production increases MMP-2 and -9 production, implying that S1P can have major effects on plaque stability¹¹². Furthermore, COX-2 induction can influence atherogenesis by providing lipid accumulation in lesional SMCs and macrophages, and (neo)angiogenesis. In addition, COX-2 derived PGs may, by virtue of their anti-proliferative and anti-migratory action, contribute to the development of a SMC-depleted and macrophage-enriched, more vulnerable plaque phenotype¹¹³. Besides the pro-atherogenic effects of COX-2 in SMCs and macrophages, it can also have atheroprotective functions by endothelial PGI₂ production, which inhibits platelet activation and cholesterol accumulation¹¹³.

A recent report of Ki and colleagues demonstrates that S1P₁, S1P₃ and S1P₅ receptor activation are necessary for maximal COX-2 induction. Therefore, it is conceivable that FTY720, which downregulates these receptors, may have additional as yet unknown effects on atherosclerotic lesion development by inhibition of COX-2 induction¹¹⁴.

Conclusions

LPA and S1P, the two major bioactive lysolipids, appear to serve as key regulators of vessel wall biology and inflammatory processes during atherosclerosis (Figure 2). LPA is an important component of moxLDL and is not only found at elevated levels in atherosclerosis but also progressively accumulates via LDL, *in situ* synthesis and release from inflammatory plaque cells (e.g. platelets, macrophages and mast cells) in the plaque during lesion development. S1P is mainly present in the circulation but its intraplaque synthesis and content remains as yet unknown. LPA not only acts thrombogenically, but was also reported to induce SMC proliferation, prime endothelial cells, enhance endothelial permeability, and aggravate T-cell, macrophage and mast cell responses. Intriguingly, it may simultaneously instruct DCs to promote a Th2-shifted immune reaction, which is generally deemed to be beneficial to ath-

erosclerosis. Although the result of this complex range of effects remains elusive, it suggests a pro-atherogenic role of LPA.

Conversely, S1P suppresses T-lymphocyte proliferation and migration and deactivates macrophages, prevents endothelial dysfunction and permeability, promotes injury repair, and induces migration and proliferation of intimal VSMCs but prevents that of healthy medial VSMCs. These responses are regarded as favorable in the context of atherosclerosis. Thus, an S1P receptor-targeted therapy demonstrated a sizable reduction in plaque development in two established animal models of atherosclerosis^{66,67}.

The pleiotropic activity and rather grave immunomodulation of both bioactive lipids may disqualify systemic intervention in S1P or LPA function as a therapeutic modality. We propose that further insight into the mode of action of these compounds on the individual cell types relevant to atherosclerosis will pave the way for more subtle approaches that partially mimic LPA- and S1P-mediated processes and thereby lead to the discovery of new lipid-targeted therapies in atherosclerosis.

Executive summary

Lysophosphatidic acid

- Lysophosphatidic acid (LPA) species accumulate in atherosclerotic lesions; as LPA, mainly alkyl-LPA, is the primary platelet-activating lipid, rupture of the LPA-containing plaque increases the risk of intra-arterial thrombus formation, leading to myocardial infarction and stroke. LPA deteriorates neointima formation in a rat model of wire injury-inflicted vascular remodeling through peroxisome proliferator-activated receptor (PPAR) γ activation.
- Lymphocytes & monocytes
 - LPA can protect T cells from apoptosis. In addition, it induces migration and matrix metalloproteinase (MMP) production in unstimulated T cells, and interleukin (IL)-2 production in stimulated T cells. Furthermore, LPA stimulates proliferation and immunoglobulin formation from B cells. In monocytes, LPA effects their activation, survival and proliferation. Moreover, it induces reactive oxygen intermediate production, the release of arachidonic acid metabolites (prostaglandin E_2 and leukotriene B_4), lipid accumulation and inhibition of vessel wall emigration.
- Dendritic cells
 - In immature dendritic cells (DCs), LPA can promote chemotaxis, while inducing IL-10 secretion and inhibiting that of IL-12 and tumor necrosis factor (TNF)- α by mature dendritic cells. LPA mediates the fine-tuning of Toll-like receptor-dependent maturation of human myeloid DC maturation, but it does not affect the ability of DCs to influence T-helper cell proliferation itself.
- Mast cells
 - In mast cells, LPA can induce histamine release causing plasma exudation and vascular leakage. Furthermore, it promotes proliferation, differentiation and release of macrophage inflammatory protein-1 β , IL-8, eotaxin and monocyte chemoattractant protein (MCP)-1, and subsequent recruitment of inflammatory cells into the arterial wall.
- Endothelial cells
 - LPA induces endothelial barrier dysfunction and upregulation of E-selectin, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, IL-8 and MCP-1 via nuclear factor κ B activation.
- Vascular smooth muscle cells
 - LPA mobilizes intracellular Ca^{2+} , which can lead to an increase in proliferation and induction of phenotypic dedifferentiation by unsaturated LPA species. Furthermore, LPA increases the expression of tissue factor, an important thrombogenic constituent of the plaque.

Sphingosine 1-phosphate

- Two very recent studies demonstrated reduced atherosclerosis in apolipoprotein E^{-/-} or low-density lipoprotein receptor^{-/-} mice treated with a synthetic sphingosine 1-phosphate (S1P) analogue, FTY720, which acts as a high-affinity agonist on S1P₁, S1P₂, S1P₃, S1P₄ and S1P₅. FTY720 displayed an unexpected direct inhibitory effect on cytosolic phospholipase A₂, independently of S1P receptors, which negatively influences the synthesis of immunomodulatory eicosanoids. S1P can induce cyclooxygenase (COX)-2 expression, which in turn promotes atherosclerotic lesion progression by induction of lipid accumulation in vascular smooth muscle cells (VSMCs) and macrophages, and neovessel formation. In addition, COX-2 induction can affect plaque stability through its antiproliferative and antimigratory effect on VSMCs contributing to the development of mainly smooth muscle cell-depleted/macrophage-enriched, and thus more vulnerable, plaques. Finally, induction of prostaglandin E_2 /cyclic AMP production, and subsequent MMP2 and MMP9 expression can lead to further plaque destabilization.
- Lymphocytes & macrophages
 - In T cells, high physiological concentrations S1P suppress proliferation and migration toward chemotactic factors, inhibits interferon- γ and IL-4 secretion, while inducing IL-10 secretion. Furthermore, S1P is required for optimal function of CD4⁺ CD25⁺ regulatory T cells to inhibit proliferation and IL-2 secretion by CD4⁺ CD25⁻ autoimmune effector cells. In addition, S1P influences migration, tissue homing and recirculation via S1P₁ receptor activation. In monocytes, exogenous S1P inhibits apoptosis.
- Dendritic cells
 - In immature DCs, S1P stimulates pertussis toxin-sensitive Ca^{2+} increase, leading to actin polymerization and chemotaxis. In mature DCs, S1P inhibits IL-12 secretion, while enhancing IL-10 secretion.
- Mast cells
 - Sphingosine kinase (SphK)1/2 become activated upon antigen-specific cross-linking of the high-affinity receptor for immunoglobulin E on mast cells. SphK1 regulates extracellular S1P, which is necessary for transactivation of S1P₁ (mast cell migration) and S1P₂ (mast cell degranulation). SphK2 is an intrinsic regulator of mast cells influencing S1P production, cytokine production and degranulation.
- Endothelial cells
 - S1P acts cytoprotectively and improves endothelial barrier function by inducing endothelial repair through stimulation of the migration and functional capacity of progenitor cells. Furthermore, it prevents endothelial dysfunction via its stimulatory effects on endothelial nitric oxide synthase activation and nitric oxide release. At physiological levels, S1P quenches TNF- α -induced ICAM-1 and VCAM-1 expression.
- Vascular smooth muscle cells
 - S1P induces proliferation of VSMCs and, depending on which receptor expression is dominant, it can either stimulate (S1P₁) or impair (S1P₂) migration.

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